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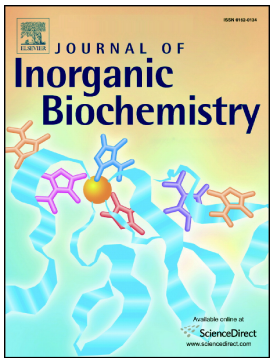


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# Bioconjugates of Co(III) Complexes with Schiff Base Ligands and Cell Penetrating Peptides: Solid Phase Synthesis, Characterization and Antiproliferative Activity

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## Abstract

In this work we synthesized a chelating Schiff base by a single condensation of salicylaldehyde with 3,4-diamino benzoic acid (**1**). This ligand was used further for complexation to $CoCl_2 \cdot 6H_2O$ under nitrogen. In the next step, three six-coordinate Co(III) complexes were synthesized by coordinating this complex with imidazole (**2**), 2-methyimidazole (**3**) and N-Boc-L-histidine methyl ester (**4**) (Boc: *tert*.-butoxycarbonyl) in axial positions with simultaneous oxidation of Co(II) to Co(III) under ambient environment. All Co(III) complexes were characterized by multinuclear NMR spectroscopy ($^{1}H$, $^{13}C$ and $^{59}Co$ NMR), FT-IR, mass spectrometry and HPLC. The Co(III) complexes were conjugated to three different cell penetrating peptides: FFFF (**P1**), RRRRRRRRRGAL (**P2**) and FFFFRRRRRRRRRGAL (**P3**). Standard solid-phase peptide chemistry was used for the synthesis of cell penetrating peptides. Coupling of N-terminal peptides with the cobalt complexes, possessing a carboxylic group on the tetradentate Schiff base ligand, afforded Co(III)-peptide bioconjugates, which were purified by semi-preparative HPLC and characterized by analytical HPLC and mass spectrometry. The antiproliferative activity of the synthesized compounds was studied against different human tumor cell lines: lung cancer A549, liver cancer HepG2 and normal human fibroblasts GM5657T, in comparison with the activity of cisplatin as a reference drug. The bioconjugate **21** containing the Co complex **4** and the combined phenylalanine and polyarginine cell penetrating sequence **P3** shows better activity against the liver cancer line HepG2 than the parent Co(III) complex **4**.

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## Introduction

Cobalt is an essential trace element in the human body where it exists exclusively in the form of vitamin $B_{12}$ (cobalamin), which is a cofactor for a number of enzymes, like isomerases, methyl transferases or dehalogenases.[1] Further, cobalamin takes part in creating neurotransmitters and stimulates the formation of erythrocytes in bone marrow.[2] Deficiency of cobalt is strongly related to the vitamin $B_{12}$ level and causes anaemia and thyroid hypofunction.[3],[4]

Recently, other cobalt compounds have gained extended attention in the field of medicinal inorganic chemistry, especially in the search for new anticancer drug candidates.[5] The severe side effects of platinum-based anticancer drugs have forced researchers towards finding metal-based anticancer drugs as alternatives.[6] From a chemical and physical point of view cobalt, similar to platinum, adopts a wide variety of coordination numbers, geometries, oxidation states, and ligand binding affinities, which makes cobalt chemistry thoroughly investigated, not only but also in the field of new anticancer agents.[7], [8] Interestingly, despite the fact that cobalt(III) complexes possess the same electron configuration ($d^6$) as platinum(IV) prodrugs, cobalt(III) complexes exhibit a different mode of action from platinum-based anticancer drugs.[9] The very inert oxidised cobalt(III) state is reduced to more labile cobalt(II), facilitating the exchange of axial ligands.[10],[11],[12]Binding of the complexes to histidine residues in or nearby the active sites of proteins causes irreversible inhibition of activity.[13],[14] Thus, Co(III) complexes inhibit histidine-containing proteins and enzymes including zinc finger transcription factors (TFs) and metalloendopeptidases. Protein inhibition occurs through a dissociative exchange of labile axial ligands for the imidazole nitrogens of histidine residues, as drawn schematically in Figure 1.[15],[16]

Moreover, cobalt is generally less toxic to humans than platinum, which creates a basis for the investigation of cobalt-containing compounds as less toxic alternatives to platinum-based anticancer drugs.[17],[18] The simple $Co^{3+}$ ion is unstable in water, but can be stabilized to hamper reduction to $Co^{2+}$ by coordination to chelating N,O or N,S donor ligands.[19] Cobalt(III) complexes derived from these donor ligands find applications as antibacterial and antiviral agents.[20] One of the most promising classes of Co(III) complexes containing N, O donor ligands are those based on chelating Schiff bases.[21] So far, among all Co(III) complexes, clinical trials have been reached only by a cobalt(III) Schiff base complex containing bis(acetylacetone)ethylenediimine (acacen), with two axially coordinated 2-

methylimidazole rings [Co(III)(acacen)(2-mimd)$_2$] (**A**).[22] The drug formulation was developed as Doxovir™ by the Redox Pharmaceutical Corporation. In 2013, Doxovir successfully completed phase II clinical trials for the treatment of Herpes Simplex Virus Type 1 (HSV-1) labialis infections and phase I clinical trials for the treatment of two viral eye infections, the major causes of blindness (ophthalmic herpetic keratitis and adenoviral conjunctivitis).[23] The promising antiviral activity of Doxovir is attributed to the direct interaction of the Co(III) Schiff base complex with its molecular target, the herpes virus maturational protease, a serine protease containing large amounts of histidine.[24]

Another promising group of cobalt(III) complexes are those with nitrogen mustards ligands, which are potent cytotoxins, utilizing the lone pair on the amine nitrogen to initiate a reaction sequence, in which DNA is cross-linked by double alkylation.[25],[26],[27] The Co(III) complex [Co(Meacac)$_2$(DCE)]$^+$ (Meacac = Methlyacetylacetonate, DCE=N,N-bis(2-chloroethyl)ethylenediamine) (**B**) had 20-times greater activity against cancer cells under hypoxic than oxic conditions, due to bioreduction of the starting Co(III) complex to Co(II) in the hypoxic regions of solid tumours and subsequent release of the cytotoxic free nitrogen mustard from the substitutionally more labile Co(II) ion.[28]

Along the same lines, the Hambley group has exploited bioreductive prodrugs of chaperone cobalt(III) complexes for the delivery of cytotoxic ligands to hypoxic solid tumours.[29] Firstly, Hambley et al. presented a bioreductively activated carrier system for the delivery and release of curcumin. The dichlorido precursor complex, [CoCl$_2$(tpa)]ClO$_4$, (tpa = tris-(2-pyridylmethyl)amine), exhibited no toxicity up to a concentration of 200 µM against the colorectal cancer cell line DLD-1, whereas the Co(III) complex with coordinated curcumin possesses an IC$_{50}$ value of 39 ± 4 µM.[30] Secondly, Hambley et al. have prepared a series of cobalt(III) complexes with the tripodal, ancillary tpa ligand as chaperones for delivery of derivatives of hydroxamic acid, which inactivates enzymes by binding to catalytic zinc ions through the hydroxamic acid moiety. The [Co(tpa)(c343ha)]ClO$_4$ complex with a fluorescent doubly deprotonated hydroxamic acid ligand (c343haH$_2$: coumarin-343 hydroxamic acid) showed enhanced antiproliferative activity against DLD-1 colon cancer cells (31 ± 2 µM) in comparison to the free c343haH$_2$ ligand (113 ± 8 µM).[31] Later, Hambley and co-workers extended the concept of Co(III) prodrugs to selectively deliver inhibitors of matrix metalloproteinases (MMP) enzymes, which are involved in the process of tumour metastasis. The Co(III) carrier system consists of the MMP inhibitor marimastat with a tetradentate tpa carrier ligand (**C**). *In vivo* antimetastatic activity tested against Balb/c mice with 4T1.2 tumour

implants of **C** showed a higher level of tumour-growth inhibition than free marimastat.[32] Meade et al. have synthesized Co(III) Schiff base-DNA conjugates targeting C2H2 transcription factors, to inhibit the Hedgehog (Hh) pathway, which regulates the activity of the Gli family of C2H2 zinc finger transcription factors in mammals. Such Co(III)-DNA bioconjugates resulted in a targeted inhibitor of the single C2H2 zinc finger transcription factor Cubitus Interruptus (Ci).[33],[34],[35] In the literature there are also examples of Co(III) conjugates with bleomycin and pepleomycin, glycopeptide antitumor antibiotics, which are used in the treatment of Hodgkin's lymphoma, carcinomas of the skin, head and neck.[36],[37] Such Co-glycopeptide conjugates bind to the DNA and under UV or visible light irradiation result in DNA cleavage.[38],[39],[40],[41]

Despite the fact that cobalt(III) complexes with small molecular bioactive ligands as described above were extensively studied as antiviral, antimicrobial and anticancer agents, the development of cobalt(III) bioconjugates with larger biomolecules and suitable drug delivery systems for them is still in its infancy. Our research is devoted to the design of peptide delivery systems for various anticancer drugs.[42],[43],[44] In the past, we have synthesized a dicobalt hexacarbonyl alkyne compound linked to the neurotensin peptide hormone. The cobalt-peptide bioconjugates showed moderate cytotoxicity against HeLa cervical cancer cells (26.4 ± 5.8 µM).[45] Thus, despite their demonstrated versatility cobalt derivatives, especially cobalt-peptide bioconjugates have not been further studied as anticancer agents. This fact and our previous work on bioconjugation techniques for inorganic pharmaceuticals,[46],[47],[48],[49] prompted us to investigate cell penetrating peptides as a drug delivery system for promising Co(III) anticancer candidates, as an alternative to platinum-based therapy.

## Results and discussion

### Synthesis and characterisation of cobalt(III) complexes

The key idea of this study is to synthesize a Schiff base ligand containing a functional group, suitable for coupling with peptides. Such covalent linking to peptides via the ligand has not been achieved before. The literature-known ligand $H_2$salophen **1** was synthesized by a single condensation of salicylaldehyde with 3,4-diamino benzoic acid (**Scheme 1**).[50],[51] The ligand was fully characterized by FT-IR, ESI-MS, HPLC, $^{1}$H- and $^{13}$C-NMR (ESI†).

In the next step, the complexation reaction of Schiff base ligand **1** to cobalt(II) was carried out under nitrogen atmosphere (**Scheme 2**). The ligand **1** was dissolved in absolute MeOH and

subsequently $CoCl_2 \cdot 6H_2O$ was added. The formation of the expected cobalt(II) complex of ligand **1** *in situ* was confirmed by electrospray mass spectrometry (see ESI†). In the full-scan mass spectra, the molecular ion peak $[M+H]^+$ (*m/z* = 417) was found and assigned to cobalt(II) complexes with ligand **1**. 3 hours were obligatory to obtain the cobalt(II) complex *in situ*. To the *in situ* formed cobalt(II) complex bearing the Schiff base ligand **1**, three different types of N-donors were added as the axial ligands (2 eq.), namely imidazol, 2-methyimidazol and N-Boc-L-histidine methyl ester (Boc: *tert*.-butoxycarbonyl). The imidazole-based ligands were chosen because of the previously noted remarkable activity of cobalt(III) Schiff base complexes with coordinated 2-methylimidazole rings.[22] In the case of N-Boc-L-histidine methyl ester, both functional groups (amino and carboxylic group) were protected to avoid any side reactions during future coupling with peptides by solid phase peptide synthesis. After adding the N-donors, the reaction mixture was opened to air to allow oxidation of cobalt(II) to cobalt(III) (Scheme 2).

All cobalt(III) complexes were characterized by FT-IR, mass spectrometry, HPLC and multinuclear NMR spectroscopy ($^1H$, $^{13}C$ and $^{59}Co$ NMR).The IR spectra of the cobalt(III) complexes **2** – **4** were compared to those of the free ligands. The IR spectral bands with their assignment observed in the region 4000-400 $cm^{-1}$ are listed in **Table 1**. The medium and broad absorption peaks in the range 3102-3356 $cm^{-1}$ were assigned to ν(O–H) stretching frequencies showing the existence of a carboxylic acid. The sharp bands indicative of NH vibrations are located at 2900-2999 $cm^{-1}$. IR spectra show that the vibration bands of the ν(C=N) imino groups were shifted to 1600–1675 $cm^{-1}$. These shifts by 15 $cm^{-1}$ compared to the free ligand **1**indicate that the nitrogen atoms of the imino groups coordinate to the cobalt centre.[52] The sharps bands of the $\nu_{as}$(COO) stretching frequencies at 1502–1633 $cm^{-1}$ and $\nu_{sym}$(COO) at 1289–1370 $cm^{-1}$ indicate the existence of carboxylate salts. All IR spectra are reproduced in the ESI†.

Electrospray ionization mass spectrometry (ESI-MS) was applied to confirm the mass of the desired compounds (**Figure 3a**). In the full-scan mass spectra, the molecular ion peaks $[M+H]^+$ were found and assigned to all complexes. The *m/z* values are consistent with the proposed constitution of the compounds. The mass spectra of cobalt(III) complexes displayed two additional peaks with moderate intensity corresponding to the $[M-L]^+$ and $[M-2L]^+$ species. This feature confirms the expected primary fragmentation pathway, which would be

characterized by the gradual loss of one and the second ligand in the axial positions. The full-scan mass spectra of complexes **2 - 4** are available in the ESI†.

To fully accomplish characterization of the Co(III) complexes also their $^{59}$Co NMR spectra were measured. Representative spectra are shown in **Figure 3**, and chemical shifts (δ) and line widths ($\nu_{1/2}$) are summarized in **Table 1**. The remaining $^{59}$Co NMR spectra are available in the ESI†. All spectra showed only one peak, which confirms the oxidation of the cobalt(II) precursor to only one cobalt(III) complex. The $^{59}$Co NMR data show that the nature of the different axial ligands affect the amount of electron density at the Co nucleus.[53] The signal is shifted downfield, when the axial ligand is altered from imidazole (**2**) to 2-methylimidazole (**3**) and to N-Boc-L-histidine methyl ester (**4**).

**Synthesis and characterisation of peptides**

To enhance the cellular uptake and hence potentially the cytotoxicity of cobalt(III) Schiff base complexes we decided to utilize cell penetrating peptides (CPPs). CPPs are usually rich in positively charged amino acids, such as arginine (Arg, R) and lysine (Lys, K).[54] Oligoarginine / arginine-rich cell-penetrating peptides have been successfully used as vectors for the intracellular delivery of small anticancer drugs without specific receptors.[55]It has been also reported that the attachment of a short peptide segment, namely hydrophobic sequences of phenylalanine residues (Phe, F) results in enhanced translocation through cell membranes of arginine-rich CPPs, due to stronger interaction with membranes.[56],[57] Therefore, we prepared two peptides containing an arginine-rich sequence, one with an additional FFFF segment at the N-terminus. A third peptide, consisting only of phenylalanine residues, was design as a control peptide for cytotoxicity experiments. Solid-phase peptide synthesis (SPPS) with Fmoc strategy (Fmoc: Fluorenylmethoxy-carbonyl) was employed to synthesize those CPPs (**Scheme 3**).[58] Peptide **P1**, which consists of only a tetraphenylalanine sequence (FFFF), was synthesized manually. The longer peptides **P2** and **P3**, were assembled on an automated, microwave-assisted synthesizer. Deprotection of temporary Fmoc protecting groups was performed with 20% piperidine in DMF. The coupling was performed with an excess of the Fmoc-amino acid (4 eq.), activated with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU, 4 eq.) in the presence of N-hydroxybenzotriazole (HOBt, 4 eq.) and an excess of diisopropylethylamine (DIEA, 6 eq.). SPPS consists of repeated cycles of N-terminal protecting group removal on

the last amino acid, followed by coupling of the next incoming amino acid. Through repetitions of a reaction cycle of peptide coupling, washing, deprotection and washing the desired peptides were obtained.

After completion of peptide synthesis and before the bioconjugation reaction with Co(III) complexes **2** – **4** the successful synthesis of peptides **P1**, **P2** and **P3** was established by HPLC and ESI-MS. For this purpose, 10 mg of the resin from each peptide was treated with 20% of piperidine in DMF in order to remove the Fmoc group from the N-terminus. Then, after washing and drying the peptides were cleaved from the resin by treatment with a mixture of TFA/phenol/$H_2O$ (95%/2.5%/2.5%). After cleavage from the resin the peptides were lyophilized. The crude peptides were purified by reverse-phase semi-preparative HPLC. Finally, the peptides were characterized by HPLC and ESI-MS measurements. The purity of the peptides was confirmed by analytical HPLC (ESI†). ESI-MS spectra confirm the molecular mass $[M+H]^+$ for peptide **P1** at *m/z* = 606, for peptide **P2** at *m/z* = 1664 and for peptide **P3** at *m/z* = 2253. Besides the signals of molecular masses, multiply charged species were formed, in the case of peptide **P2** $(M+2H)^{2+}$ at *m/z* = 832, $(M+3H)^{3+}$ at *m/z* = 555, $(M+4H)^{4+}$ at *m/z* = 416, and for peptide **P3** $(M+2H)^{2+}$ at *m/z* = 1127 and $(M+3H)^{3+}$ at *m/z* = 752 (ESI†).

**Synthesis and characterisation of bioconjugates**

In the last synthesis step, the peptides were coupled to our Co(III) complexes which contain a free carboxylic acid group for bioconjugation. The coupling of Co(III) complexes **2, 3** and **4** with the Fmoc-deprotected peptides **P1**, **P2**, **P3** was performed with the fully side-chain protected peptides on the resin in order to avoid any undesired side reactions (**Scheme 4**). The carboxylic acid group on the Schiff base ligand provided a suitable functionality for coupling by SPPS. The metal complex was dissolved in DMF and coupling was performed with an excess of the cobalt complexes (4 eq.), activated with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU, 4 eq.) in the presence of N-hydroxybenzotriazole (HOBt, 4 eq.) and an excess of diisopropylethylamine (DIEA, 8 eq.). After 24 h, an excess of reagents was removed by filtration, the resin was washed with DMF, $CH_2Cl_2$, $Et_2O$, and then dried under vacuum. Cleavage from the resin and deprotection of the pbf side-chain protecting groups of arginine (pdf: 2,2,4,6,7-pentamethylhydrobenzofuran-5-

sulfonyl), for peptides **P2** and **P3,** was achieved with 95% TFA. The obtained metal-peptide bioconjugates were purified by preparative HPLC to obtain the desired products. All metal-peptide bioconjugates were characterized by analytical HPLC, ESI-MS and MALDI-MS. Analysis by RP-HPLC showed main peaks (ESI†), which were isolated and characterized by ESI mass spectrometry. After purification by semi-preparative HPLC and lyophilization, all bioconjugates were obtained as pale brown solids. ESI-MS analysis of the conjugates showed *m/z* values that were consistent with that of the charged species $[M+H]^+$, $[M+Na]^+$ and doubly charged species $[M + 2H]^{2+}$, also showing the expected isotopic mass distribution patterns (ESI†). The observed *m/z* values are listed in **Table 2**.

**Cytotoxicity**

Our next objective was to evaluate the anti-proliferative activity of Co(III) complexes and cobalt-peptide bioconjugates against human liver cancer cells (HepG2), human lung cancer cells (A549) and normal human fibroblast cells (GM5657T) by the MTT assay (MTT assay: A cytotoxicity assays based upon formation of a purple-coloured precipitated from the yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye after reduction in metabolically active cells). The choice of cell lines was guided by our previous studies on platinum, gold and metallocene bioconjugates.[44],[45],[46],[59] Cytotoxicity results are expressed as $IC_{50}$ values (half-maximal inhibitory concentration) and are summarized in **Table 3**. Dose-response curves are shown in the ESI†. For comparison, the $IC_{50}$ value of cisplatin was also determined using the same assay, and identical conditions. As often in the field, stock solutions of our compounds were prepared in DMSO for solubility reasons, however final DMSO concentrations were in all cases well below 1 %. Even though not all compounds were insoluble in water (or buffer) at the required concentrations, and certainly cisplatin is soluble enough, we still utilized DMSO as the solvent for coherence, so that all compounds are treated in an absolutely identical fashion. While we are aware of the effect of DMSO on cisplatin activity as reported by Hall et al.,[60] we did not observe a similar influence of DMSO on ligand exchange in our Co complexes by $^{59}$Co NMR.

The effect of solvent and, more generally, assay conditions deserves further discussion. It is well known that DMSO exhibits a significant, diminishing effect on the cytotoxicity of cisplatin.[60] In our studies we used DMSO stock solutions in order to compare the activity of bioconjugates and cobalt(III) complexes with cisplatin in a range of different cell lines. According to the literature,[61] cisplatin dissolved in $H_2O$ alone has an $IC_{50}$ value of 3.6 ± 0.4

μM against A549 human lung cancer cells, whereas in our experiments its $IC_{50}$ value was 25.3 ± 2.3 μM (dissolved in DMSO). Against HepG2 human liver cancer cells, the values for cisplatin were (2.09 ± 0.15 μM) in water,[62] compared to our experiments (13.3 ± 1.4 μM) with DMSO present. However, other differences must also be taken into account, such as assay type, incubation time, and probably general conditions of the cell line (e.g. no. of passages for a given cell line). In this case, we note that the MTT assay was used in all studies discussed above. However, while we incubate our compounds with cells routinely for 48 h, Yao et al. incubated the A549 cells for 24 h only with cisplatin, then the media with compounds were replaced by fresh media, and cells were cultured for another 48 h. As normally no additional information is available on the age or "fitness" of the cell line (and also it can only be assumed that identity checks of cell lines are regularly performed), such data can be compared only with appropriate caution, and there is clearly not one "true" $IC_{50}$ value for a given cell line. For the same reason, we feel most comfortable with performing all cytotoxicity assays at the same time, on the same cell lines with identical treatment of all samples including negative and positive controls – knowing that especially the conditions for cisplatin may be considered "sub-optimal".

Firstly, we investigated the cytotoxic activity of all cobalt complexes. As indicated in **Table 3** all Co(III) complexes display moderate *in vitro* activity. HepG2 liver cancer cells appeared to be the most sensitive to cobalt complexes among all testes cell lines. Complex **4** was the most active towards HepG2 human liver cancer cells (13.2±0.8 μM) comparable with cisplatin (13.3±1.4 μM). The GM5657T non-cancerous human fibroblast cells were the most sensitive (22.0±0.9 μM) to complex **2**.

Secondly, we investigated the influence of cell penetrating peptides attached to the cobalt(III) complexes on *in vitro* cytotoxicity (**Table 3**). Since complex **4** appeared to be the most potent cytotoxic agent among all cobalt complexes, only bioconjugates containing this complex (**15**, **18**, **21**) were investigated by the MTT assay. Bioconjugate **15** with the short phenylalanine sequence showed only moderate activity towards all cancer cells, lower than complex **4**. Bioconjugate **18** consisting of an polyarginine domain showed activity similar to the parent Co(III) complex. Finally, bioconjugate **21** possessing the combined polyarginine sequence and a short uptake-accelerating tetra-phenylalanine sequence on the N-terminus shows slightly improved activity in comparison to complex **4** against all tested cells. Similar observations were made by Takayama et al., who observed that the attachment of a small

hydrophobic peptide segment to arginine rich CPPs improves cellular uptake.[63] Also Sadler et al. described the improved cytotoxic activity of osmium(II) complexes after conjugation to polyarginine peptides.[64] Since cell penetrating peptides without any particular selectivity were used in this work cancer cells as well as normal human fibroblast cells were about equally affected.

In a control experiment we investigated the anti-proliferative impact of the cell penetrating peptides alone, i.e. without cobalt(III) Schiff base complexes attached. Together, the metal-free peptides **P1**, **P2** and **P3** were examined by MTT assays using the same cell lines and conditionsas for the parent bioconjugates (**Table 3**). These peptides exhibit no in vitro activity at concentrations of up to 250 μM. These results are in agreement with observations made by Gross et al.,[44] where similar peptides with only a slight C-terminal modification appeared to be inactive as well.

## Conclusion

Cobalt complexes emerge as a promising alternative to classical platinum-based anticancer drugs. Here we present the synthesis of Co(III) complexes with salen-type Schiff base ligand as chelating donor and three different imidazole derivatives containing N-donors as axial ligands. Notably, a free carboxylic group on the salen-type ligand provides a handle for the synthesis of covalent bioconjugates. By reaction with this carboxylate group, cobalt(III)-bioconjugates with cell penetrating peptides have been synthesized and characterized for the first time. First, the cell penetrating peptides were synthesized by solid phase peptide synthesis (SPPS). Then, an SPPS reactions scheme of resin-bound peptides and the Co(III) complexes with one free carboxylate group afforded monoconjugated cobalt-peptide species, which were purified by RP-HPLC and characterized by ESI-MS. All Co complexes and selected Co-peptide bioconjugates were screened for their cytotoxicity against selected cancer cell lines. The Co(III) complexes alone show promising cytotoxicity in the mid-to-low micromolar range. Among all bioconjugates, the bioconjugate which consists of a CPP-polyarginine peptide sequence with four addition phenylalanine residues (**21**), was found to be most active against HepG2 liver cancer cells. It is noteworthy that the peptide sequences chosen here are not know for any particular intra-cellular targeting other than uptake through lysosomes. On the other hand, adding a peptide amplifies the molecular weight of the (small)

Co complex significantly, and this might potentially alter its propensity to interact with the intracellular target. On the other hand, the fact that we do see acceptable bioactivity of the Co-peptide conjugates in our work indicates that at least a fraction of the Co complex will reach the intracellular target and exert its activity. Further investigations as to what this target would be shall be carried out and the results reported subsequently.

**Experimental section**

**Materials and reagents**

All reagents and chemicals were purchased from commercial sources and used without further purification. Salicylaldehyde, 3,4-diamino benzoic acid and $CoCl_2 \cdot 6H_2O$ were purchased from Merck. All Fmoc-protected amino acids were purchased from Iris Biotech GmbH. All the materials and organic solvents including absolute methanol, ethanol, dimethyl sulfoxide and dimethylformamide were commercially obtained from Merck, Aldrich or Fluka. Unless otherwise noted, all manipulations were performed using standard Schlenk techniques under nitrogen atmosphere. For the biological experiments, Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum, penicillin/streptomycin mixture, trypsin/EDTA, and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich. Compounds were dissolved in DMSO and diluted with the tissue culture medium before use.

**General Methods:** Solvents were dried according to standard procedures and stored over molecular sieves (4 Å). Solid-supported reactions were performed in 5 mL plastic syringes with a porous polypropylene disc as filter. HPLC was performed by using two buffer systems (buffer A: $H_2O$/MeCN/TFA, 95:5:0.1, v/v/v; buffer B: MeCN/$H_2O$/TFA, 95:5:0.1, v/v/v) as the mobile phase. Preparative HPLC runs were performed by using a Dr. Maisch reprosil C18 reversed-phase column (250 × 20 mm) at a flow rate of 10 mL/min with a linear gradient of buffer B (100 % in 40 min) from 100 % buffer A with a total run time of 60 min. Analytical HPLC runs were performed by using a Knauer Eurospher-II C18 reversed-phase column (250 × 4.6 mm) at a flow rate of 1.0 mL/min with a linear gradient of buffer B (100 % in 20 min) from 100 % buffer A with a total run time of 40 min. FT-IR spectra were recorded with ATR technique on a Spectrum 65 PerkinElmer instrument. NMR spectra were recorded at room temperature with a Bruker Avance 300 Digital ($^1$H at 300 MHz) spectrometer. $^1$H and $^{13}$C NMR spectra were referenced using the residual solvent chemical shift (DMSO-$d_6$). Mass spectra were recorded with a Bruker Esquire 6000 (ESI-MS) spectrometer. UV-Vis absorption spectra were recorded on a Spectrophotometer Microplate Reader (Berthold Detection System).

**Synthesis of the ligand**

(**1**): Salicylaldehyde (1.60 g, 0.13 mmol) was mixed with ethanol (10.0 mL) and simultaneously 3,4-diamino benzoic acid (1.00 g, 0,01 mmol) with the same solvent (10.0 mL). This solution of 3,4-diamino benzoic acid was added dropwise to the salicylaldehyde solution and the reaction mixture was stirred overnight at room temperature. After 24h, the orange precipitation was collected and washed three times with $Et_2O$. The product was dissolved and purified by preparative HPLC using 0.01% TFA as the aqueous mobile phase and acetonitrile as the organic mobile phase with a linear gradient from 10% to 90% of the organic mobile phase over 20 min. The final product was dried by rotary evaporation, followed by lyophilization, to give a pale yellow oil. Yield: (2.09 g, 52%). Anal. Calc. for $C_{21}H_{16}N_2O_4$: C, 69.99; H, 4.48; N, 7.77. Found: C, 70.11; H, 5.03; N, 7.51. MS (ESI-MS, pos. mode, m/z): obs.: 361.00 $[M+H]^+$; calc.: 361.11 $[M+H]^+$, obs.: 383.00 $[M+Na]^+$; calc.: 383.10 $[M+Na]^+$; obs.: 255.40 $[M-L]^+$; calc.: 254.07 $[M-L]^+$. 1H NMR (300 MHz, DMSO-$d_6$) δ: 8.94 (s, 2H), 8.13 (d, 2H), 7.80 (ddd, 2H), 7.71 (m, 4H), 7.65 (d, 2H), 7.35 (d, 2H), 7.32 (s, 2H), 6.93 (t, 4H). 13C NMR (126 MHz, DMSO-$d_6$) δ: 163.6, 161.1, 160.0, 144.6, 132.4, 132.1, 130.1, 127.2, 123.6, 123.5, 121.4, 120.5, 117.8.

**Synthesis of Co(III) complexes**

(**2**): To a methanolic suspension (5 mL) of ligand **1** (40 mg, 0.0989 mmol), $CoCl_2 \cdot 6H_2O$ complex (51.7 μL, 0.297 mmol) was added directly under nitrogen atmosphere at room temperature. After 2 hours, 2 eq. of imidazol (12.54 mg, 0.0989 mmol) was added to the reaction. The solution was opened to air and the reaction was stirred for 12 h to produce a brown solution. The solution was concentrated. Addition of diethyl ether precipitated a brown solid. The product was filtered and washed with cold diethyl ether. Yield: (0.657 g, 49%). Anal. Calc. for $C_{27}H_{22}ClCoN_6O_4$: C, 55.07; H, 3.77; N, 14.27. Found: C, 55.42; H, 3.83; N, 14.49. MS (ESI-MS, pos. mode, m/z): obs.: 564.80 $[M+Na]^+$; calc.: 574.09 $[M+Na]^+$, obs.: 485.90 $[M-L]^+$; calc.: 484.06 $[M-L]^+$, obs.: 485.90 $[M-2L]^+$; calc.: 417.03 $[M-2L]^+$. 1H NMR (300 MHz, DMSO-$d_6$) δ: 8.94 (s, 2H), 8.13 (d, 2H), 7.80 (ddd, 2H), 7.71 (m, 4H), 7.65 (d, 2H), 7.35 (d, 2H), 7.32 (s, 2H), 6.93 (t, 4H). 13C NMR (126 MHz, DMSO-$d_6$) δ: 163.6, 161.1, 160.0, 144.6, 132.4, 132.1, 130.1, 127.2, 123.6, 123.5, 121.4, 120.5, 117.8.

(**3**): To a solution of the ligand **1** (0.50 g, 2.45 mmol) in MeOH (5.0 mL) was added directly solid $CoCl_2 \cdot 6H_2O$ complex (0.24 g, 1.025 mmol) under nitrogen atmosphere at room temperature with continuous stirring. The mixture was stirred at room temperature for 2 h. After that, 2 eq. of 2-methylimidazol (12.54 mg, 0.0989 mmol) was added to the reaction. The reaction was subjected to stirring for 18 h in an open-air environment to produce a bright brown precipitation. Finally, the precipitate was filtered, washed with $Et_2O$ (5.0 mL) and dried. Yield: (0.657 g, 49%). Anal. Calc. for $C_{29}H_{26}ClCoN_6O_4$: C, 56.46; H, 4.25; N, 13.62. Found: C, 56.86; H, 4.33; N, 14.01. MS (ESI-MS, pos. mode, m/z): obs.: 582.00 $[M+H]^+$; calc.: 581.48 $[M+H]^+$, obs.: 499.80 $[M-L]^+$; calc.: 498.70.10 $[M-L]^+$, obs.: 416.90 $[M-2L]^+$; calc.: 417.03 $[M-2L]^+$. 1H NMR (300 MHz, DMSO-$d_6$) δ: 8.94 (s, 2H), 8.13 (d, 2H), 7.80 (ddd, 2H), 7.71 (m, 4H), 7.65 (d, 2H), 7.35 (d, 2H), 7.32 (s, 2H), 6.93 (t, 4H). 13C NMR (126 MHz, DMSO-$d_6$) δ: 163.6, 161.1, 160.0, 144.6, 132.4, 132.1, 130.1, 127.2, 123.6, 123.5, 121.4, 120.5, 117.8.

(**4**): The ligand **1** (0.31 g, 1.5 mmol) was suspended in MeOH (10 mL) and to the suspension was added immediately solid $CoCl_2 \cdot 6H_2O$ complex (0.24 g, 1.025 mmol) under nitrogen atmosphere at room temperature with continuous stirring. The reaction was carried out for 3 h, and then 2 eq. of N-Boc-L-histidine methyl ester (12.54 mg, 0.0989 mmol) was added to the reaction. The solution was opened to air and an immediate colour change to deep brown was observed. After stirring at room temperature for 24 h, the resulting solution was allowed to evaporate resulting in the precipitation of a deep brown solid, which was collected by filtration and washed with $Et_2O$. Yield: (0.657 g, 49%). Anal. Calc. for $C_{45}H_{52}ClCoN_8O_{12}$: C, 54.52; H, 5.29; N, 11.30. Found: C, 54.92; H, 5.37; N, 11.46. MS (ESI-MS, pos. mode, m/z): obs.: 458.88 $[M+H]^+$; calc.: 458.11 $[M+H]^+$; obs.: 480.82 $[M+Na]^+$; calc.: 481.10 $[M+Na]^+$; (ESI-MS, neg. mode, m/z): obs.: 456.85 $[M-H]^-$; calc.: 457.10 $[M-H]^-$; obs.: 478.73 $[M+Na-H]^-$; calc.: 480.09 $[M+Na-H]^-$. 1H NMR (300 MHz, DMSO-$d_6$) δ: 8.94 (s, 2H), 8.13 (d, 2H), 7.80 (ddd, 2H), 7.71 (m, 4H), 7.65 (d, 2H), 7.35 (d, 2H), 7.32 (s, 2H), 6.93 (t, 4H). 13C NMR (126 MHz, DMSO-$d_6$) δ: 163.6, 161.1, 160.0, 144.6, 132.4, 132.1, 130.1, 127.2, 123.6, 123.5, 121.4, 120.5, 117.8.

**Peptide Synthesis**: The FFFF peptide (**P1**) was synthesized manually by means of the Fmoc protocol on an Fmoc-Phe-Wang resin (0.64 mmol/g). This peptide was synthesized on a 0.25 mmol scale. Each synthetic cycle consisted of the following steps: Fmoc removal, washing, coupling, washing. Fmoc removal: The resin (357 mg, 0.250 mmol) was treated with a 20 % solution of piperidine in DMF (3.0 mL; 2 × 10 min). The solution was removed and the resin

washed with DMF (3.0 mL; 3 × 1 min) and $CH_2Cl_2$ (3.0 mL; 3 × 1 min). The presence of free α-amino functionalities was checked by the bromophenol blue (BPB) test (blue-green beads). Coupling of standard amino acids: A mixture of Fmoc-Xxx-OH (1.00 mmol, 4 equiv.), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU, 1.00 mmol, 4 eq.), N-hydroxybenzotriazole (HOBt, 1.00 mmol, 4 eq.) and DIEA (1.5 mmol, 6 equiv.) in DMF (3.0 mL) was added to the resin and the suspension mixed by shaking at room temperature for 45 min. Reagents and solvents were removed by filtration, and the resin was subsequently washed with DMF (3.0 mL; 3 × 1 min) and $CH_2Cl_2$ (3.0 mL; 3 × 1 min). Completion of the coupling (as monitored by the absence of free α-amino functionalities) was verified either by the Kaiser test or the BPB test (BPB: bromophenol blue, colorless beads in both cases). Then, the resin was washed with $CH_2Cl_2$ (3.0 mL; 3 × 1 min), $Et_2O$ (3.0 mL; 3 × 1 min), and finally was dried under vacuum. The resin bound polyarginine peptides RRRRRRRRRGAL (**P2**) and FFFFRRRRRRRRRGAL (**P3**) were synthesized on a CEMLibertyBlue™ automated microwave peptide synthesizer using standard protocols (amino acid coupling: TBTU in DMF (0.5 M), HOBt in DMF (0.5 M), DIEA in NMP (2 M) and amino acids in DMF (0.2 M); arginine coupling: 25 min, 75 1C, 0 W followed by 5 min, 75 1C, 25 W; standard amino acid coupling: 5 min, 75 1C, 24 W. For deprotection: 20% piperidine in DMF: initial deprotection: 0.5 min, 75 1C, 30 W followed by deprotection: 3 min, 75 1C, 50 W). Afterwards, aliquots of 100 mg of peptide-containing resin were transferred into a filter-containing syringe for further derivatization.

**Deprotection and Cleavage**. The Fmoc deprotection was performed twice by treating the Fmoc protected peptide with 2 mL of 20% piperidine in DMF (each time 10 min). For the synthesis of all compounds, the N-terminal Fmoc group was deprotected, and after each step the resin was washed 5 times using 2 mL of DMF. TFA cleavage: The resin was swirled in a mixture of TFA/phenol/$H_2O$ (2.0 mL; 95:2.5:2.5, v/v/v) at room temperature for 1 h. Then the resin was removed and the residual TFA solution transferred to a 50 mL falcon tube and an ice-cold mixture of $Et_2O$/hexane (20 mL; 1:1 v/v) was added to precipitate the peptide. The supernatant was removed after centrifugation, and the peptide pellets were washed twice with $Et_2O$/hexane. The crude peptides were dissolved in MeCN/$H_2O$ (1:1, v/v) and lyophilized. Crude peptides were purified by preparative HPLC, and the pure fractions were pooled and lyophilized. Finally, the peptides were analyzed by analytical HPLC and characterized by ESI-MS.

**Cell culture. General procedure**. Cells were grown in RPMI 1640 cell culture medium with 1% sodium pyruvate, 1% L-glutamine, 100 units per mL Pen Strep, 10% fetal bovine serum. The cells were maintained at 37°C in a humidified incubator under an atmosphere containing 5% $CO_2$.

**Cytotoxicity experiments:** Dulbecco's Modified Eagle's Medium (DMEM), containing 10% fetal calf serum, 1% penicillin and streptomycin, was used as growth medium. A549, HepG2 and GM5657T cells were detached from the wells with trypsin and EDTA, harvested by centrifugation and re-suspended again in cell culture medium. The assays were carried out on 96 well plates with 6000 cells per well for all cell lines: human lung cancer (A549), human liver cancer (HepG2) and normal human fibroblast (GM5657T). After 24 h of incubation at 37.8 °C and 10 % $CO_2$, the cells were treated with the compounds (with final DMSO concentrations of 0.5 %) with a final volume of 200 mL per well. For a negative control, one series of cells was left untreated. The cells were incubated for 48 h followed by adding 50 mL MTT (2.5 mg $mL^{-1}$). After an incubation time of 2 h, the medium was removed and 200 mL of DMSO were added. The formazan crystals were dissolved and the absorption was measured at 550 nm, using a reference wavelength of 620 nm. Each test was repeated in quadruplicates in three independent experiments for each cell line.

## Conflicts of interest

The authors declare no conflict of interest.

## Author Contributions (CRediT)

D.S.: Methodology, Investigation, Visualization, Writing – Original Draft. N.M.N.: Conceptualization, Visualization, Writing – Review & Editing, Supervision, Project Administration, Funding Acquisition.

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## FIGURES AND SCHEMES

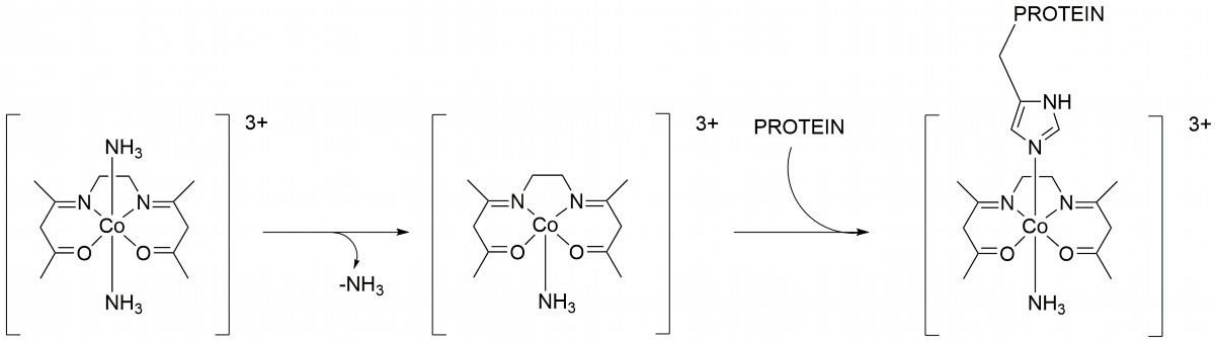



**Figure 1.** Cobalt binding to histidine residues through dissociative axial ligand exchange.

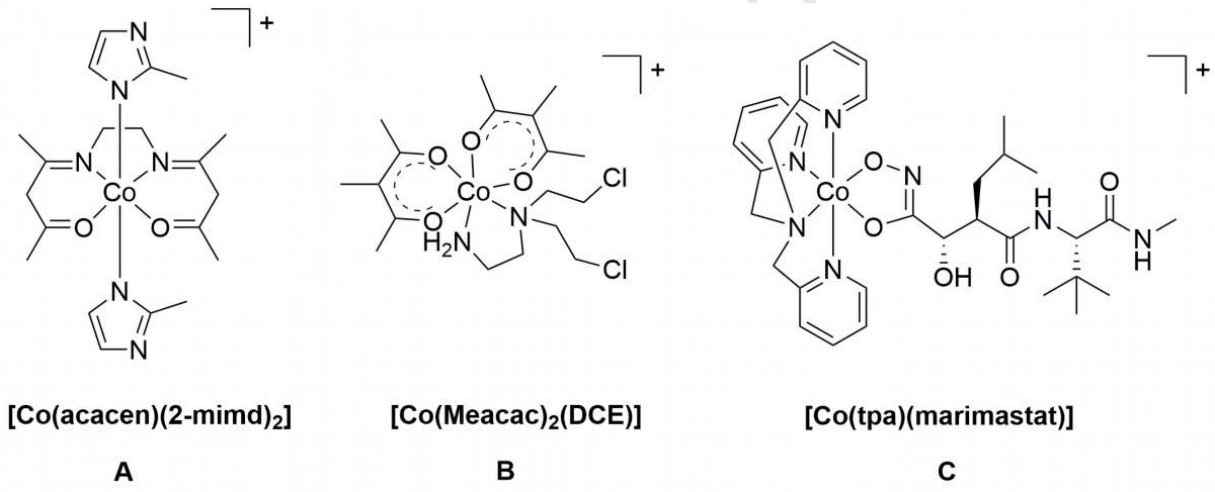
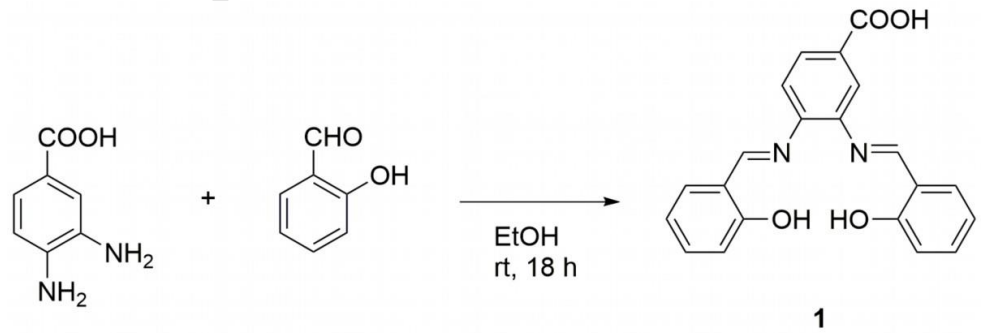
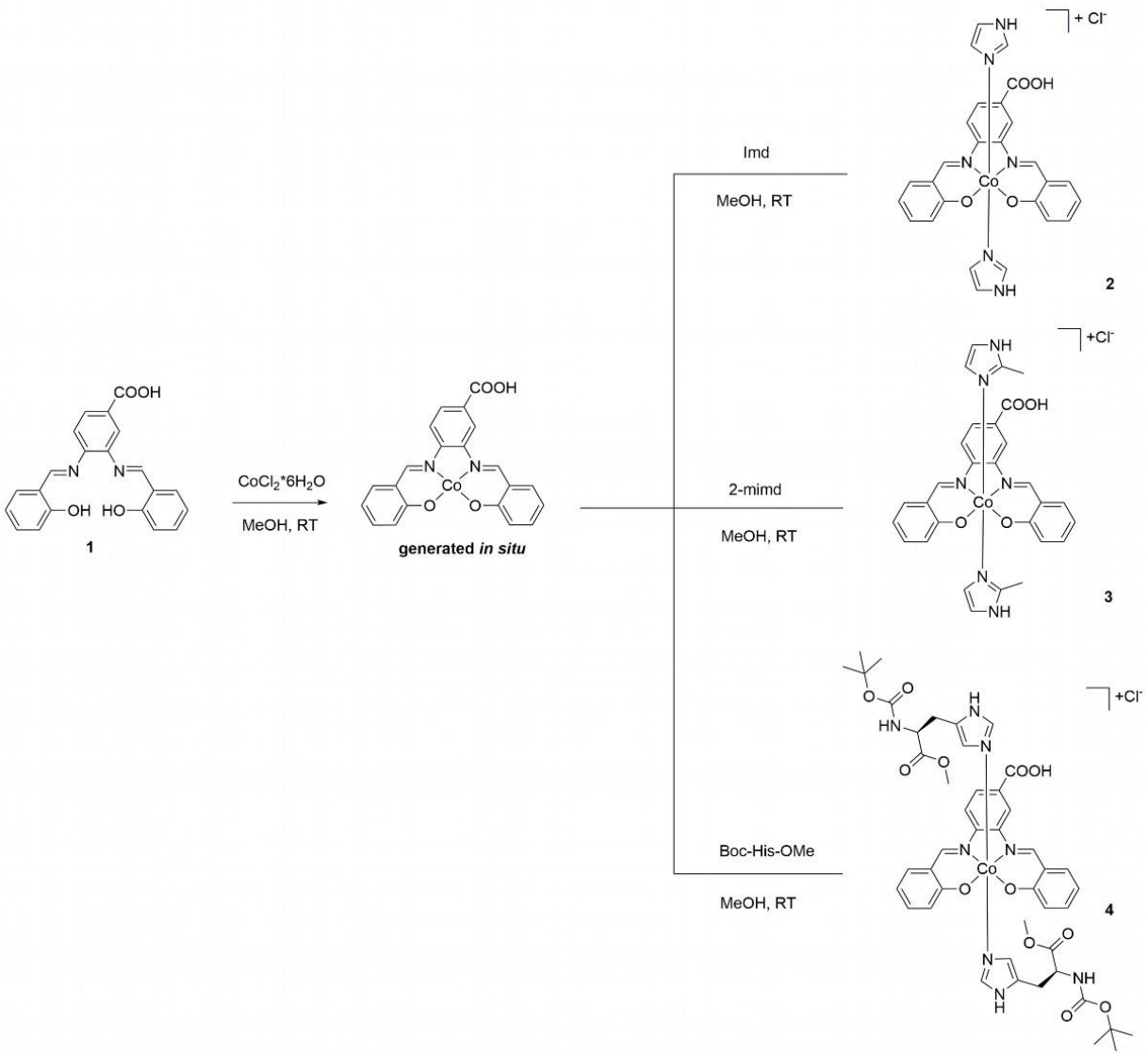



**Figure 2.** Examples of cobalt(III) inorganic drug candidates.




**Scheme 1.** Synthesis of Schiff base ligand**1**.




**Scheme 2.** Synthesis of cobalt(III) complexes (Imd: imidazole; 2-mimd: 2-methyl-imidazole. RT: room temp.).

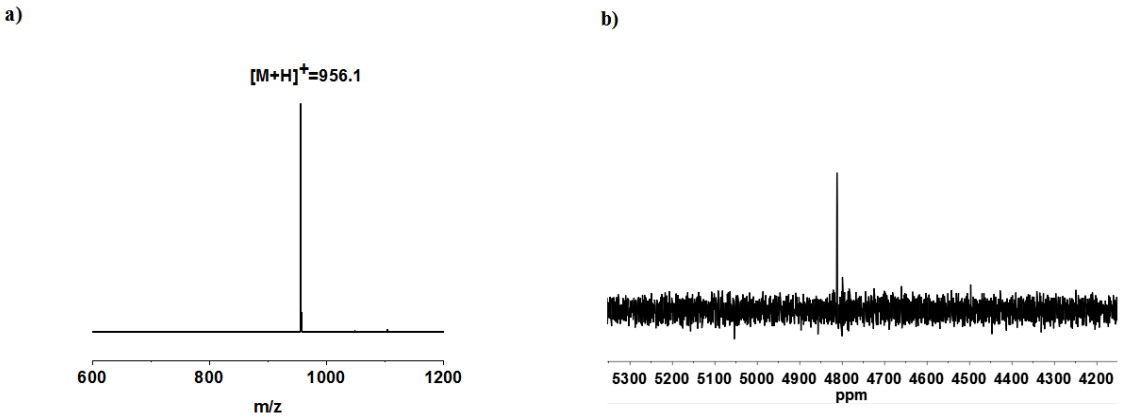
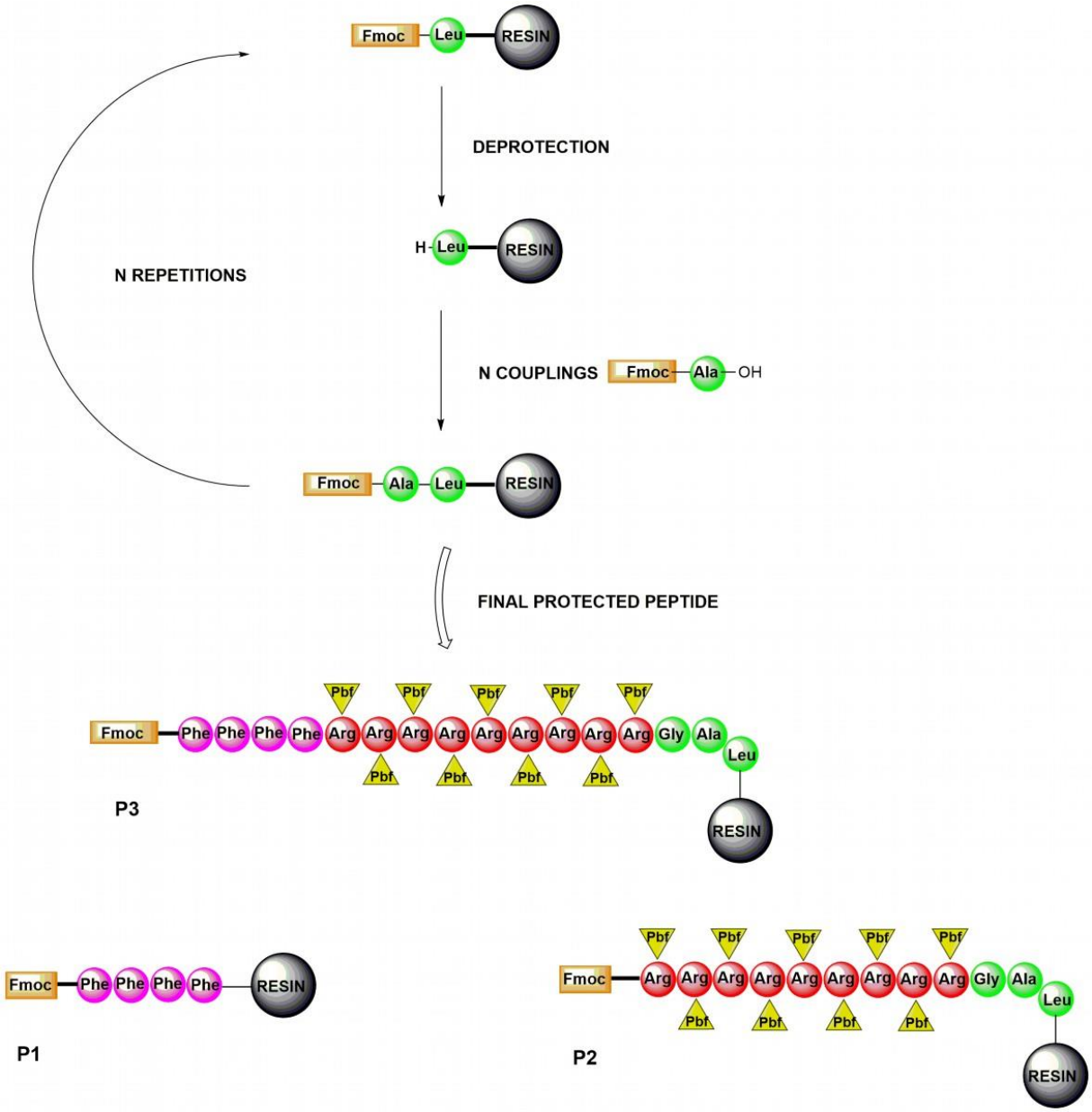
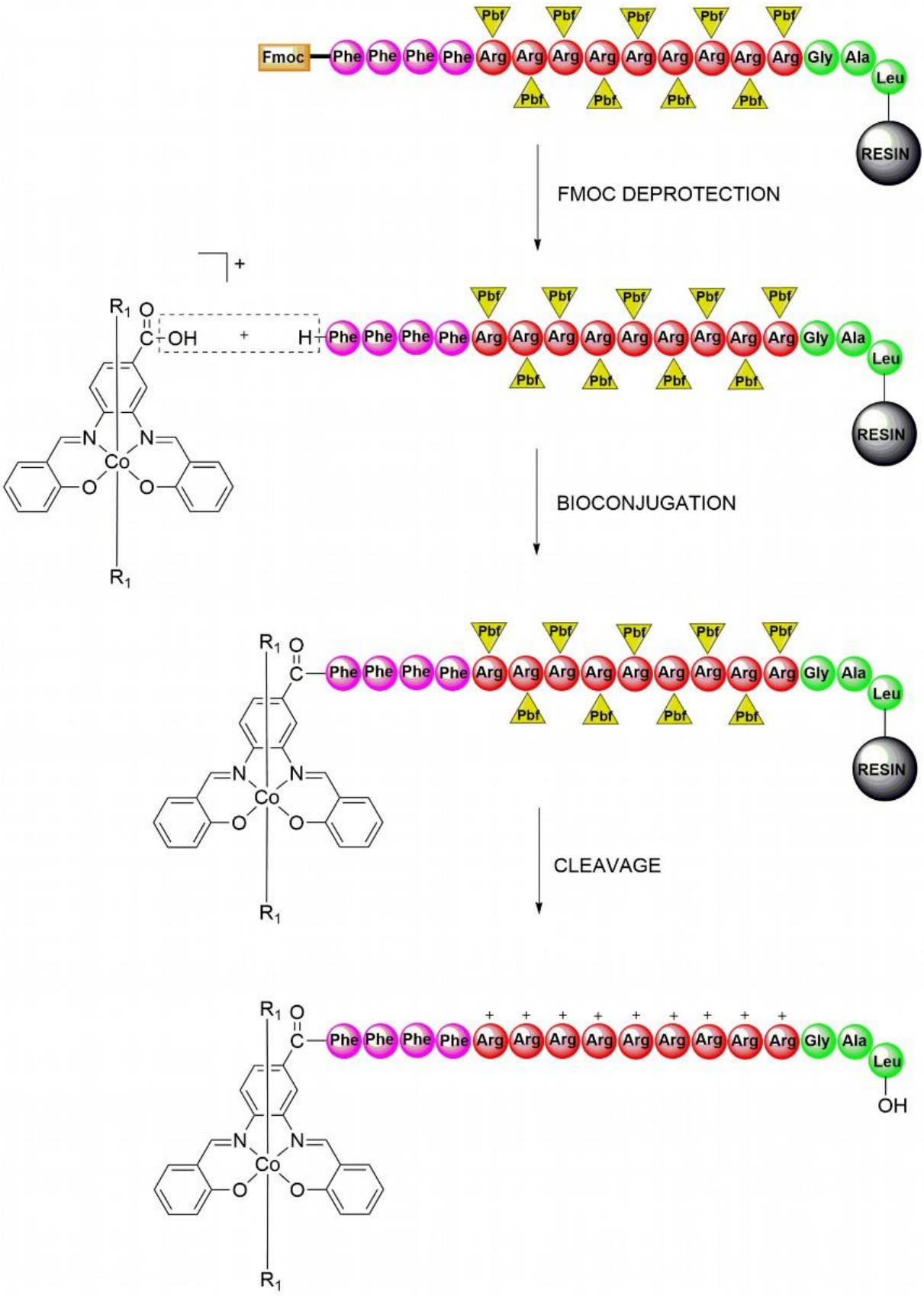



**Figure 3.** a) ESI-MS spectrum of complex **4**; b) $^{59}$Co NMR spectra of complex **4** in dmso-$d_6$.




**Scheme 3.** Synthesis of peptides **P1**, **P2** and **P3** by SPPS.




**Scheme 4**. Synthesis of Co(III)-peptide bioconjugates.

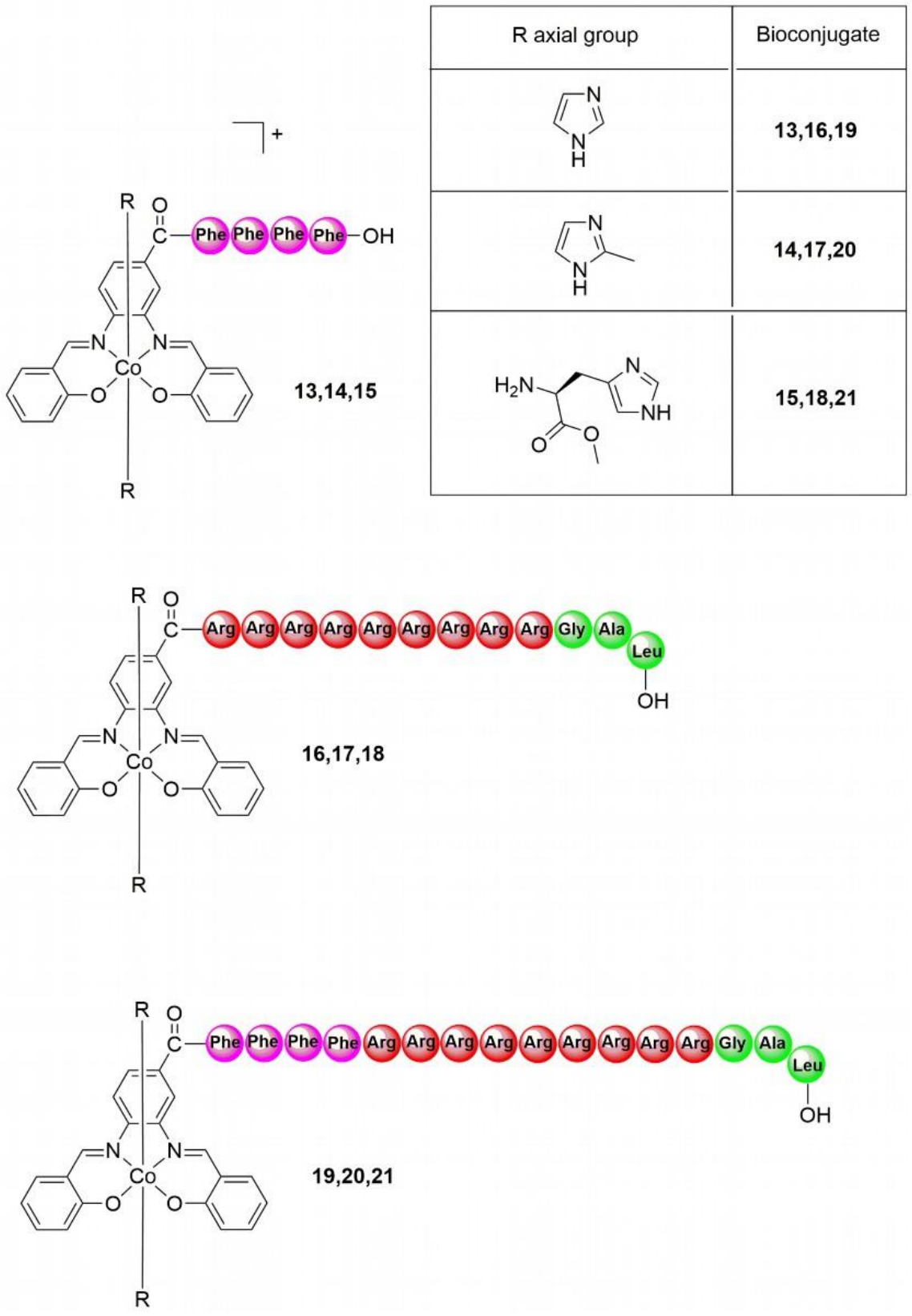



**Figure 4.** Structures of Co(III)-peptide bioconjugates.

## TABLES

**Table 1.** Principal IR bands, assignments, and $^{59}$Co NMR chemical shifts.

| Compound | IR assignment | | | | | $^{59}$Co NMR | |
|---|---|---|---|---|---|---|---|
| | $\nu$(O-H) | $\nu$(N-H) | $\nu$(C=N) | $\nu_{as}(COO^-)$ | $\nu_s(COO^-)$ | δ (ppm) | $\nu_{1/2}$ (Hz) |
| **1** | 3344 | - | 1670 | 1633 | 1340 | - | - |
| **2** | 3230 | 2988 | 1680 | 1537 | 1324 | 4550 | 5200 |
| **3** | 3223 | 2999 | 1688 | 1566 | 1354 | 4670 | 2900 |
| **4** | 3220 | 2987 | 1695 | 1588 | 1365 | 4812 | 2400 |

**Table 2.** Characterization of peptides and bioconjugates after purification by semi-preparative HPLC.

| Compound | Yield [%] | $m/z_{exptl}$ | $m/z_{calcd}[M+H]^+$ | $t_R$ [min] |
|---|---|---|---|---|
| **P1** | 44 | 606.40 | 606.28 | 6.12 |
| **P2** | 38 | 1664.03 | 1664.06 | 9.30 |
| **P3** | 41 | 2252.87 | 2252.34 | 9.80 |
| **13** | 12 | 1140.02 | 1139.09 | 7.19 |
| **14** | 25 | 1168.08 | 1167.12 | 10.30 |
| **15** | 23 | 1342.55 | 1341.46 | 11.50 |
| **16** | 18 | 2198.42 | 2197.09 | 7.30 |
| **17** | 32 | 2226.00 | 2225.12 | 10.72 |
| **18** | 17 | 2400.09 | 2399.24 | 12.10 |
| **19** | 36 | 2785.40 | 2785.43 | 8.20 |
| **20** | 33 | 2813.80 | 2813.46 | 12.60 |
| **21** | 22 | 2969.90 | 2969.52 | 13.25 |

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**Table 3.** Cytotoxicity data of cisplatin, Co complexes, metal-free peptides and Co-peptide conjugates determined by the MTT assays after 48 h of incubation in our lab, all under identical conditions.

| $IC_{50}$ [μM][a] | Cell lines | | |
|---|---|---|---|
| | A549 | HepG2 | GM5657T |
| **Cisplatin** | 25.3±2.3 | 13.3±1.4 | 9.0±2.2 |
| **2** | 43.8±1.6 | 28.7±0.9 | 22.0±0.9 |
| **3** | 65.3±0.9 | 43.0±1.5 | 46.5±1.3 |
| **4** | 32.0±2.2 | 13.2±0.8 | 51.2±1.1 |
| **P1** | >250 | >250 | >250 |
| **P2** | >250 | >250 | >250 |
| **P3** | >250 | >250 | >250 |
| **15** | 64.3±2.8 | 49.3±1.9 | 72.4±3.3 |
| **18** | 35.8±1.1 | 20.0±0.9 | 61.0±3.1 |
| **21** | 25.3±1.9 | 9.0±1.2 | 36.4±1.6 |

[a] The $IC_{50}$ values are averages of three independent determinations. All compounds were dissolved in DMSO for stock solutions (final concentration 0.5 %).

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# Graphical Abstract

# Bioconjugates of Co(III) Complexes with Schiff Base Ligands and Cell Penetrating Peptides: Solid Phase Synthesis, Characterization and Antiproliferative Activity

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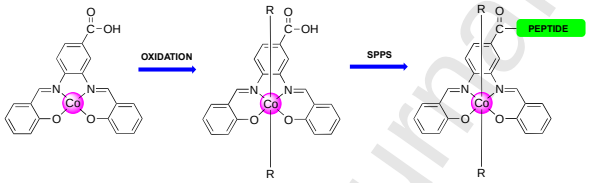
**GA Text:** Bioconjugates of octahedral Co(III) complexes with various cell targeting peptides were prepared by solid phase peptide synthesis (SPPS), and a first structure-activity relationship with regard to the peptide sequence was derived from cytotoxicity assays.

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# Highlights

## Bioconjugates of Co(III) Complexes with Schiff Base Ligands and Cell Penetrating Peptides: Solid Phase Synthesis, Characterization and Antiproliferative Activity

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- Selective oxidation of Co(II) to Co(III) complexes with biocompatible ligands
- Synthesis of octahedral Co(III) complexes with conjugated cell targeting peptides
- Co-peptide conjugates prepared by solid phase peptide synthesis (SPPS)
- Comprehensive characterization and stability studies of Co-peptide conjugates
- Structure-activity relationship with regard to peptide sequence from cytotoxicity assay

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