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# Organoruthenium and -osmium Complexes of 2-Pyridinecarbothioamides Functionalized with a Sulfonamide motif: Synthesis, Cytotoxicity and Biomolecule Interaction

Jahanzaib Arshad,[a,b] Muhammad Hanif,[a,*] Ayesha Zafar,[a] Sanam Movassaghi,[a] Kelvin K. H. Tong,[a] Jóhannes Reynisson,[a] Mario Kubanik,[a] Amir Waseem,[b] Tilo Söhnel,[a] Stephen M. F. Jamieson,[c] Christian G. Hartinger[a,*]

[a] School of Chemical Sciences, University of Auckland, Private Bag 92019, Auckland 1142, New Zealand.

[b] Department of Chemistry, Quaid-i-Azam University, Islamabad 45320, Pakistan.

[c] Auckland Cancer Society Research Centre, University of Auckland, Private Bag 92019, Auckland 1142, New Zealand

* School of Chemical Sciences, University of Auckland, Private Bag 92019, Auckland 1142, New Zealand. http://hartinger.auckland.ac.nz/

E-mail: c.hartinger@auckland.ac.nz; m.hanif@auckland.ac.nz; Fax: (+64) 9 373 7599 ext 87422

## Abstract

Anticancer active $Ru^{II}(\eta^6$-*p*-cymene) complexes of bioactive 2-pyridinecarbothioamide ligands (PCAs) were shown to have high selectivity for plectin and can be administered orally (Chem. Sci., **2013**, *4*, 1837–1846 and Angew. Chem. Int. Ed., **2017**, *56*, 8267 – 8271). Herein, we report the functionalization of the PCA ligand with a sulfonamide group and its conversion into $M(\eta^6$-*p*-cymene) complexes (M = Ru, Os). The presence of the sulfonamide motif in many organic drugs and metal complexes endowed these agents with interesting biological properties and may result in the latter case in multitargeted agents. The compounds were characterized with standard methods and the *in vitro* anticancer activity data was compared with studies on the hydrolytic stability of the complexes and their reactivity to small biomolecules. A molecular modelling study against carbonic anhydrase II revealed plausible binding modes of the complexes in the catalytic pocket.



## Keywords

Anticancer Activity; Sulfonamide; Organoruthenium Compounds; Bioorganometallics; 2-Pyridinecarbothiamide Ligands.

## Introduction

Sulfonamides constitute an important class of pharmacologically active agents. Drugs featuring this pharmacophore have been used for the treatment of a variety of conditions, from infectious diseases to antiepileptic or antiobesity drugs.[1-4] The sulfonamide group is known to form adducts with $Zn^{2+}$ ions present in active sites of metalloenzymes, particular in those that are overexpressed in diseased conditions. For example, carbonic anhydrases (CAs)[5] and histone deacetylases (HDACs) are Zn-containing metalloenzymes overexpressed in many tumors. These enzymes are considered important targets in anticancer drug discovery.[6] Sulfonamides have been extensively investigated to inhibit the activity of these enzymes, in particular CAs. Under the basic conditions used in the enzyme inhibition assay, the deprotonated nitrogen atom of the sulfamoyl moiety of these compounds binds to the Zn ion in the active site of the enzyme and disrupts its catalytic process.[5-7] The remaining components of the drugs' structures are involved in various hydrophilic and/or hydrophobic interactions with amino acid residues of the active site and/or water molecules. This was demonstrated by X-ray crystallographic analysis of the adduct formation between various CAs and many representatives of sulfonamide-based inhibitors.[5,8-13] The sulfonamide acetazolamide and its derivatives were evaluated both in *in vitro* and *in vivo* assays as human carbonic anhydrase IX (*h*-CA IX) targeted anticancer agents,[14] whereas several examples including indisulam (E7070) and SLC-0111 entered clinical trials for the treatment of various advanced solid tumors.[15,16]

Sulfonamides are versatile chelating ligands and, depending on their structure, they can act as mono-, bi- or tridentate donor systems to transition metals ions.[1,6,17,18] The coordination complexes of different clinically-used sulfonamides with $Ag^{I}$, $Co^{II}$, $Ni^{II}$, $Cu^{II}$ and $Zn^{II}$ have been evaluated for their biological properties.[19-21] In many examples, the enzyme inhibitory activity of these metal complexes was better than of their ligands alone, possibly due to synergistic effects between the metal ion and the sulfonamide by interacting with different areas of the active site of the enzyme.[6,17] Similarly, Re and $^{99m}$Tc complexes of sulfonamides were developed for molecular imaging of *h*-CA IX-expressing tumors (Figure 1).[22,23] Some of these compounds demonstrated nanomolar affinities for the pharmaceutically-relevant isozymes *h*-CA IX and *h*-CA XII, which was much higher than that of acetazolamide, a benchmark organic inhibitor for

CAs. A co-crystal structure of a Re complex with *h*-CA II showed that the deprotonated nitrogen of the sulfonamide group bound to the catalytically-active Zn center and the [CpRe(CO)$_3$] moiety showed hydrophobic interactions with Phe131, Leu198, and Pro202.[24] Biological activity and structure-activity relationships (SAR) for metallocenes functionalized with the sulfonamide pharmacophore through triazole, triazole-ester, triazole-amide, amide and urea linkers were reported. These compounds showed moderate to good inhibitory activity *in vitro* and some examples demonstrated high selectivity for cancer-associated CA IX and CA XII compared to off-target CA I and II.[25]

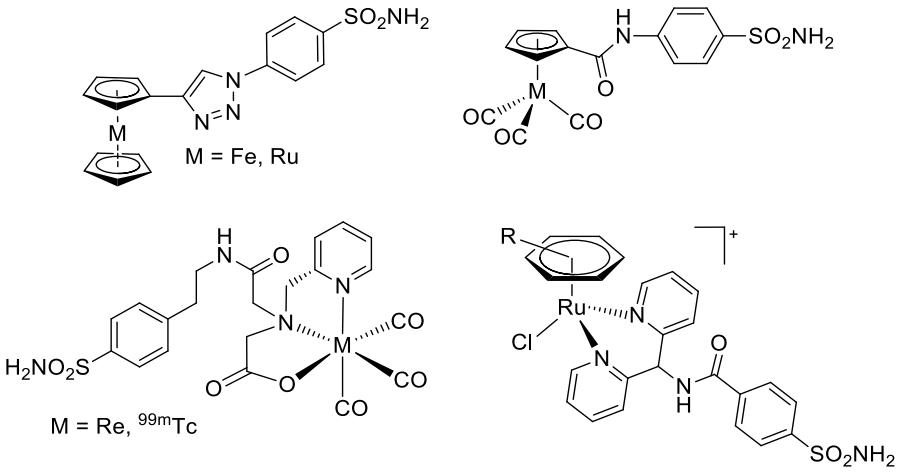


**Figure 1.** The structures of lead bioactive metal complexes bearing a sulfonamide pharmacophore.

Ruthenium half-sandwich complexes of the general structure [($\eta^6$-arene)Ru(bipy)Cl]$^+$ displayed very high affinity towards *h*-CA II.[26] The co-crystal structure of *h*-CA II with the Ru(arene) complex revealed that the complex bound to the catalytic zinc site through the sulfonamide moiety. The aryl spacer formed close contacts with the hydrophobic residues of the enzymes and the Ru(arene) scaffold was positioned at the entrance of the cavity. Interestingly, there was no direct interaction between the ruthenium center and the protein, despite the presence of a labile chlorido ligand.[26]

We have recently developed organometallic anticancer complexes of 2-pyridinecarbothioamide ligands (PCAs).[27-31] The Ru complex termed plecstatin-1 demonstrated target selectivity for plectin in an invasive B16 melanoma tumor model.[30] Herein, we report the functionalization of the PCA scaffold with the sulfonamide pharmacophore and its coordination to Ru$^{II}$/Os$^{II}$(cym) (cym = $\eta^6$-*p*-cymene)

organometallics. The compounds were evaluated for their tumor-inhibition potential against a panel of human cancer cell lines and their stability in solution as well as their reactivity toward small biomolecules. Their interaction with CA was studied by molecular modelling.

## Results and Discussion

Bioactive PCAs can act as *S,N*-bidentate ligands to metal ions to access a library of organometallic and coordination compounds.[27,32,33] We functionalized a PCA ligand with a sulfonamide, a motif found in many drugs and involved in interactions with the active sites of CAs. The sulfonamide-substituted PCA **1** was prepared in a one-pot synthesis by refluxing *p*-phenylenediamine sulfanilamide and elemental sulfur in 2-picoline for 18 h with a catalytic amount of sodium sulfide (Scheme 1). After work up and recrystallization from acetonitrile, **1** was obtained in a good yield of 67%. The ligand was characterized by NMR spectroscopy, ESI-MS, elemental analysis and single crystal X-ray diffraction. In the $^{1}$H NMR spectrum of **1**, the thioamide proton was detected at 12.48 ppm. This accounts for a downfield shift of *ca.* 2 ppm as compared to the amide proton of picolinamide ligands.[34] The protons of the pyridine ring were observed in the range of 7.6–8.7 ppm, while the signals assigned to the aromatic phenyl protons were detected in the range of 7.8–8.2 ppm. In the $^{13}$C{H} NMR spectrum the pyridine ring carbon atoms were detected in the range of 124–153 ppm while the carbons of the aromatic ring resonated between 124.3 and 141.5 ppm. The ESI-mass spectrum of the ligand featured the pseudomolecular ion [**1** + Na]$^{+}$ at *m/z* 316.0157 which is in close agreement with the calculated value.

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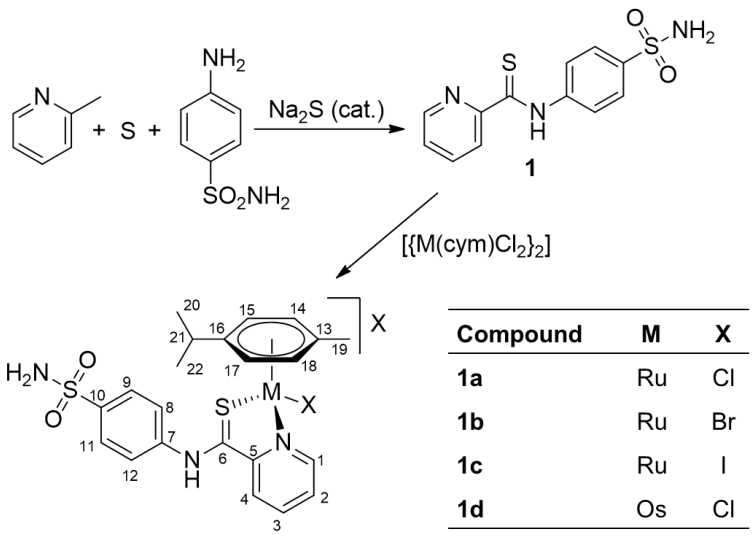


| Compound | M | X |
|---|---|---|
| **1a** | Ru | Cl |
| **1b** | Ru | Br |
| **1c** | Ru | I |
| **1d** | Os | Cl |

**Scheme 1.** Synthetic route to *N*-(4-sulfamoylphenyl)pyridine-2-carbothioamide **1** and its organometallic $Ru^{II}$ and $Os^{II}$ complexes **1a**–**1d** with the numbering scheme used to assign the signals in the NMR spectra.

The molecular structure of *N*-(4-sulfamoylphenyl)pyridine-2-carbothioamide **1** was determined by single crystal X-ray diffraction analysis (Figure 2). Crystals were grown by slow evaporation from a methanol-dichloromethane mixture at room temperature. PCA **1** crystallized in the monoclinic space group *Cc* (compare Table 3 for the crystallographic parameters). The hydrogen and oxygen atoms of the sulfonamide group were involved in intermolecular H bonds with other molecules of **1**. The pyridine and benzene rings were found to be disordered indicating a strong displacement along the S2-C10-C7-N2 and C6-C5-C2 axes in the molecule.

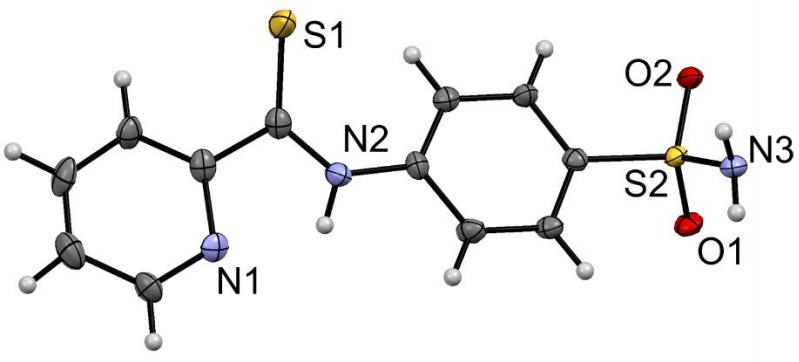


**Figure 2.** Molecular structure of *N*-(4-sulfamoylphenyl)pyridine-2-carbothioamide **1** drawn at 50% probability level.

Compound **1** was converted into the corresponding $Ru^{II}$(cym) and $Os^{II}$(cym) complexes **1a**–**1d** in good yields (53–88%). The reactions were performed under nitrogen atmosphere by reacting **1** (2 eq.) with $[Ru/Os(cym)X_2]_2$ (1 eq.) in a mixture of tetrahydrofuran and dichloromethane at 40 °C for 4 h (Scheme 1). The red to dark red/black products were obtained after filtration[27] and were characterized by 1D and 2D NMR spectroscopy, ESI-MS and elemental analysis. The $^1$H NMR spectra of all complexes were recorded in *d*$_4$-MeOD (Figures S1–S4). Due to the fast H/D exchange in protic deuterated solvents, the thioamide proton was not detected while the spectra recorded for **1a** and **1d** in DMSO-*d*$_6$ featured peaks at around 7.3 ppm absent in the former (Figure S5). The H4 and H1 protons of the pyridine ring were deshielded due to coordination of the pyridine nitrogen atom causing a shift by *ca.* 1 ppm. The nature of the metal ion had only a slight effect on the $^1$H and $^{13}C\{^1H\}$ NMR chemical shifts of the PCA ligand. The $^{13}C\{^1H\}$ spectra (Figures S6–S9) contained most of the expected peaks but some of the quaternary carbon atoms were not detected, presumably because of too low concentration of the samples. Importantly, the spectra showed significant differences for the aromatic *p*-cymene C–H atoms for the Ru complex **1a** as compared to its Os counterpart **1b**. These carbon atoms resonated about 10 ppm downfield in case of **1a** as compared **1b**. Similar shifts have been observed for related compounds while in other cases the shifts were less pronounced.[35-37]

The molecular structure of a single crystal formed from slow diffusion of diethyl ether into methanol solution of **1d** was determined by single crystal X-ray diffraction analysis (Figure 3; compare Table 3 for the crystallographic parameters). The Os center adopted a pseudooctahedral coordination geometry and **1** coordinated to the metal ion as an anionic *N,S*-bidentate ligand after deprotonation of the amide group. Therefore, we label this compound as **1d$^{neutral}$**. This is in contrast to all other molecular structures of related Ru and Os complexes where the PCA ligand was neutral and a complex cation was formed.[27-29] The Os–cym$_{centroid}$ and Os–Cl distances were 1.671 Å and 2.442(4) Å and therefore similar to those reported for related complexes.[27-29] The Os–S1 and Os–N1 bond lengths were 2.355(4) and 2.133(1) Å. The C6–S1 bond (1.754(15) Å in **1d$^{neutral}$**) was elongated as compared to 1.655(5) Å for **1**, indicating a higher single bond character. The C6–N2 distance of 1.251(19) Å in **1d$^{neutral}$** was slightly shorter compared to a bond length of 1.345(6) Å in **1**, demonstrating increased double bond character upon coordination of the Os center to

the S atom and deprotonation of the amide group. The latter bond is hardly modified when PCA coordinates as a neutral ligand to a metal center.[31]

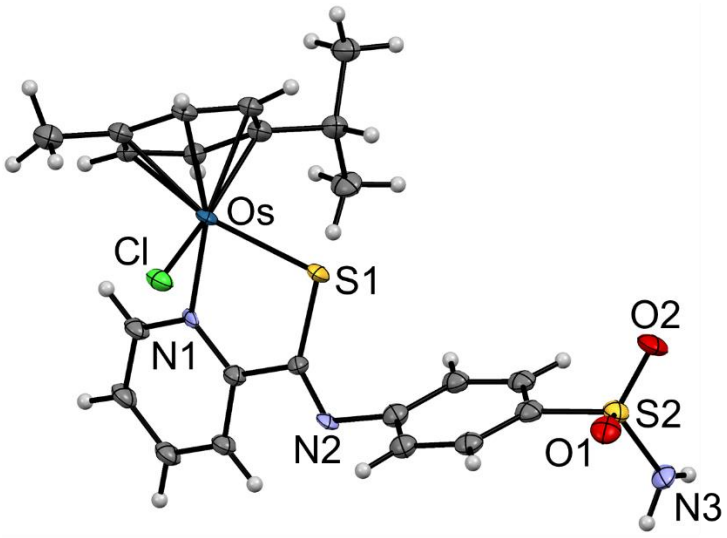


**Figure 3.** Molecular structure of **1d**$^{neutral}$ drawn at 50% probability level.

To confirm the ionic nature of the complexes, conductivity measurements were performed for **1** and its complexes **1a–d** in acetonitrile. All the complexes showed higher conductivity than the neutral ligand (Table S1), indicating their ionic nature. However, it should be noted that the conversion of the cationic form into the neutral form may be accompanied by the release of HCl.

The formation of the complexes was also confirmed by ESI-MS. In light of the molecular structure of **1d**$^{neutral}$, which features the PCA ligand in its deprotonated form coordinated to Os, it is interesting to note that the mass spectrum of **1d** recorded in positive ion mode featured a peak at an *m/z* value assigned to $[M - Cl]^+$ ions but the most abundant peak was from a $[M– 2Cl – H]^+$ species, which was the only peak found for the Ru complexes. The elemental analysis data of the complexes were in close agreement with the theoretical values for the protonated complexes with a chlorido counterion.

**Stability in aqueous solution and reactivity toward amino acids**

The aqueous stability of complexes **1a**–**1d** was determined by NMR spectroscopy and ESI-MS. The compounds were dissolved in $D_2O$ and $^1H$ NMR spectra were recorded

after 0.25, 1, 3, 24, 48, 72, 96 and 120 h. The compounds underwent chlorido/aqua ligand exchange reactions within 15 min of incubation in $D_2O$. There was no change in the spectrum over a period of 120 h, indicating the high stability of the formed aqua species.

Depending on the nature of metal ion and co-ligands, metal complexes are prone to undergo ligand exchange when encountered with biomolecules such as proteins. In order to understand the nature of such interactions, reactions of **1a** and **1d** with the amino acids L-cysteine (Cys), L-methionine (Met), and L-histidine (His) were monitored by $^1$H NMR spectroscopy in $D_2O$. Despite that both **1a** and **1d**, undergo immediate hydrolysis, they did not react with amino acids within 24 h of incubation at 1 : 1 and 1 : 2 (complex : amino acid) molar ratio (Figure 4 for His), after which another equivalent of amino acid was added and the reaction was followed for another 96 h. The $^1$H NMR spectra however remained largely unchanged with only a minor amount of another species (< 5%) forming, possibly due to adduct formation with the amino acids. This low reactivity was further confirmed by ESI-MS, where no adduct formation was observed with amino acids. The relative high stability of the aqua species of these complexes is unique compared to that of analogous Ru PCA complexes.

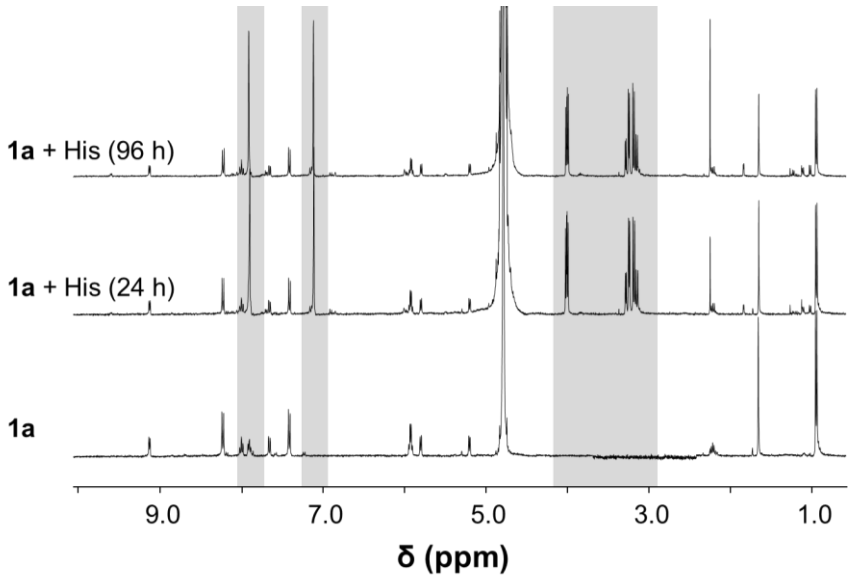


**Figure 4**. $^1$H NMR spectroscopic study of the reaction between **1a** and His in $D_2O$, monitored for 72 h. The peaks of His are highlighted in grey boxes.

### *In vitro* anticancer activity

The antiproliferative activity of ligand **1** and its respective complexes **1a**–**1d** was determined in human HCT116 colorectal, H460 non-small cell lung, SiHa cervical, and SW480 colon carcinoma cells (Table 1). The sulfonamide-substituted PCA ligand **1** was moderately active only in the HCT116 cancer cell line with an $IC_{50}$ value of 105 µM. The Ru(cym) and Os(cym) complexes were inactive in all tested cancer cell lines. This is surprising given the fact that plecstatin-1 and other related derivatives were highly cytotoxic (Table 1).[27,29,30] The low potency may be related to the comparatively low lipophilicity of ligand **1** (clog*P* = -0.148) as compared to *N*-(4-fluorophenyl)pyridine-2-carbothioamide in F-SN (clog*P* = 1.832),[29] possibly interfering with efficient accumulation in cancer cells. Another explanation may be that the sulfonamide substituent hinders the interaction of the complex with plectin, which was identified as the target for plecstatin-1.[30]

**Table 1.** *In vitro* anticancer activity ($IC_{50}$ values) of ligands **1,** its respective Ru/Os(cym) complexes **1a, 1b, 1c** and **1d**, and related compounds F-SN and plecstatin-1 in human colorectal (HCT116), non-small cell lung (NCI-H460) cervical (SiHa) and colon carcinoma (SW480) cells (exposure time 72 h). The clog*P* values for the PCAs **1** and F-SN are also given.

| **Compound** | **$IC_{50}$ value (µM)** | | | | **clog*P*** |
|---|---|---|---|---|---|
| | **HCT116** | **NCI-H460** | **SiHA** | **SW480** | |
| **1** | 105 ± 3 | >300 | >300 | >300 | - 0.148 |
| **1a** | >211 | >300 | >300 | >300 | - |
| **1b** | >300 | >300 | >300 | >300 | - |
| **1c** | >300 | >300 | >300 | >300 | - |
| **1d** | >300 | >300 | >300 | >300 | - |
| F-SN [29] | 5.7 ± 0.7 | 7.8 ± 1.8 | 16 ± 6 | 33 ± 2 | 1.832 |
| plecstatin-1 [29] | 6.5 ± 0.3 | 10 ± 2 | 8.3 ± 0.7 | 9.9 ± 0.7 | - |

### Molecular Modelling

As crystal structure of *h*-CA II with a co-crystallized Ru complex (SRX) featuring a sulfonamide functional group has been reported (PDB ID: 3PYK),[38] we modelled ligand **1** and both possible enantiomers of its chiral Ru and Os complexes **1a** (**1a$^{E1}$** and **1a$^{E2}$**) and **1d** (**1d$^{E1}$** and **1d$^{E2}$**), respectively, into the catalytic pocket using a

molecular dynamics approach. The results were compared to that of a co-crystallized Ru complex (SRX) with a sulfonamide functional group. All the compounds were found to interact through H bonds with Thr residues in close proximity to the Zn ion in the active site, to which the sulfonamide moieties bound (Table 2). In addition, they formed lipophilic interactions with Val121, Leu60, and Leu198, as did SRX (in addition to Pro202). The ligand and its complexes practically adopted the same conformation, independent of the chirality at the metal center. The predicted pose of **1a**$^{E2}$ is shown in Figure 5a with its hydrogen bonds with Thr199 and Thr200 *via* the oxygen atom of the sulfonamide group. Complex **1a**$^{E2}$ is residing deep in the catalytic site of the enzyme showing an excellent fit (Figure 5b), as did all the other complexes, and blocks access to the Zn ion coordinated to His94, His96, and His119. This demonstrates that the enzyme is a viable target, which however would have to be verified experimentally.

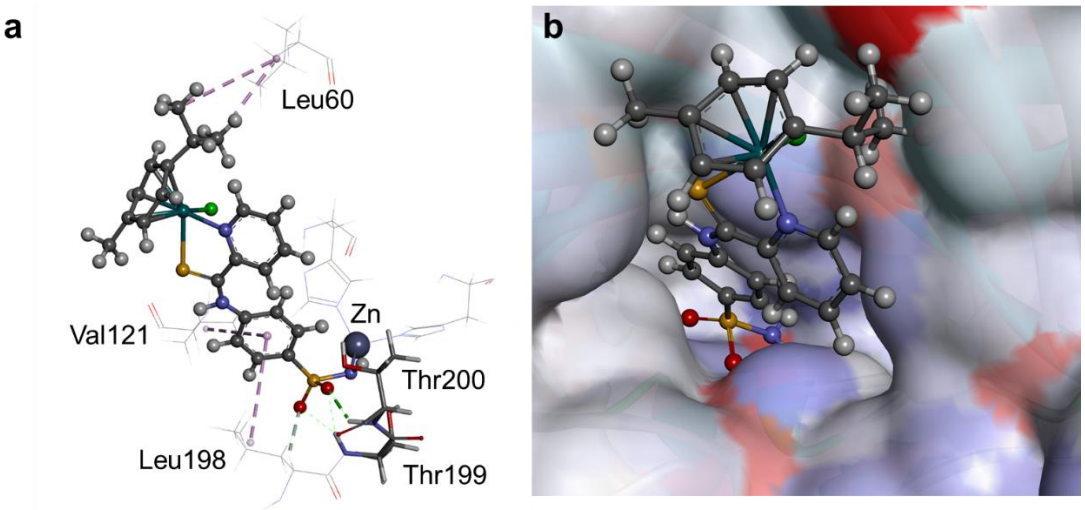


**Figure 5.** The modelled configuration of **1a**$^{E2}$ in the catalytic site of carbonic anhydrase II (PDB ID 3PYK). a) Hydrogen bonds are depicted as green dotted lines between the metal complex and the amino acids Thr199, and Thr200. Lipophilic interactions are represented as purple dotted lines with Val121, Leu60 and Leu198. b) The enantiomer **1a**$^{E2}$ is shown in the binding pocket with the protein surface rendered. Red depicts a negative partial charge on the surface, blue depicts a positive partial charge and grey shows neutral/lipophilic areas.

**Table 2.** The H bonds and lipophilic interactions of the modelled compounds with amino acid residues of carbonic anhydrase II.

| Compound | H bonds | Lipophilic interactions |
|---|---|---|
| **SRX** | Thr199 | Val121, Leu198, Pro202 |
| **1** | Thr200 | Val121, Leu198 |
| **1a$^{E1}$** | Thr199, Thr200 | Val121, Leu198 |
| **1a$^{E2}$** | Thr199, Thr200 | Val121, Leu198, Leu60 |
| **1d$^{E1}$** | Thr199, Thr200 | Val121, Leu198 |
| **1d$^{E2}$** | Thr200 | Val121, Leu198, Leu60 |

## Conclusions

We describe in this paper an approach where we borrowed the PCA pharmacophore for functionalization with a sulfonamide and the preparation of its half sandwich complexes to target the enzyme carbonic anhydrase. The Ru(cym) and Os(cym) complexes were synthesized and thoroughly characterized. Interestingly, the molecular structure of **1d** suggests deprotonation of the carbothioamide moiety, while similar structures crystallized in the protonated form, as did ligand **1**. We evaluated the compounds for their stability in aqueous solution and reactivity with biomolecules. The compounds undergo a quick chlorido/aqua ligand exchange but are surprisingly unreactive to amino acids. The antiproliferative activity was assayed in a small panel of human cancer cell lines and an $IC_{50}$ value could only be determined for ligand **1** in HCT116 cells. While binding to CA II, as determined by molecular modelling studies, may not result in anticancer activity, this shows that the compounds are still capable of interacting with the Zn ion in the catalytic site of CA II.

## Acknowledgments

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## Experimental

*Materials and methods*

All reactions were carried out under nitrogen atmosphere using standard Schlenk techniques. Chemicals obtained from commercial suppliers were used as received and were of analytical grade. Tetrahydrofuran (THF) and dichloromethane (DCM) were first dried through a solvent purification system (LC Technology Solutions Inc., SP-1 solvent purifier), degassed under a $N_2$ flow, and stored in a Schlenk flask. Methanol was dried using standard procedures and stored over activated molecular sieves (3 Å).

α-Terpinene, 2-picoline, and $Na_2S{\cdot}9H_2O$ were purchased from Merck, 4-aminobenzenesulfonamide, sulfur, and $OsO_4$ from Sigma-Aldrich, L-histidine, L-methionine and L-cysteine from AK Scientific, and $RuCl_3{\cdot}3H_2O$ (99%) from Precious Metals Online.

The dimers bis[dichlorido(η$^6$-*p*-cymene)ruthenium(II)],[39] bis[dibromido(η$^6$-*p*-cymene)ruthenium(II)],[40] bis[diiodido(η$^6$-*p*-cymene)ruthenium(II)],[40] and bis[dichlorido(η$^6$-*p*-cymene)osmium(II)][41,42] were synthesized by adapting reported procedures.

$^1$H and $^{13}$C{$^1$H} and 2D (COSY, HSQC, HMBC) NMR spectra were recorded on a Bruker Avance AVIII 400 MHz NMR spectrometer at ambient temperature at 400.13 MHz ($^1$H) or 100.61 MHz ($^{13}$C{$^1$H}). Chemical shifts are reported versus $SiMe_4$ and were determined by reference to the residual solvent peaks.

High resolution mass spectra were recorded on a Bruker micrOTOF-QII mass spectrometer in positive electrospray ionization (ESI) mode. Elemental analyses were carried out on an Exeter Analytical Inc-CE-440 Elemental Analyser and were performed at the Campbell Microanalytical Laboratory, The University of Otago. X-ray diffraction measurements of single crystals were carried out on a Bruker SMART APEX2 diffractometer with a CCD area detector using graphite monochromated Mo-Kα radiation (λ = 0.71073 Å). The molecular structures were solved and refined with the SHELXL-2016 [43] and Olex2[44,45] program packages. The molecular structures were visualized using Mercury 3.9.

**Table 3.** X-ray diffraction measurement parameters for **1** and **1d**$^{neutral}$.

| | **1** | **1d**$^{neutral}$ |
|---|---|---|
| CCDC | 1829882 | 1829883 |
| Formula | $C_{12}H_{11}O_2N_2S_2$ | $C_{22}H_{24}ClN_3O_2OsS_2$ |
| Molecular weight (g mol$^{-1}$) | 293.36 | 652.21 |
| Crystal size (mm) | 0.32 × 0.10 × 0.08 | 0.26 × 0.10 × 0.08 |
| Wavelength (Å) | 0.71073 | 0.71073 |
| Temperature (K) | 100(2) | 100(2) |
| Crystal system | monoclinic | monoclinic |
| Space group | *Cc* | *P-1* |
| *a* (Å) | 4.8844(6) | 6.9829(7) |
| *b* (Å) | 28.476(3) | 12.2144(10) |
| *c* (Å) | 8.8935(9) | 13.5379(12) |
| *α* (°) | 90 | 79.167(5) |
| *β* (°) | 94.869(7) | 83.956(6) |
| *γ* (°) | 90 | 82.303(6) |
| Volume (Å$^3$) | 1232.5(2) | 1120.06(18) |
| Z | 4 | 2 |
| Calculated Density (mg/mm$^3$) | 1.581 | 1.934 |
| Absorption coefficient (mm$^{-1}$) | 0.433 | 6.024 |
| F(000) | 608 | 636 |
| Theta range (°) | 25.233 | 24.403 |
| Number of Parameters / Reflections (all) | 204 / 2214 | 289 / 3613 |
| Final R indices [I > 2σ(I)] | $R_1$ = 0.0412<br>$wR_2$ = 0.0741 | $R_1$= 0.0844<br>$wR_2$ = 0.1594 |
| R indices (all data) | $R_1$ = 0.0514<br>$wR_2$ = 0.0774 | $R_1$ = 0.1071<br>$wR_2$ = 0.1668 |
| Goodness-of-fit on $F^2$ | 1.050 | 1.116 |

*General procedure for the synthesis of organo-Ru and -Os complexes*

A solution of $[M(cym)Cl_2]_2$ (M = Ru, Os) in dry DCM was added to a stirred solution of **1** in dry THF. The reaction mixture was stirred for 4 h at 40 °C under nitrogen atmosphere. A change in color from brown to deep red was observed immediately after the addition of the dimeric precursor. The solvent was evaporated and the residue was dissolved in a minimal volume of DCM, followed by addition of *n*-hexane that resulted in immediate precipitation. After placing it in the fridge overnight, the precipitate was filtered, and dried under reduced pressure.

*[Chlorido($\eta^6$-p-cymene)(N-(4-sulfamoylphenyl)pyridine-2-carbothioamide)ruthenium(II)] chloride **1a***

The synthesis of **1a** was performed following the general complexation procedure, using *N*-(4-sulfamoylphenyl)pyridine-2-carbothioamide (120 mg, 0.41 mmol) and $[Ru(cym)Cl_2]_2$ (124 mg, 0.20 mmol). After completion of the reaction, the solvent was concentrated in vacuum up to 5 mL and *n*-hexane was added for further precipitation in the fridge. The solid product was filtered, followed by washing with dichloromethane (2 × 10 mL) and drying in vacuum. Yield: 130 mg (53%), red solid. Elemental analysis found: C, 39.84; H, 3.89; N, 5.81, calculated for $C_{22}H_{25}Cl_2N_3O_2RuS_2 \cdot 0.7CH_2Cl_2 \cdot 1.25H_2O$: C, 40.00; H, 4.27; N, 6.17. MS ($ESI^+$): $m/z_{calc}$ 528.0353 [**1a** – 2Cl – H]$^+$ (*m*/z 528.0356). $^1$H NMR (400.13 MHz, $d_4$-MeOD, 25 °C): δ = 9.66 (d, $^3J_{(H1,H2)}$ = 6 Hz, 1H, H-1), 8.43 (d, $^3J_{(H4,H3)}$ = 8 Hz, 1H, H-4), 8.29 (td, $^3J_{(H3,H4)/(H3,H2)}$ = 8 Hz, $^4J_{(H3,H1)}$ = 2 Hz, 1H, H-3), 8.09 (d, $^3J_{(H9,H8)/(H11,H12)}$ = 9 Hz, 2H, H-9/H-11), 7.85 (t, $^3J_{(H2,H3)/(H2,H1)}$ = 8 Hz, 1H, H-2), 7.76 (d, $^3J_{(H8,H9)/(H12,H11)}$= 9 Hz, 2H, H-8/H-12), 6.05 (d, $^3J_{(H15,H14)}$ = 6 Hz, 1H, H-15), 5.94 (d, $^3J_{(H17,H18)}$ = 6 Hz, 1H, H-17), 5.91 (d, $^3J_{(H18,H17)}$ = 6 Hz, 1H, H-18), 5.65 (d, $^3J_{(H14,H15)}$ = 6 Hz, 1H, H-14), 2.74 (sept, $^3J_{(H21,H20)/(H21,H22)}$ = 7 Hz, 1H, H-21), 2.21 (s, 3H, H-19), 1.21 (d, $^3J_{(H20,H21)}$ = 7 Hz, 3H, H-20), 1.13 (d, $^3J_{(H22,H21)}$ = 7 Hz, 3H, H-22) ppm. $^{13}C\{^1H\}$ NMR (100.61 MHz, $CDCl_3$ [0.3 mL] / $d_4$-MeOD [0.1 mL], 25 °C): δ = 159.0 (C-5), 153.4 (C-1), 140.3 (C-10), 139.81 (C-3), 129.3 (C-9/C-11), 127.4 (C-2), 125.3 (C-4), 125.1 (C-8/C-12) 106.3 (C-16), 103.6 (C-13), 88.1 (C-15), 87.8 (C-17), 85.4 (C-18), 84.3 (C-14), 31.4 (C-21), 22.8 (C-20), 21.7 (C-22), 18.3 (C-19) ppm.

*[Bromido($\eta^6$-p-cymene)(N-(4-sulfamoylphenyl)pyridine-2-carbothioamide)ruthenium(II)] bromide* **1b**

The synthesis of **1b** was performed following the general complexation procedure, using *N*-(4-sulfamoylphenyl)pyridine-2-carbothioamide (100 mg, 0.34 mmol) and $[Ru(cym)Br_2]_2$ (125 mg, 0.17 mmol). After completion of the reaction, the solvent was concentrated in vacuum up to 5 mL and *n*-hexane was added for further precipitation in the fridge. The solid product was filtered, followed by washing with dichloromethane (2 × 10 mL) and drying in vacuum. Yield: 145 mg (62%), red solid. Elemental analysis found: C, 39.31; H, 3.71; N, 5.75, calculated for $C_{22}H_{25}Br_2N_3O_2RuS_2 \cdot 0.2C_4H_8O$: C, 38.96; H, 3.81; N, 5.98. MS ($ESI^+$): $m/z_{calc}$ 528.0353 [**1b** – 2Br – H]$^+$ ($m/z$ 528.0340). $^1$H NMR (400.13 MHz, $d_4$-MeOD, 25 °C): δ = 9.66 (d, $^3J_{(H1,H2)}$ = 6 Hz, 1H, H-1), 8.45 (d, $^3J_{(H4,H3)}$ = 8Hz, 1H, H-4), 8.30 (td, $^3J_{(H3,H4)/(H3,H2)}$ = 8 Hz, $^4J_{(H3,H1)}$ = 2 Hz, 1H, H-3), 8.11 (d, $^3J_{(H9,H8)/(H11,H12)}$ = 9 Hz, 2H, H-9/H-11), 7.83 (m, 3H, H-2/H-8/H-12), 6.05 (d, $^3J_{(H15,H14)}$ = 6 Hz, 1H, H-15), 5.94 (d, $^3J_{(H17,H18)}$ = 7 Hz, 1H, H-17), 5.90 (d, $^3J_{(H18,H17)}$ = 6 Hz, 1H, H-18), 5.69 (d, $^3J_{(H14,H15)}$ = 6 Hz, 1H, H-14), 2.81 (sept, $^3J_{(H21,H20)/(H21,H22)}$ = 7 Hz, 1H, H-21), 2.28 (s, 3H, H-19), 1.21 (d, $^3J_{(H20,H21)}$ = 7 Hz, 3H, H-20), 1.15 (d, $^3J_{(H22,H21)}$ = 7 Hz, 3H, H-22) ppm. $^{13}C\{^1H\}$ NMR (100.61 MHz, $CDCl_3$ [0.3 mL] / $d_4$-MeOD [0.1 mL], 25 °C): δ = 158.8 (C-1), 153.3 (C-7), 142.9 (C-10), 140.0 (C-3), 129.5 (C-9/C-11), 127.5 (C-2), 125.9 (C-4), 125.7 (C-8/C-12) 107.6 (C-16), 103.4 (C-13), 87.8 (C-15), 87.4 (C-17/C-18), 85.1 (C-14), 31.3 (C-21), 22.5 (C-20), 21.6 (C-22), 18.9 (C-19) ppm.

[Iodido($\eta^6$-p-cymene)(*N*-(4-sulfamoylphenyl)pyridine-2-carbothioamide)ruthenium(II)] iodide **1c**

The synthesis of **1c** was performed following the general complexation procedure, using *N*-(4-sulfamoylphenyl)pyridine-2-carbothioamide (80 mg, 0.27 mmol) and $[Ru(cym)I_2]_2$ (133 mg, 0.14 mmol). After completion of the reaction, the solid product was filtered, followed by washing with dichloromethane (2 × 10 mL) and tetrahydrofuran (1 × mL) and drying in vacuum. Yield: 187 mg (88%), Red solid. Elemental analysis found: C, 35.99; H, 3.72; N, 4.72, calculated for $C_{22}H_{25}I_2N_3O_2RuS_2 \cdot 0.75\ C_4H_8O$: C, 35.89; H, 3.74; N, 5.02. MS ($ESI^+$): $m/z_{calc}$ 528.0353 [**1c** – 2I – H]$^+$ ($m/z$ 528.0340). $^1$H NMR (400.13 MHz, $d_4$-MeOD, 25 °C): δ = 9.63 (d, $^3J_{(H1,H2)}$ = 6 Hz, 1H, H-1), 8.42 (d, $^3J_{(H4,H3)}$ = 8Hz, 1H, H-4), 8.25 (td, $^3J_{(H3,H4)/(H3,H2)}$ = 8 Hz, $^4J_{(H3,H1)}$ = 2 Hz, 1H, H-3), 8.09 (d, $^3J_{(H9,H8)/(H11,H12)}$ = 9 Hz, 2H, H-9/H-11), 7.77 (m,

3H, H-2/H-8/H-12), 6.03 (d, $^{3}J_{(H15,H14)}$ = 6 Hz, 1H, H-15), 5.88 (d, $^{3}J_{(H17,H18)}$ = 7 Hz, 1H, H-17), 5.85 (d, $^{3}J_{(H18,H17)}$ = 7 Hz, 1H, H-18), 5.70 (d, $^{3}J_{(H14,H15)}$ = 6 Hz, 1H, H-14), 2.89 (sept, $^{3}J_{(H21,H20)/(H21,H22)}$ = 7 Hz, 1H, H-21), 2.37 (s, 3H, H-19), 1.21 (d, $^{3}J_{(H20,H21)}$ = 7 Hz, 3H, H-20), 1.17 (d, $^{3}J_{(H22,H21)}$ = 7 Hz, 3H, H-22) ppm. $^{13}C\{^{1}H\}$ NMR (100.61 MHz, $CDCl_3$ [0.3 mL] / *d*$_4$-MeOD [0.1 mL], 25 °C): δ = 159.7 (C-1), 139.2 (C-3), 128.7 (C-9/C-11), 128.2 (C-2), 127.5 (C-4), 124.9 (C-8), 124.6 (C-12), 87.7 (C-15), 87.4 (C-17), 85.7 (C-18), 85.2 (C-14), 31.5 (C-21), 22.4 (C-20), 21.6 (C-22), 19.4 (C-19) ppm.

*[Chlorido(η$^{6}$-p-cymene)(N-(4-sulfamoylphenyl)pyridine-2-carbothioamide)osmium(II)] chloride* **1d**

The synthesis of **1d** was performed following the general complexation procedure, using *N*-(4-sulfamoylphenyl)pyridine-2-carbothioamide (90 mg, 0.31 mmol) and $[Os(cym)Cl_2]_2$ (121 mg, 0.15 mmol). After work up the solid product was washed with dichloromethane (2 × 10 mL) and the solvent was removed on a rotary evaporator. Yield: 168 mg (80%), black solid. Elemental analysis found: C, 39.28; H, 3.94; N, 5.87; S, 8.96, calculated for $C_{22}H_{25}Cl_2N_3O_2OsS_2 \cdot 0.1C_6H_{14}$: C, 38.93; H, 3.82; N, 6.03; S, 9.20. MS (ESI$^+$): *m/z*$_{calc}$ 618.0925 [**1d** – 2Cl – H]$^+$ (*m/z* 618.0918), *m/z*$_{calc}$ 654.0692 [**1d** –Cl]$^+$ (*m/z* 654.0665). $^{1}H$ NMR (400.13 MHz, *d*$_4$-MeOD, 25 °C): δ = 9.50 (d, $^{3}J_{(H1,H2)}$ = 6 Hz, 1H, H-1), 8.43 (d, $^{3}J_{(H4,H3)}$ = 9 Hz, 1H, H-4), 8.21 (t, $^{3}J_{(H3,H4)/(H3,H2)}$ = 8 Hz, 1H, H-3), 8.04 (d, $^{3}J_{(H9,H8)/(H11,H12)}$ = 9 Hz, 2H, H-9/H-11), 7.73 (d, $^{3}J_{(H2,H3)/(H2,H1)}$ = 8 Hz, 1H, H-2), 7.63 (d, $^{3}J_{(H8,H9)/(H12,H11)}$ = 9 Hz, 2H, H-8/H-12), 6.14 (d, $^{3}J_{(H15,H14)}$ = 6 Hz, 1H, H-15), 6.06 (d, $^{3}J_{(H17,H18)}$ = 6 Hz, 1H, H-17), 6.02 (d, $^{3}J_{(H18,H17)}$ = 6 Hz, 1H, H-18), 5.75 (d, $^{3}J_{(H14,H15)}$ = 6 Hz, 1H, H-14), 2.64 (sept, $^{3}J_{(H21,H20)/(H21,H22)}$ = 7 Hz, 1H, H-21), 2.27 (s, 3H, H-19), 1.19 (d, $^{3}J_{(H20,H21)}$= 7 Hz, 3H, H-20), 1.08 (d, $^{3}J_{(H22,H21)}$ = 7 Hz, 3H, H-22) ppm. $^{13}C\{^{1}H\}$ NMR (100.61 MHz, $CDCl_3$ [0.3 mL] / *d*$_4$-MeOD [0.1 mL], 25 °C): δ = 158.4 (C-1), 139.5 (C-3), 129.5 (C-9/C-11), 127.3 (C-2), 125.2 (C-4), 124.2 (C-8/C-12), 96.3 (C-13), 79.4 (C-15), 78.9 (C-17), 76.3 (C-18), 73.7 (C-14), 31.0 (C-21), 22.6 (C-20), 21.5 (C-22), 18.1 (C-19) ppm.

**Stability in aqueous solution and reactivity with amino acids**

The hydrolytic stability of **1a**–**1d** was studied by dissolving the compounds (1–2 mg/mL) in $D_2O$. $^{1}H$ NMR spectra were recorded after 0.5, 2, 24, 48, 72, 96 and 120 h and ESI-mass spectra after 0.5, 24, 96 h and 7 d. To determine the reactivity with the

amino acids Cys, His and Met, **1a** or **1d** (1–2 mg/mL) was dissolved in $D_2O$ and 2 equivalents of the respective amino acids were added. The incubation mixture was analyzed by $^1$H NMR spectroscopy and ESI-MS after 0.5, 2, 24, 48, 72, and 96 h.

**Sulforhodamine B Cytotoxicity Assay**

HCT116, SW480 and NCI-H460 cells were supplied by ATCC, while SiHa cells were from Dr. David Cowan, Ontario Cancer Institute, Canada. The cells were grown in αMEM (Life Technologies) supplemented with 5% fetal calf serum (Moregate Biotech) at 37 °C in a humidified incubator with 5% $CO_2$.

The cells were seeded at 750 (HCT116, NCI-H460), 4000 (SiHa) or 5000 (SW480) cells/well in 96-well plates and left to settle for 24 h. The compounds were added to the plates in a series of 3-fold dilutions, containing a maximum of 0.5% DMSO at the highest concentration. The assay was terminated after 72 h by addition of 10% trichloroacetic acid (Merck Millipore) at 4 °C for 1 h. The cells were stained with 0.4% sulforhodamine B (Sigma-Aldrich) in 1% acetic acid for 30 min in the dark at room temperature and then washed with 1% acetic acid to remove unbound dye. The stain was dissolved in unbuffered Tris base (10 mM; Serva) for 30 min on a plate shaker in the dark and quantified on a BioTek EL808 microplate reader at an absorbance wavelength of 490 nm with 450 nm as the reference wavelength to determine the percentage of cell growth inhibition by determining the absorbance of each sample relative to a negative (no inhibitor) and a no-growth control (day 0). The $IC_{50}$ values were calculated with SigmaPlot 12.5 using a three-parameter logistic sigmoidal dose−response curve between the calculated growth inhibition and the compound concentration. The presented $IC_{50}$ values are the mean of at least 3 independent experiments, where 10 concentrations were tested in duplicate for each compound.

**Conductivity measurements**

The conductivity in acetonitrile was determined for ligand **1** and complexes **1a–d** (0.1 mM) on an Oakton CON 700 Conductivity/°C/°F Benchtop Meter at room temperature.

### Calculated logarithmic octanol/water partition coefficient (clog*P*)

ChemBioDrawUltra 15.0 was used to determine the calculated logarithmic octanol-water partition coefficient (clog*P*) of **1**.

### Molecular Modelling

Scigress Ultra version F.J 2.6[46] was used for the modelling of the ligands into the crystal structure of human carbonic anhydrase II (PDB ID 3PYK).[38] Hydrogen atoms were added to the structures and the ligands were built into the binding pocket based on co-crystallized [chlorido{N-[di(pyridin-2-yl-κN)methyl]-4-sulfamoylbenzamide}{(1,2,3,4,5,6-η)-(1R,2R,3R,4S,5S,6S)-1,2,3,4,5,6-hexamethylcyclohexane-1,2,3,4,5,6-hexayl}ruthenium(II)]. The ligands were first structurally optimized followed by short 1 ps molecular dynamics simulations using the MM2 force field.[47]

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