

X-ray absorption spectroscopy of an investigational anticancer gallium(III) drug: Interaction with serum proteins, elemental distribution pattern and coordination of the compound in tissue

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Abstract

Tris(8-quinolinolato)gallium(III) (**1**, KP46) is a very promising investigational anticancer drug. Its interaction with serum proteins as well as the elemental distribution and coordination in tissue were investigated with X-ray absorption (XAS) methods. Model compounds with mixed O, N and/or S donor atoms are reported. The coordination and structure of **1** in cell culture medium (minimum essential medium, MEM) and fetal calf serum (FCS) was probed by XANES and EXAFS. The interaction of **1** with the serum proteins apotransferrin (apoTf) and human serum albumin (HSA) was addressed as well. Applying micro-XAS to tissue samples from mice treated with **1**, the gallium distribution pattern was analyzed and compared to those of physiological trace elements. The complex **1** turned out to be very stable under physiological conditions, in cell culture media and in tissue samples. The coordination environment of the metal center remains intact in the presence of apoTf and HSA. The gallium distribution pattern in tumor and liver tissue revealed high similarities to the distribution patterns of Zn and Fe, minor similarities to Cu and Ni, and none to Ca.

Keywords

X-ray absorption spectroscopy, gallium compound, anticancer drug, serum protein, elemental distribution pattern

Introduction

Tris(8-quinolinolato)gallium(III) (**1**, KP46)^{1,2} (Figure 1) is a metal complex currently studied as an anticancer drug, which shows promise to be superior to simple gallium salts in terms of antitumor activity.²⁻⁴ The anticancer properties of gallium were discovered in 1971^{5,6} and in the mid 1970's gallium nitrate had entered first clinical trials.⁷ The main drawbacks of a therapy with gallium nitrate and other gallium salts was the need for slow, long-term infusion to avoid toxicities associated with high plasma levels of gallium, the poor bioavailability when given via the oral route and the loss of tumor selectivity when present in higher concentrations in plasma.⁸ These issues could be circumvented by the application of novel gallium complexes like tris(3-hydroxy-2-methyl-4H-pyran-4-onato)gallium(III) (gallium maltolate) and **1**.^{1,9,10} With these complexes higher tissue and serum concentrations with a smaller number of applications could be achieved.¹¹ Furthermore, **1** circumvented unicellular and multicellular resistances¹² and showed antiproliferative activity in cell lines expressing multidrug resistance-associated protein (MRP) and lung resistance protein (LRP).⁸ In a phase I clinical trial seven patients were treated with **1** out of those 4 with renal cell carcinoma and 3 with other carcinomas (head and neck, stomach, ovaries).³ Most remarkably, orally administered **1** yielded long-lasting disease stabilization on two patients and one partial remission of renal cell carcinoma.^{3,13} Furthermore, preclinical studies suggest its suitability for the treatment of malignant melanoma.¹⁴

The uptake pathway and mode of action is relatively well understood for gallium nitrate and gallium chloride,^{8,15} but remains elusive for the gallium complexes. The uptake of gallium nitrate through the transferrin receptor into the cell and the subsequent inhibition of ribonucleotide reductase are considered as the critical steps in the mechanism of action.^{16,17} Gallium is bound to the iron dependent M2 subunit of ribonucleotide reductase and causes a halt in the production of deoxynucleoside triphosphates (dNTPs) which in turn stops DNA synthesis and arrests the cell

cycle in the S-phase.⁸ **1** on the other hand is a relatively stable coordination compound and therefore different uptake pathways are conceivable.¹⁵ High concentrations of iron in the cell culture medium induced resistance to gallium salts.¹⁸ The resistant and sensitive cells showed the same rate of Ga uptake, but the resistant cells were able to increase the iron level significantly and overcome inhibition through Ga in this way. The resistance to Ga salts was ascribed to an up-regulation of the transferrin receptor.¹⁸ Considerable differences in the mode of action of **1** in comparison to gallium salts or more soluble complexes are likely.

X-ray absorption spectroscopy (XAS)¹⁹ allows for the investigation of the oxidation state, the coordination environment of a metal site and the elemental distribution of metals in biological systems. This method has two main advantages, firstly it needs no long range order and secondly it offers the possibility to investigate spectroscopically silent elements.²⁰ Hence, metals like Au(I), Zn(II) and Ga(III) possessing a full or empty d-shell are ideal candidates for the investigation by XAS. Especially gallium is well suited for investigation by this method, as it does not occur naturally in biological tissues. This fact makes it possible to study the overall average oxidation state and coordination environment by bulk XAS techniques. Micro-XAS mapping, using a highly focused X-ray beam, gives us the opportunity to study the gallium distribution pattern in different tissues and regions thereof. At selected spots in the tissue micro-XANES gives information about the local coordination environment. The low concentration of **1** in mouse tissue was one of the major obstacles encountered in biological experiments, in particular in the micro-XAS regime. Using a 3rd generation X-ray light source in combination with an ultra low temperature sample space and a highly sensitive multi element solid state detector enabled the study of this compound in biological tissue. While the high photon flux (about 4×10^{12} ph/s) of a 3rd generation synchrotron is an advantage in terms of very dilute samples it also incorporates the danger of photoreduction of the metal site under study and

increased overall sample degradation.²¹⁻²³ To overcome or minimize sample degradation low temperature sample environments are used. The lower photon flux of a 2nd generation facility causes lesser X-ray damage but lowers the signal to noise ratio.²¹ In this study spectra were collected on both types of synchrotrons, the 2nd generation DESY and the 3rd generation DLS, allowing to directly compare data taken at different generations of synchrotrons and to reveal the real situation in a biological environment.

In this study we report the X-ray absorption near edge spectroscopy (XANES) and extended X-ray absorption fine structure (EXAFS) spectra of gallium model compounds with known coordination spheres. XANES was used to investigate the oxidation state and coordination motif whereas EXAFS was used to determine the type, amount and distance of the 1st shell atoms. The coordination environment of **1** in the presence of different serum proteins and in tissue samples from mice treated with **1** was investigated with XAS. Micro-XAS mapping of the tissue samples was performed to resolve the elemental distribution. At gallium hot spots on the tissue XANES spectra were recorded. Acquiring information about the possible interaction of **1** with serum proteins and the determination of the coordination environment of gallium(III) in mouse tissue are the main targets in this study.

Figure 1

Results

XANES

Model compounds

XANES spectroscopy has been applied to the ionic compound Ga(NO₃)₃, the tetrahedral [GaCl₄]⁻ complex anion and five octahedral complexes with mixed S/N/O first shell environments. Following model compounds were investigated by XAS measurements: **1** (with 1st coordination sphere GaO₃N₃),^{1,2} tris(1-Phenyl-1,3-butanedionato-O,O')gallium(III) (**2**, KP47,

GaO₆),²⁴ bis{*N*-(9-bromo-7,12-dihydroindolo[3,2-*d*][1]benzazepin-6-ylidene)-*N'*-(2-oxidobenzylidene)hydrazone-*N,N',O*}-gallium(III) chloride (**3**, KP1438, GaO₂N₄),²⁵ bis(2-acetylpyridine *N,N'*-dimethylthiosemicarbazonato)-*N,N,S*-gallium(III) hexafluorophosphate (**4**, KP1485, GaS₂N₄),²⁶ tris(*N,N*-diethyldithiocarbamato-*S,S'*)gallium(III) (**5**, KP1135, GaS₆),^{27,28} tetraethylammonium tetrachlorogallate (**6**, [GaCl₄][−]), and gallium nitrate decahydrate (**7**, Ga(NO₃)₃•10H₂O).^{29–31} Structural formulas are shown in Figure 1.

The XANES spectra of the model compounds and their 1st derivatives are shown in Figure 2. With increasing electronegativity of the surrounding atoms the edge positions are shifted to higher energies. The edge position of **1** was determined to be 10375.5 eV by the maximum in the 1st derivative. The spectrum shows no pre-edge feature, has a sharply rising white line and an additional feature at about 10385 eV past the edge. The peak at 10385 eV is also seen in the reference compound β-Ga₂O₃, where it was ascribed to multiple scattering events occurring during the absorption process.³² In comparison to **1** (GaO₃N₃), the tetrahedral **6** anion has the lowest energy position (−2.8 eV), whereas **2** (GaO₆) exhibits the highest energy position shifted by +0.5 eV (see Table 1). The edge positions of **3** (GaO₂N₄) and **1** (GaO₃N₃) are the same, reflected by their nearly identical coordination environment (O₂N₄ and O₃N₃).

The white line peaks are all about 2.5 abs units except for **6**. For **6** the number of 1st shell scatterers is reduced to four chlorine atoms with a tetrahedral configuration. The finding of a lowered energy position is in agreement with a study of Nishi et al.³² where gallium oxides containing different coordination environments were investigated (tetrahedral Ga(t) and octahedral Ga(o)). The edge position of **6** was determined to be 10372.7 eV, which is even 1 eV below the one for Ga(t) (10373.6 eV). In addition the spectra of Ga(t) and **6** are very similar in shape.

The sulfur containing model compounds have the edge at lower energy: 10373.6 eV for **5** (GaS₆) and 10374.1 eV for **4** (GaS₂N₄). There are no significant additional features in the spectra except post edge shoulders at about 10377 eV for **5** (GaS₆) and 10382 eV for **4** (GaS₂N₄). The edge position of the ionic **7** occurs at 10375.0 eV.

Figure 2 Table 1

KP46 in presence of MEM, FCS, apoTf and HSA

In comparison to well-known metal based drug candidates such as 2-acetylpyridine-4,4-dimethyl-3-thiosemicarbazonato-N,N,S-dichlorogallium(III) (KP1492, logP -1.40)²⁶ and 2-acetylpyridine-N-pyrrolidinyl-3-thiosemicarbazonato-N,N,S-dichlorogallium(III) (KP1497, logP -0.69)²⁶, **1** is a lipophilic compound with its logP value of 0.88.^{8,33}

Therefore the mode of action of **1** is supposed to be different from the gallium salts.⁸ Fetal calf serum (FCS) and minimum essential medium (MEM) are widely used in cell culture. FCS and MEM were used to model the chemical environment during the application *in vitro*. The main components of MEM are amino acids, salts (Ca, K, Mg, Na) and glucose while FCS mainly consists of bovine albumin and growth factors like α -, β - and γ -globulines with a low concentration in antibodies.^{34,35}

Two transport proteins were envisaged as binding partners of **1** potentially involved in the transport via the blood stream to the target site: apo-transferrin (apoTf) and human serum albumin (HSA). These two are the most relevant transport proteins for transition metals *in vivo*.³⁶

XANES spectra have been collected for **1** in MEM, in FCS and in the presence of apoTf and HSA. The normalized XANES spectra of these four samples and their 1st derivatives are shown in Figure 2. Again the edge position was determined over the maximum in the 1st derivative. The edge positions of **1** in MEM and FCS and of **1** in the presence of apoTf and HSA are reported in Table 1. For all four solution spectra the edge energies vary only between 10375.4 and 10375.5

eV, which indicates no edge shifts. The heights of the white lines are about 2.7 abs units. All spectra show a post edge peak at around 10385 eV, typical for **1**. Moreover the XANES patterns of all liquid samples resembled that of solid **1**. The absence of any significant changes in the spectra of **1** in solution implies an intact octahedral coordination geometry throughout the different samples. **1** is a relatively stable complex, with gallium forming strong bonds to its ligands via oxygen and nitrogen atoms and no ligand dissociation could be observed under these mild conditions. Therefore a direct binding to the Ga metal site is ruled out. As XAS measures the average signals from all Ga atoms, this is valid for the bulk of the Ga atoms present in the sample.

EXAFS

Model compounds

The extracted fine structures and the Fourier transforms are shown in Figure 3. In the top row of Figure 3 the fine structure and Fourier transform (FT) of Cl and S containing compounds are shown. The phase shift between **5** (GaS_6) and **4** (GaS_2N_4) is a result of the differing amounts of sulfur scatterers. The heavy scatterer S ($Z = 16$) and the light scatterer N ($Z = 7$) are out of phase.³⁷ **6** exhibiting a tetrahedral coordination geometry with four heavy chlorine scatterers ($Z = 17$) is phase shifted to both S and/or N containing fine structures. The difference in phase between **6** and **5** is mainly due to the differing Ga–Cl (2.16 Å) and Ga–S (2.43 Å) distances and the 1st shell peak of **5** (GaS_6) in the FT is at higher R than for chlorine. Both spectra (**5** and **6**) show very uniform EXAFS oscillations over the whole k-range indicating a uniformly distributed 1st shell made up of one type of atom.

1, **2** and **3** are octahedral complexes with only O/N light scatterers in the first coordination shell exhibiting similar fine structures. With increasing amount of oxygen ligands the signal in the high

k range becomes stronger and the same is true for the amplitudes in the FT. This is in agreement with the reported oxygen and nitrogen distances for the different compounds.

The fitting analysis using FEFF^{38,39} was restricted to the 1st coordination shell extracted from the first peak in the FT. The known information from the crystallographic data was taken as a starting point for the fits. The amount of scatterers was fixed not to exceed the number of fitting parameters. The results of the 1st shell fits of the model compounds using theoretical amplitudes and phases provided by the FEFF code are presented in Table 2. The results for the DL-EXCURV⁴⁰ fits are given in Table 3. The distances are given as the average fitted distances for each atom type/shell (O/N, S and C).

The curve fitting results obtained by FEFF and DL-EXCURV are both in good agreement with the crystallographic data. Thus, the reliability of the amplitude and phase shift extracted from **1** is confirmed.

Figure 3

Table 2 Table 3

KP46 in presence of MEM, FCS, apoTf and HSA

The k³ weighted EXAFS spectra and the corresponding FT of **1** in the presence of MEM, FCS, apoTf and HSA are shown in Figure 4. The extracted fine structures and FT for all five samples look almost identical. The results of the XANES analysis are confirmed by the EXAFS data (Figure 4). The fits of the FEFF calculated theoretical spectra to the experimental ones are presented in Table 4. In Table 5 the results of the DL-EXCURV analysis are presented. The same structural model was used for fitting the experimental spectra of **1** in BN and in solution. The fits of the solution samples resemble the crystallographic data in the same way as shown in the fit for solid **1**.

Figure 4 Table 4

Table 5

KP46 in mice tumor and liver tissue

XANES analysis

XANES spectra of tumor and liver samples from mice treated with **1** were collected at the synchrotrons in Hamburg (DESY) and Didcot (DLS). Due to the low concentration of gallium in these samples no EXAFS signals could be collected. The XANES spectra from DLS were collected at spots high in Ga, identified with micro-XAS as described in the next paragraph. The tissue samples at DESY were collected with an unfocused beam. For the tumor and liver spectra measured at DLS, several spectra were collected at positions indicated by the crossing yellow lines in Figure 6 for the tumor and in Figure 7 for the liver sample.

The normalized XANES spectra and the corresponding 1st derivatives of the tumor and liver samples are coplotted with **1** in MEM in Figure 5. The edge positions of the XANES spectra taken from the tumor and liver material are all at 10375.5 eV within 0.2 eV deviation. In respect of the low signal strength the changes in the edge position are within the statistical error and there is no evidence for a substantial change throughout the probed tissue samples. In comparison to the solid **1** in boron nitride and to **1** in MEM no significant shift in energy could be observed.

The white lines of the Ga XANES spectra from the tissue samples are slightly broadened and the feature at about 10385 eV, typical for **1**, is strongly attenuated. Similar features in other publications are assigned to multiple scattering effects,³² its attenuation might be due to the higher degree of disorder in the complex tissue environment and the low signal to noise ratio.

The line shapes and spectral features are the same for tumor and liver samples treated with **1**. Different coordination of **1** in liver and tumor tissue is not assumed. From a XANES point of view, the three dimensional coordination of the gallium from **1** in the tissue is nearly identical to **1** in boron nitride. Differences in the white line heights are attributed to damping of the

fluorescence signals because of the inhomogeneity of the tissue samples (thickness effects and pinholes).

Figure 5

Micro-XAS analysis

Micro-XAS images of murine tumor and liver tissue were collected during two beamtimes in 2008 and 2011 to analyze the elemental distribution in the tissue samples, identifying regions of high gallium concentration. Simultaneously to the collection of gallium maps the distribution patterns of Ca, Fe, Ni, Cu, and Zn were obtained, which made it possible to study the correlation of these elements with the Ga distribution pattern. In Figure 6 the micro-XAS maps of a murine tumor (sarcoma 180) and in Figure 7 the maps for a liver sample from the same individual are shown.

The maps show the distribution of Ga, Zn, Fe, Ca, Cu and Ni. The tumor sample exhibits the same distribution pattern for Ga and Zn. The Fe distribution differs from Ga in the tumor tissue. The opposite is true for the liver sample where the Fe and Ga patterns are very similar, but the Zn distribution is different. The distributions of Cu and Ni are similar in both samples but the signals are much weaker. In contrast Ca shows a completely different distribution in both organs.

Figure 6

Figure 7

Discussion

XAS methods were used to identify any changes in the coordination environment of **1** in the presence of two different serum proteins (HSA and apoTf), dissolved in culture medium (MEM) and fetal calf serum (FCS) and *in vivo* by analyzing liver and tumor samples originating from mice treated with **1**.

KP46 in presence of MEM, FCS, apoTf and HSA

MEM and FCS are widely used in cell culture. Therefore the integrity and stability of **1** in these media is relevant for the conduction of reliable *in vitro* tests. The main constituents of MEM are inorganic salts, glucose, amino acids and vitamins.³⁴ FCS comprises a variety of different amino acids³⁵ making up a large set of possible binding partners to the metal center. Since gallium reacts as a hard Lewis acid, oxygen and nitrogen should be preferential binding partners to Ga via the dissociation of one or more quinolinolato ligands or hydration through aqua ligands. Both events imply substantial changes in the coordination environment of gallium(III) and would have been clearly visible in the XANES and EXAFS spectra, as shown for the model compounds. The measured spectra are identical to each other and to the **1** boron nitride preparation.

The interaction of **1** with HSA and apoTf was previously investigated in other studies with capillary zone electrophoresis coupled with UV/VIS detection (CZE-UV/VIS)³³ and with capillary electrophoresis coupled to induced coupled plasma mass spectrometry (CE-ICP-MS) and electrospray ionization MS (CE-ESI-MS).⁴¹ The hydrolytic stability and lipophilicity of the complex were tested with CZE-UV/VIS and water/n-octanol partitioning tests,³³ respectively. The complex was stable under physiological conditions (at pH 7.4 in 10 mM NaH₂PO₄/Na₂HPO₄, 100 mM NaCl) with a half life of $\tau_{1/2} = 14.2$ h. The propensity to be more soluble in n-octanol than in water is caused by the rather lipophilic quinolinolato ligands. This opens the possibility of a non-transferrin mediated uptake of the drug into the cell through hydrophobic interactions. **1** was more reactive towards apoTf than towards HSA, showing very little reactivity towards the latter.⁴¹ The overall affinity towards HSA and apoTf was very low in comparison to dissolved Ga salts.⁴¹ The detection method used did not address the metal center directly but detected the formed adducts as a whole. Besides a covalent binding of the metal center to these molecules a hydrophobic interaction is conceivable as well.

The XAS experiments in this study address the Ga center directly and provide valuable information about the possible binding situation. For the first time it is possible to address the atomic neighborhood of Ga in **1** in the presence of HSA and apoTf. Applying XAS no direct interaction or covalent bonding of the metal center towards HSA and apoTf could be found. This would have involved the dissociation of a ligand, easily detectable by XAS spectroscopy. Nonetheless an adduct formation between **1** and HSA or apoTf is not foreclosed by these results. More likely a non-covalent more hydrophobic interaction could be the foundation of the underlying interaction mechanism. The stability of the complex under physiological conditions is underlined by this XAS study. This counts for the majority of the molecules of **1**, since XAS measurements only describe the average coordination environment of all Ga atoms present in the sample.

KP46 in tissue samples

Up to date no studies on the coordination of the metal center of **1** in biological tissue have been undertaken. In references ⁴² and ⁴³ a connection between the treatment of tumor cell lines with Ga nitrate and the internal Zn pool of these cells was drawn. In reference ⁴² gallium nitrate resistant and gallium nitrate sensitive cell lines were analyzed regarding their difference in the gene expression pattern of genes regulating the transition metal metabolism. It could be shown that gallium resistant cells showed a significantly higher expression of the metallothionein-2A gene (MT2A) and the zinc efflux transporter (ZnT-1). Both proteins are involved in the cellular handling of Zn. MT2A is an intracellular cystein-rich protein correlated to the handling of Zn, Cd and certain other metals. ZnT-1 is a membrane based zinc efflux transporter. Gallium induces the expression of these proteins by the formation of reactive oxygen species (ROS). The increased oxidative stress initializes the metal-responsive transcription factor (MTF-1) related pathway which in turn activates MT2A and ZnT-1 gene transcription.⁴³ These two proteins have a crucial

impact on the intracellular Zn concentration, and Ga interfered with the expression patterns of these genes through the creation of ROS. Normally the metallothionein-2A metabolism is strongly related to the divalent metals Zn and Cd. The metallothionein-2A expression is induced by Ga as well, a trivalent metal not occurring in the divalent state.

The results presented in this study demonstrate the correlation of Ga with the Zn distribution in tumor tissue. In the liver sample the Ga concentration was correlated to the Fe distribution pattern, which is not very surprisingly as these two ions are very similar. Similarities to other transition metals like Cu and Ni are given but to a minor degree. On the other side Ga patterns are absolutely independent from the Ca distribution (see Figure 6 and Figure 7). The XANES spectra collected at the low flux beamline D2 at DESY (unfocused beam) are the same as the spectra collected at the high flux beamline I18 at DLS ($5 \times 5 \mu\text{m}^2$ focused beam) (Figure 5). A substitution of one ligand through a sulfur containing amino acid might have been a possible scenario in the biological samples and would have been clearly visible in the XANES spectra. In none of the collected XANES spectra (focused at DLS and unfocused at DESY) any substantial change in the immediate atomic environment of the metal center was observed.

Conclusions

XAS spectroscopy was applied to study **1** *in vitro* and *in vivo*. The basis of the analysis was provided by the examination of Ga model compounds with known coordination environments. The characterization of the model compounds was in good agreement with other studies undertaken on similarly coordinated Ga species in the past. No direct binding of the majority of the metal centers of **1** to the serum proteins HSA and apoTf could be detected. XAS further proved the stability of the complex under physiological conditions. The relative inertness of the metal center throughout the course from application to the target site in conjunction with the most likely drug carriers and reaction partners could be pointed out. With micro-XAS it was possible

to investigate the distribution pattern of **1** *in vivo* and correlate it to the distribution patterns of other biological important metals like Ca, Fe, Cu, Ni and Zn.

XANES spectra were collected at spots with a high content of Ga, revealing the local coordination of the metal center *in vivo*. The integrity of the majority of the molecules of **1** in mouse tumor and liver tissue was confirmed.

XAS has proven to be a very powerful tool for the investigation of metallodrugs in a variety of test systems missing any long range order. The sensitivity of the method to small changes in the ligand environment and the ability to detect the elements of question in trace amounts was approved.

Experimental section

Sample preparation

The model compounds were diluted in boron nitride (Sigma Aldrich, CAS 10043-11-5, 99.5% BN) to a calculated absorption of about 1 abs following standard procedures.⁴⁴ The samples were filled into aluminum sample holders and sealed with Kapton foil.

The solution samples were prepared according to previously described procedures.⁴¹ Human serum albumin (HSA) from plasma, human apo-transferrin (apoTf), minimum essential medium (MEM), fetal calf serum (FCS), sodium bicarbonate, disodium hydrogenphosphate, sodium chloride, glycerol and dimethylsulfoxide (DMSO) were purchased from Sigma Aldrich. Phosphate buffered saline (PBS) (pH = 7.4) was prepared according to standard procedures.⁴¹ Stock solutions of **1** 10 mM in DMSO, apoTf 1.5 mM in PBS and HSA 1.5 mM in PBS were prepared. The final solutions of **1** were prepared by mixing 10% (v/v) **1** in DMSO, 70% (v/v) of the biomolecules stock solutions and 20% (v/v) of glycerol. Before the glycerol was added the solutions were incubated at 37°C for 30 min. After the incubation the solutions were filled into aluminum sample holders sealed with Kapton foil, flash frozen in liquid nitrogen and stored at

−85 °C. Following solutions were prepared: **1** 1 mM in MEM, **1** 1 mM in FCS, **1**+apoTf 1:1 mM in DMSO/PBS, **1**+HSA 1:1 mM in DMSO/PBS.

The tissue samples were taken from experiments performed in accordance with the European Community Guidelines for the use of experimental animals in the animal facility at the Cancer Research Institute, Slovak Academy of Sciences, Bratislava, Slovak Republic. BALB/c mice bearing the subcutaneous sarcoma 180 were treated with **1** in the following way: **1** was suspended in a Tween-60/H₂O 1:4 solution and applied per orally in a dosage of 200 mg/kg/day on six consecutive days. Tumor and liver were collected one day after the last drug administration and stored at −80 °C. Samples of these were filled into aluminum sample holders, sealed with Kapton foil and flash frozen in liquid nitrogen. Following tissue samples were measured: tumor at DLS, tumor at DESY, liver at DLS, liver at DESY.

XAS experiments

The XANES and EXAFS experiments were carried out at two different synchrotron radiation facilities and beamlines, beamline D2 at the Deutsche Elektronen Synchrotron (DESY) in Hamburg (Germany) and the beamline I18 at the Diamond Light Source (DLS) in Didcot (England). The micro-XAS maps of the tissue samples were collected at DLS.

The model compounds were measured in transmission mode, all other spectra and the micro-XAS maps were collected in fluorescence mode. Dead time correction was applied to the fluorescence spectra. The beamline D2 at DESY was equipped with a Si(111) double crystal monochromator and a Canberra 13 element solid state Ge detector for fluorescence measurements. The DORIS synchrotron was operated at 4.5 GeV. The beamline I18 at DLS was equipped with a double crystal Si(111) monochromator and 27 mm period undulator giving an energy range of 2 – 20.7 keV (flux of 4×10^{12} ph/s). Higher harmonics were rejected using two plane mirrors with Rh and Ni stripes. For the micro-XAS maps and XANES of the tissue samples

the beam was focused with mechanical Kirkpatrick-Baez mirrors with Si and Rh stripes to a spot size of $5 \times 5 \mu\text{m}^2$. The signal was detected with ionization chambers in transmission and a 9 element solid state Ge detector in fluorescence mode. The Diamond synchrotron was operated at 3 GeV with an electron beam current of 250 mA to 150 mA. All tissue and solution samples were measured at cryogenic temperatures (about 50 K) using an Oxford Instruments MicroRes He cryostat. The model BN preparations were measured at room temperature whereas at DESY all measurements were conducted at cryogenic temperatures around 20 K.

The XANES signals were measured at the Ga K-edge with a pre-edge region from 10217 eV to 10340 eV with a step size of 6 eV, an edge region from 10340 eV to 10402 eV with a step size of 0.5 eV. The scanning times were set to 1 sec in the pre-edge and 2 sec in the edge region. The k-space was measured from 3 to 8 \AA^{-1} with a step size of 0.05 \AA^{-1} . The post edge scanning time was increased according to a predefined exponential like curve from 1 to 6 sec.

The EXAFS modulations were measured at the Ga K-edge with pre-edge and edge settings as used in the XANES measurements. The k-space was measured from 3 to 14 \AA^{-1} with a step size of 0.04 \AA^{-1} . The scanning times were 1 sec in the pre-edge (10217 eV – 10240 eV), 2 sec in the edge (10340 eV – 10402 eV) and 2-8 sec (10402 eV – 11110 eV), increasing according to a predefined curve, in the post-edge region.

The micro-XAS maps were collected at beamline I18 at DLS with a fixed energy of 12 keV. The beam was focused to a spot size of $5 \times 5 \mu\text{m}^2$ and the fluorescence signals were collected for 3 sec at each spot. The XANES spectra of the tissue samples from DLS were measured with a focused beam as well. The spots high in gallium where the XANES spectra were taken are marked in Figure 6 and Figure 7 (crossing yellow lines).

Energy calibration

The monochromator stability was proven and the energy calibration was performed by measuring gallium oxide (**8**, β -Ga₂O₃) every two sample scans. **8** was purchased from Sigma Aldrich (Puratronic 99.999%, CAS: 12024-21-4) and measured as BN preparation.

In accordance with previous studies^{32,45} on **8** three significant features in the XANES spectra could be determined: an edge shoulder at 10375.0 eV, a post edge peak at 10379.2 eV and a third small peak at about 10385 eV (Figure 8). The edge position lies at 10373.5 eV and was determined as the inflection point on the rising edge of the XANES spectrum. The spectrum of **8** constitutes an overlay of tetrahedral (Ga(t)) and octahedral (Ga(o)) gallium.³² The absorption maximum of Ga(t) lies at 10375.0 eV, represented as an edge shoulder in Figure 8, the one of Ga(o) lies at 10379.2 eV, also seen as a post edge peak in Figure 8.

The energy calibration was done using the post edge peak at 10379.2 eV by applying the software packages ATHENA⁴⁶ and IFEFFIT.⁴⁷ In Figure 8 the normalized spectrum and the 1st derivative of **8** are shown.

Figure 8

XAS analysis

The program packages ATHENA, ARTEMIS,⁴⁶ FEFF,^{38,39} EXPROG,⁴⁸ PySpline,⁴⁹ and DL-EXCURV⁴⁰ were applied for XAS data analysis.

XANES analysis

The pre-edge background was removed by a linear approximation in the range of -50 eV to -150 eV before the edge. This baseline was subtracted from the entire spectrum. The normalization was accomplished fitting a quadratic polynomial in the post edge region from 150 eV to 240 eV. The function was extrapolated to the first absorption maximum and the absorbance was set to unity there. The edge position was determined as the maximum in the 1st derivative of

the spectrum (inflection point of the steeply rising edge). The spectra were shifted according to the observed shift for the standard compound **8** ($E_0 = 10373.6$ eV, see Figure 8).

EXAFS analysis

FEFF analysis:

The pre-edge background subtraction and the normalization were performed as described for the XANES spectra. The normalized EXAFS signals were extracted using the AUTOBK algorithm incorporated into IFEFFIT.⁴⁷ The background function is constructed using cubic splines with evenly spaced knots throughout the data region. The number of splines is defined as $(2R_{\text{bkg}}\Delta k)/\pi$. Where the R_{bkg} value defines the frequencies which are allowed to be removed by the smoothly varying background spline, and is usually set to one half of the 1st shell distance (longer frequencies will be removed). In our case R_{bkg} was set to 1 Å and the spline range Δk to 0.5 – 13.5 Å⁻¹. The Fourier transform was constructed using a Hanning window from 2 – 10 Å⁻¹ and a δk of 2 Å⁻¹. The back-Fourier transform was extracted using a Hanning window from 1 – 2 Å and a δR of 0 Å.

FEFF 7⁵⁰ was used for the calculation of the theoretical EXAFS amplitudes and phases. The input file for the FEFF code was created out of the structural model of **1**¹ described below and the crystallographic 1st shell data of the model compounds, respectively. The program package ARTEMIS⁴⁶ was used to make the least square fits of the theoretical spectra to the experimental ones. The shift in the edge position (E_0) and the mean square relative displacement (σ^2 , Debye-Waller factor) were refined for all scattering paths. The absorber – scatterer distance (R) was defined over a contraction expansion factor α (alpha) relative to the starting values of the structural models. The amplitude reduction factor S_0^2 was set to a fixed value of 1.0, which allows the correct comparison of the different fits. The coordination number N was set according

to the crystallographic value to not exceed the number of free parameters. The 1st shell fits were performed on the backtransformed, Fourier filtered spectra.

DL-EXCURV analysis:

The k^3 weighted EXAFS signals were obtained using the program PySpline. The edge energy was set to the values as determined in the XANES analysis. The pre edge background was fitted through a straight line and subtracted from the entire spectrum. The post edge background was fitted using a three segment spline build up of third order polynomials. The FT was constructed using a Kaiser-Bessel window with $\delta k = 0.1 \text{ \AA}^{-1}$. DL-EXCURV calculated the theoretical EXAFS spectra for the defined structural model based on the curved wave method for single scattering. The ground- and excited state exchange contributions to the effective potential were calculated with the von Barth method.⁵¹ The structural parameters atomic distance (R), Debye-Waller factor ($2\sigma^2$) and the residual shift (EF) of the edge energy (E_0) were refined to obtain the best fit between experiment and theory. The amplitude reduction factor (AFAC) and the number of scatterers (N) were fixed. The quality of the fit was indicated by the R-factor.⁵² The number of free parameters (N_P) was always inspected to be less than the number of independent data points (N_{IND}).⁵³ The fits were performed on the raw k^3 weighted EXAFS data.

Structural model:

The minimal distinguishable distances are determined according to the formula $\delta R = \pi/(2 \times k_{\max})$,^{54,55} where k_{\max} represents the upper limit used for the Fourier transformation. For **1** $\delta R = 0.157 \text{ \AA}$ and is in the same range as the most distant O and N atoms in our crystallographic model ($\delta R_{\text{eff}} = 0.162 \text{ \AA}$). This makes it possible to distinguish these two first shell atom species and perform sub shell fits for O and N and determine their mean distance to the central Ga atom. The 1st shell was built up of the 3 O atoms at distances from 1.937 \AA to 1.950 \AA and 3 N atoms

with distances from 2.061 Å to 2.099 Å. The calculated mean distances Ga–O and Ga–N for **1** were 1.944 Å and 2.077 Å, respectively.

Micro-XAS analysis

The micro-XAS maps were analyzed using the program package PyMCA.⁵⁶ The maps were constructed by normalizing the fluorescence signals with the incoming X-ray beam intensity I_0 . The upper limit for the signals was set to the 99 percentile to remove unwanted signal peaks due to detector glitches.

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Notes

The authors declare no competing financial interest.

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A Ga fluorescence map was taken on a mouse liver sample treated with **1** at the Swiss Light Source with a spot size of $2 \times 2 \mu\text{m}^2$ for initial experiments. EXAFS data were taken at a high concentration spot on the map (data not shown). The EXAFS on the hot spot showed the characteristic spectrum of **1**. We highly acknowledge the support of Dr. D. Grolimund and Dr. C. Borca Swiss Light Source, Paul Scherrer Institute, Villigen, Switzerland.

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Abbreviations used

XAS, X-ray absorption spectroscopy; XANES, X-ray absorption near edge structure; EXAFS, extended X-ray absorption fine structure; micro-XAS, micro X-ray absorption spectroscopy; BN, boron nitride; MEM, minimum essential medium; HSA, human serum albumin, DMSO, dimethyl sulfoxide; DESY, Deutsches Elektronen Synchrotron; DLS, Diamond Light Source Ltd.; FT, Fourier transform.

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Tables

Table 1: Model compounds and edge shifts

Table 2: 1st shell fits of the model compounds using theoretical amplitudes and phases provided by the FEFF code. N_{fix} is the fixed coordination number, R is the average distance, R_{cryst} is the crystallographic value, ΔR is the difference between R and R_{cryst} , σ^2 is the Debye Waller factor, E_0 is the residual shift of the edge energy.

Table 3: DL-EXCURV EXAFS fits of the model compounds. N_{fix} is the fixed coordination number, R is the average distance, R_{cryst} is the crystallographic value, ΔR is the difference between R and R_{cryst} , σ^2 is the Debye Waller factor, E_0 is the residual shift of the edge energy, R_{fit} is the quality of the fit, fit index is the sum of the square of the residuals.

Table 4: 1st shell q-space fits of **1** in presence of MEM, FCS, apoTf and HSA with amplitudes and phases provided by the FEFF code. N_{fix} is the fixed coordination number, R is the average distance, σ^2 is the Debye Waller factor, E_0 is the residual shift of the edge energy.

Table 5: DL-EXCURV EXAFS fits of **1** in presence of MEM, FCS, apoTf and HSA. N_{fix} is the fixed coordination number, R is the average distance, σ^2 is the Debye Waller factor, E_0 is the residual shift of the edge energy, R_{fit} is the quality of the fit, fit index is sum of the square of the residuals.

Figures

Figure 1: Gallium model compounds

Figure 2: Normalized spectra and 1st derivataives of the model compounds and of **1** in the presence of minimum essential medium, fetal calf serum, apo-transferrin, human serum albumin and as BN preparation.

Figure 4: Extracted fine structures and Fourier transforms of the model compounds

Figure 5: Extracted fine structures and Fourier transforms of **1** in the presence of minimum essential medium, fetal calf serum, apo-transferrin, human serum albumin and as BN preparation.

Figure 6: Tumor and liver XANES data and corresponding normalized 1st derivatives in comparison to **1** in MEM.

Figure 7: Normalized micro-XAS maps of tumor tissue, upper limit of the color maps is the 99 percentile. Taken at beamline I18 at Diamond Light Source (UK)

Figure 8: Normalized micro-XAS maps of liver tissue, upper cut-off of the color maps is the 99 percentile. Taken at beamline I18 at Diamond Light Source (UK).

Figure 9: Normalized spectrum and 1st derivative of **8**.

Tables

compounds	1 st coord. shell	coord. conc. [mM]	edge energy [eV]	shift to 1 [eV]
6	GaCl ₄	tetrahedral	10372.7	- 2.8
5	GaS ₆	octahedral	10373.6	- 1.9
4	GaS ₂ N ₄	octahedral	10374.1	- 1.4
3	GaO ₂ N ₄	octahedral	10375.5	+ 0.0
1	GaO ₃ N ₃	octahedral	10375.5	+ 0.0
2	GaO ₆	octahedral	10375.9	+ 0.5
7	GaO ₆	ionic comp.	10375.0	- 0.5
1 in MEM		1 mM	10375.5	
1 in FCS		1 mM	10375.4	
1 + HSA		1:1 mM	10375.4	
1 + apoTf		1:1 mM	10375.4	
tumor DLS			10375.4	
tumor DESY			10375.5	
liver DLS			10375.3	
liver DESY			10375.5	

Table 1: Model compounds and edge shifts

comp.	path	N_{fix}	R [Å]	R_{cryst} [Å]	ΔR [Å]	σ^2 [Å ² 10 ⁻³]	E_0 [eV]	fit index [%]	cryst. ref.
6	Ga-Cl	4	2.167(3)	2.163	0.004	3.12 ± 1.22	3.0 ± 1.9	1.3	29
5	Ga-S	6	2.437(4)	2.433	0.004	2.35 ± 0.90	2.6 ± 1.4	2.3	28
4	Ga-S	2	2.40(6)	2.344	0.056	3.47 ± 1.22	2.5 ± 1.7	1.2	26
	Ga-N	4	2.09(6)	2.095	-0.005				
3	Ga-O	2	1.916(5)	1.912	0.004	2.49 ± 1.25	2.7 ± 1.6	1.3	25
	Ga-N	4	2.080(5)	2.075	0.005				
1	Ga-O	3	1.97(3)	1.944	0.026	2.41 ± 1.68	3.8 ± 2.7	2.1	2
	Ga-N	3	2.09(3)	2.077	0.013				
2	Ga-O	6	1.97(5)	1.946	0.024	4.53 ± 1.12	3.1 ± 1.7	0.9	24

Table 2: 1st shell fits of the model compounds using theoretical amplitudes and phases provided by the FEFF code. N_{fix} is the fixed coordination number, R is the average distance, R_{cryst} is the crystallographic value, ΔR is the difference between R and R_{cryst} , σ^2 is the Debye Waller factor, E_0 is the residual shift of the edge energy.

comp.	path	N_{fix}	R [Å]	R_{cryst} [Å]	ΔR [Å]	σ^2 [Å ² 10 ⁻³]	E_0 [eV]	R_{fit} [%]	fit index	cryst. ref.
6	Ga-Cl	4	2.159(3)	2.163	-0.004	3.38 ± 0.17	-6.8 ± 0.6	22.4	0.15	²⁹
5	Ga-S	6	2.425(3)	2.433	-0.008	3.21 ± 0.19	-9.9 ± 0.5	24.4	0.15	²⁸
4	Ga-N	4	2.08(1)	2.095	-0.015	5.85 ± 1.06	-8.4 ± 0.6	29.8	0.67	²⁶
	Ga-S	2	2.376(7)	2.344	0.032	3.59 ± 0.71				
	Ga-C	4	3.03(2)	2.928	0.102	4.08 ± 2.06				
3	Ga-O	2	1.91(1)	1.912	-0.002	3.26 ± 1.26	-6.2 ± 0.7	26.4	0.50	²⁵
	Ga-N	4	2.07(1)	2.075	-0.005	5.64 ± 1.42				
	Ga-N	2	2.52(3)	2.875	-0.355	9.95 ± 3.80				
	Ga-C	6	2.94(1)	2.943	-0.003	5.75 ± 1.32				
1	Ga-O	3	1.946(4)	1.944	0.002	1.84 ± 0.57	-7.2 ± 0.3	26.5	0.16	²
	Ga-N	3	2.077(6)	2.077	0.000	1.61 ± 0.71				
	Ga-C	6	2.811(5)	2.803	0.008	2.80 ± 0.42				
	Ga-C	9	3.94(1)	4.245	-0.305	7.27 ± 1.50				
2	Ga-O	6	1.957(5)	1.946	0.011	4.47 ± 0.47	-5.7 ± 0.6	29.5	0.57	²⁴
	Ga-C	6	2.92(3)	2.891	0.029	9.93 ± 3.77				

Table 3: DL-EXCURV EXAFS fits of the model compounds. N_{fix} is the fixed coordination number, R is the average distance, R_{cryst} is the crystallographic value, ΔR is the difference between R and R_{cryst} , σ^2 is the Debye Waller factor, E_0 is the residual shift of the edge energy, R_{fit} is the quality of the fit, fit index is the sum of the square of the residuals.

comp.	path	N_{fix}	R [Å]	σ^2 [Å ² 10 ⁻³]	E_0 [eV]	fit index [%]
1	Ga-O	3	1.97(3)	2.41 ± 1.68	3.8 ± 2.7	2.1
	Ga-N	3	2.09(3)			
1 + FCS	Ga-O	3	1.98(3)	2.25 ± 1.81	3.6 ± 2.5	2.4
	Ga-N	3	2.10(4)			
1 + MEM	Ga-O	3	1.98(3)	2.62 ± 1.99	3.6 ± 2.7	2.8
	Ga-N	3	2.10(3)			
1 + HSA 1:1	Ga-O	3	1.98(3)	2.13 ± 1.65	4.4 ± 2.3	2.1
	Ga-N	3	2.10(3)			
1 + apoTf 1:1	Ga-O	3	1.98(3)	2.78 ± 1.96	3.2 ± 2.9	2.4
	Ga-N	3	2.09(3)			

Table 4: 1st shell q-space fits of **1** in presence of MEM, FCS, apoTf and HSA with amplitudes and phases provided by the FEFF code. N_{fix} is the fixed coordination number, R is the average distance, σ^2 is the Debye Waller factor, E_0 is the residual shift of the edge energy.

comp.	path	N_{fix}	R [Å]	σ^2 [Å ² 10 ⁻³]	E_0 [eV]	R_{fit} [%]	fit index
1	Ga-O	3	1.946(4)	1.84 ± 0.57	-7.2 ± 0.3	26.5	0.16
	Ga-N	3	2.077(6)	1.61 ± 0.71			
	Ga-C	6	2.811(5)	2.80 ± 0.42			
	Ga-C	9	3.94(1)	7.27 ± 1.50			
1 + FCS	Ga-O	3	1.943(4)	1.38 ± 0.42	-7.3 ± 0.4	29.9	0.22
	Ga-N	3	2.083(7)	1.03 ± 0.23			
	Ga-C	9	2.830(7)	4.21 ± 0.72			
	Ga-C	3	3.94(2)	7.83 ± 2.11			
1 + MEM	Ga-O	3	1.938(6)	2.22 ± 0.73	-7.2 ± 0.4	30.2	0.23
	Ga-N	3	2.079(7)	1.59 ± 0.94			
	Ga-C	9	2.821(7)	4.85 ± 0.76			
	Ga-C	3	3.94(2)	8.08 ± 2.09			
1 + HSA 1:1	Ga-O	3	1.943(5)	1.45 ± 0.65	-7.2 ± 0.4	28.0	0.19
	Ga-N	3	2.081(6)	1.02 ± 0.86			
	Ga-C	9	2.819(7)	4.28 ± 0.67			
	Ga-C	3	1.93(2)	8.78 ± 2.22			
1 + apoTf 1:1	Ga-O	3	1.946(5)	2.41 ± 0.86	-7.2 ± 0.4	30.1	0.21
	Ga-N	3	2.076(9)	2.16 ± 1.14			
	Ga-C	9	2.834(7)	4.60 ± 0.74			
	Ga-C	3	3.92(2)	8.78 ± 2.26			

Table 5: DL-EXCURV EXAFS fits of **1** in presence of MEM, FCS, apoTf and HSA. N_{fix} is the fixed coordination number, R is the average distance, σ^2 is the Debye Waller factor, E_0 is the residual shift of the edge energy, R_{fit} is the quality of the fit, fit index is sum of the square of the residuals.

Charts and figures:

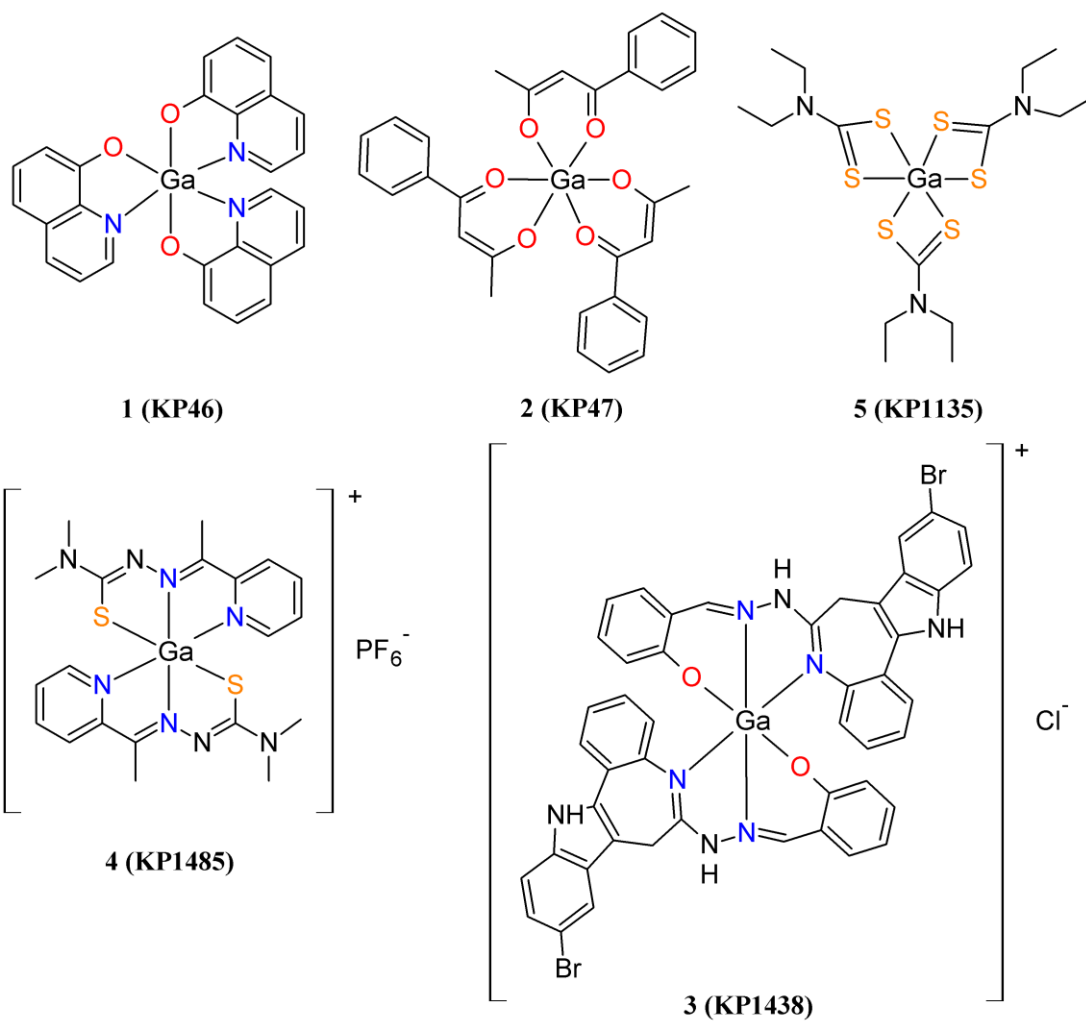


Figure 1: Gallium model compounds

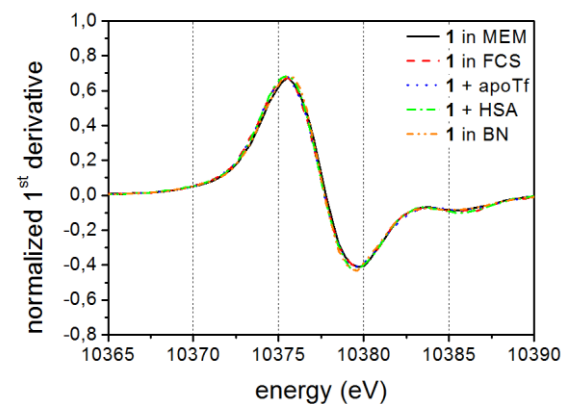
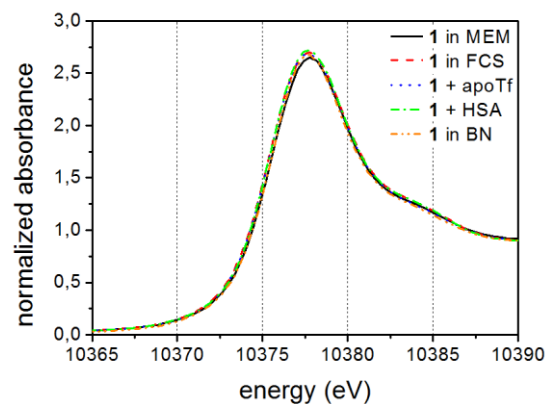
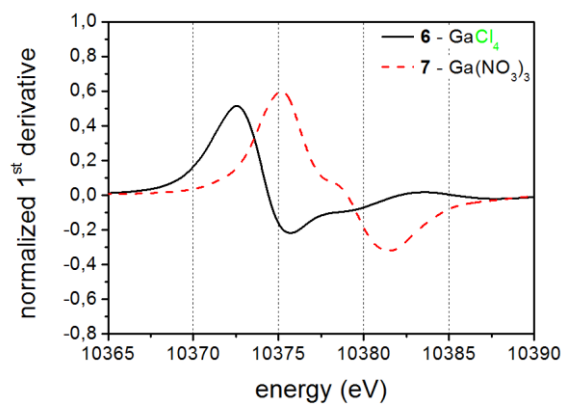
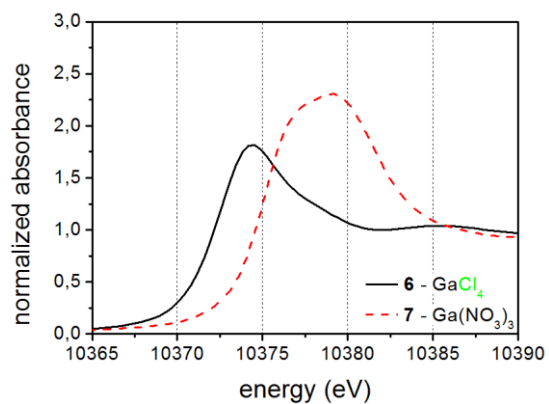
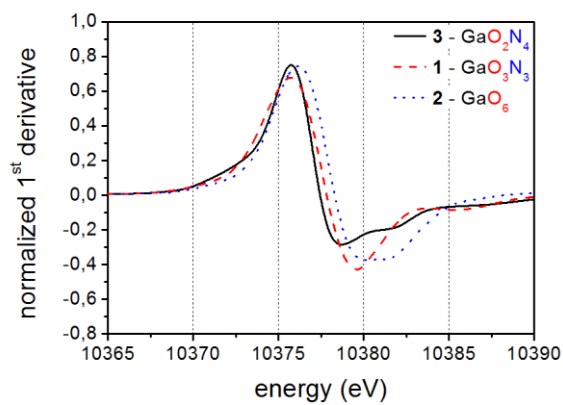
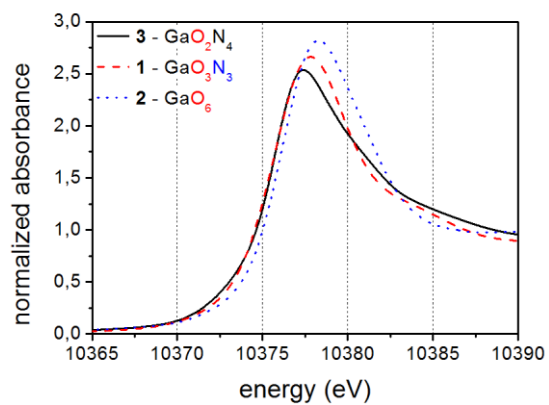
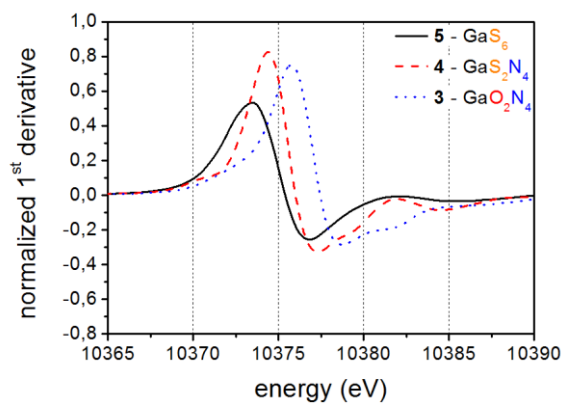
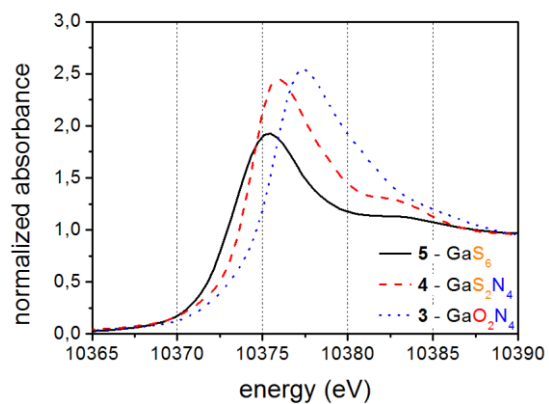


Figure 2: Normalized spectra and 1st derivatives of the model compounds and of **1** in the presence of minimum essential medium, fetal calf serum, apo-transferrin, human serum albumin and as BN preparation.

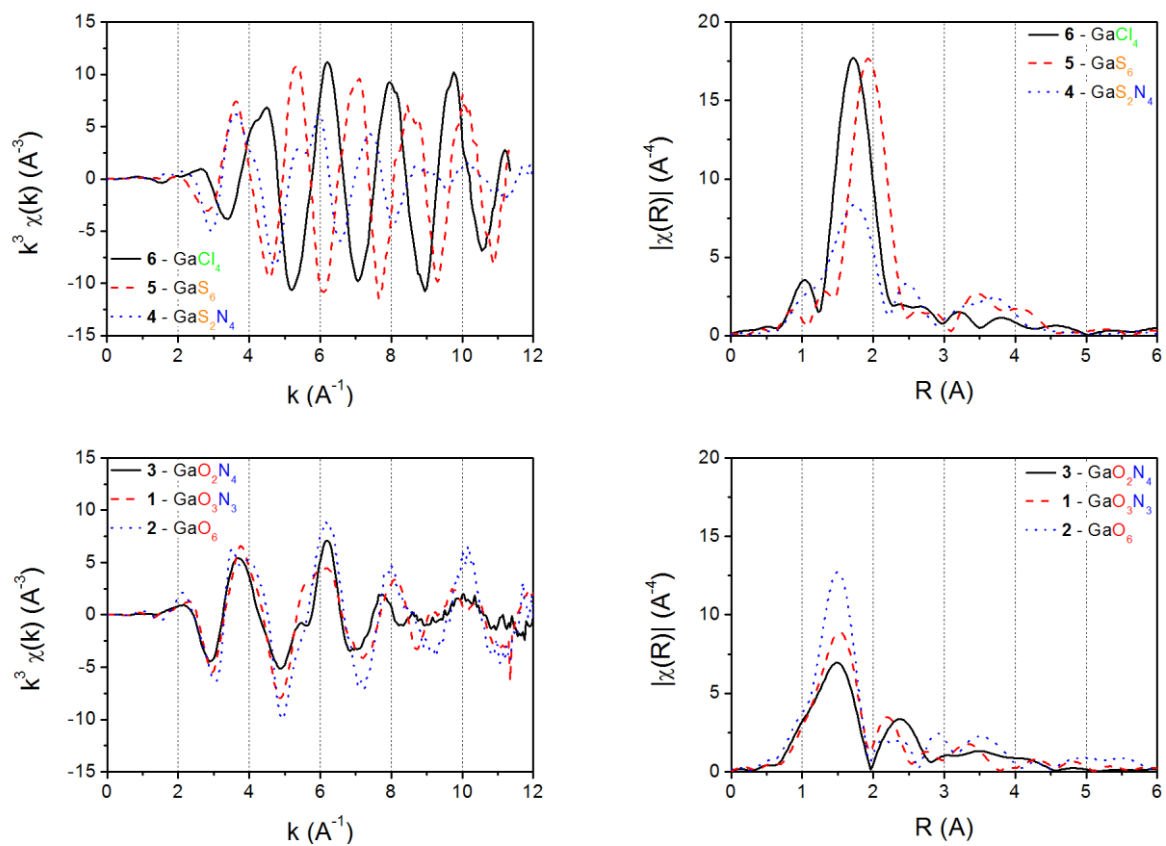


Figure 3: Extracted fine structures and Fourier transforms of the model compounds

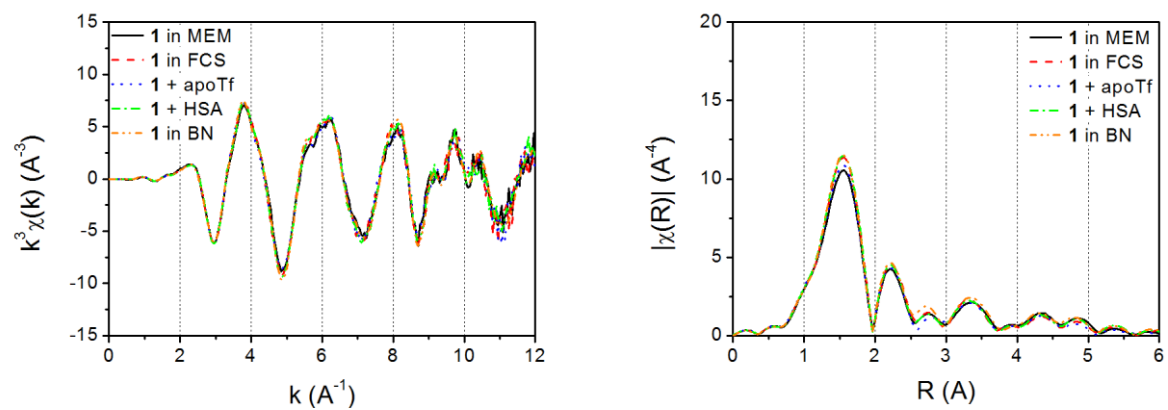


Figure 4: Extracted fine structures and Fourier transforms of **1** in the presence of minimum essential medium, fetal calf serum, apo-transferrin, human serum albumin and as BN preparation.

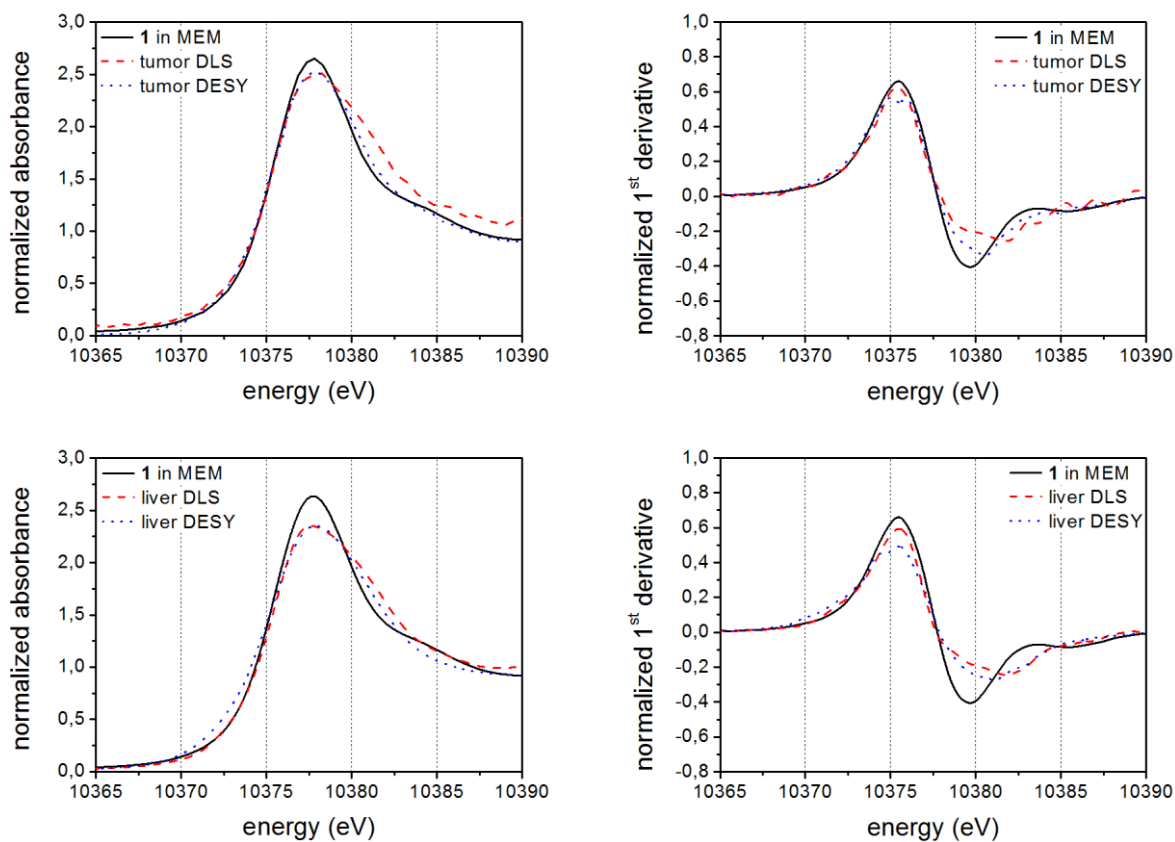


Figure 5: Tumor and liver XANES data and corresponding normalized 1st derivatives in comparison to **1** in MEM.

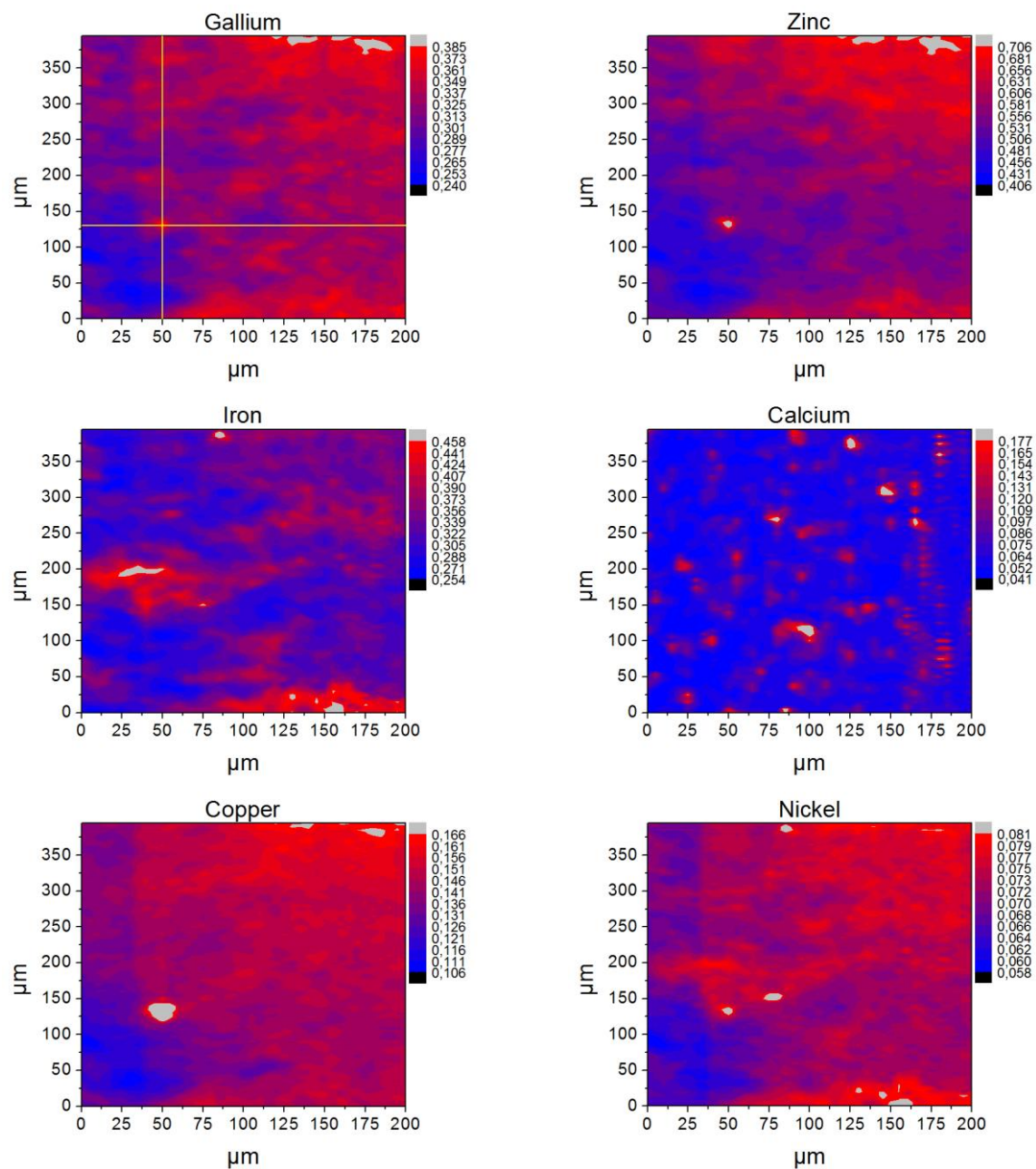


Figure 6: Normalized micro-XAS maps of tumor tissue, upper limit of the color maps is the 99 percentile. Taken at beamline I18 at Diamond Light Source (UK)

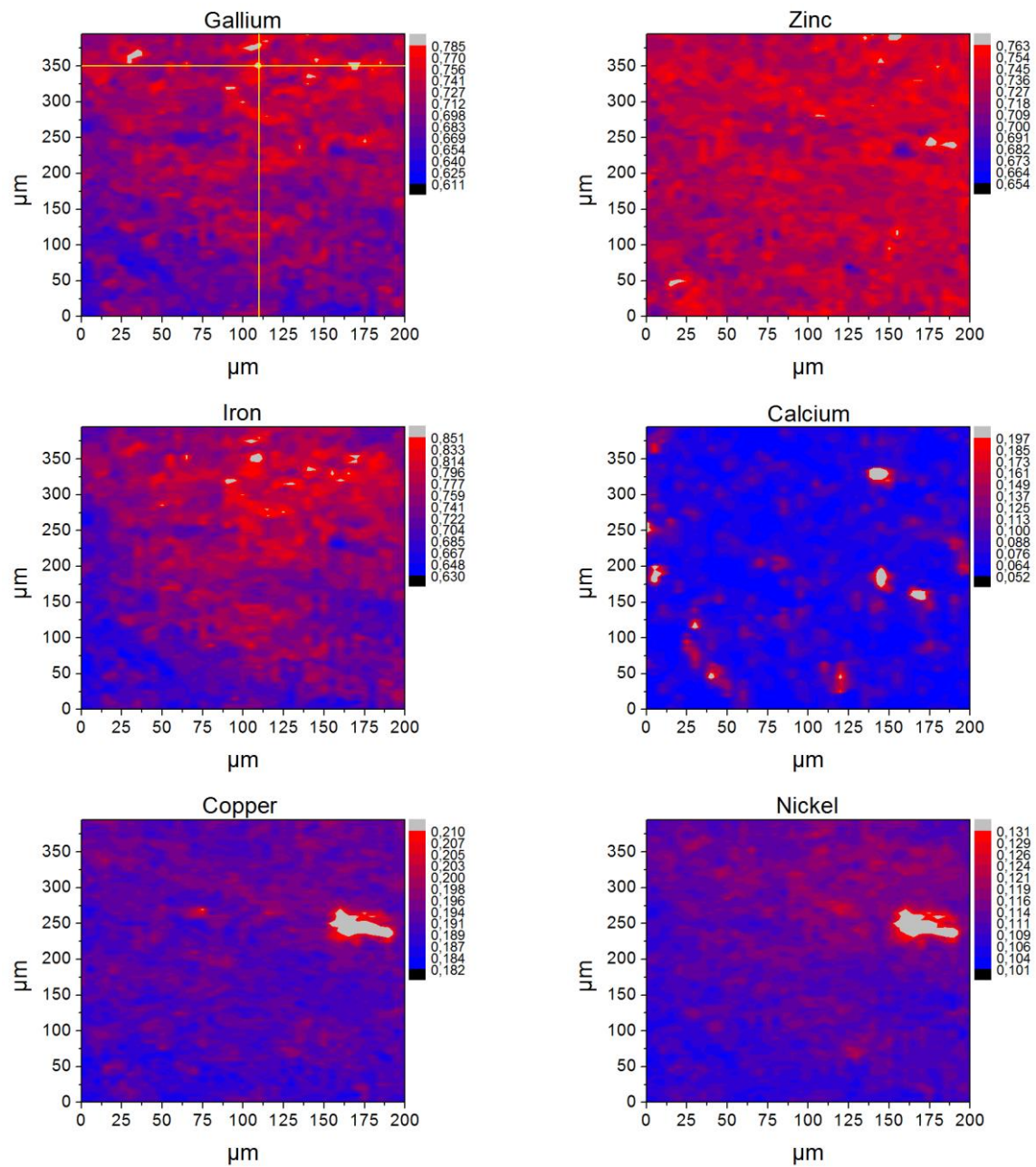


Figure 7: Normalized micro-XAS maps of liver tissue, upper cut-off of the color maps is the 99 percentile. Taken at beamline I18 at Diamond Light Source (UK).

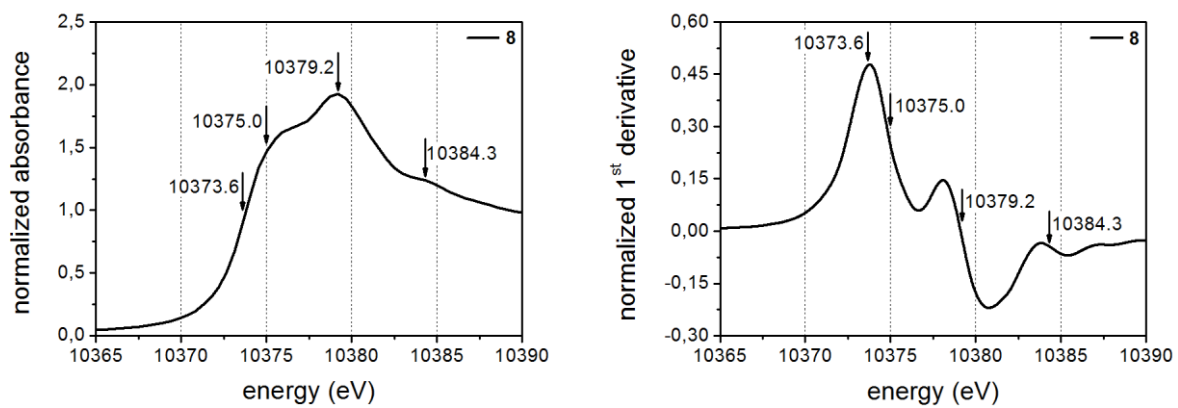


Figure 8: Normalized spectrum and 1st derivative of **8**.

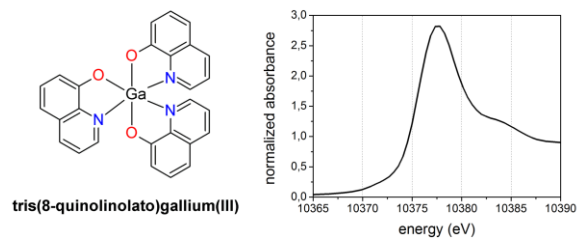


Table of Contents graphic

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