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ESI–MS Characterisation of Protein Adducts of Anticancer Ruthenium(II)-Arene PTA (RAPTA) Complexes

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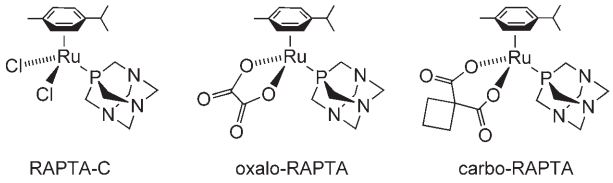
Metal-based drugs are playing an increasing role in the field of modern anticancer pharmacology. Indeed, following the clinical success of cisplatin in the treatment of various cancer forms, several other metal complexes, both platinum and nonplatinum, were designed, prepared, and tested as experimental anticancer drugs.[1] Among them, ruthenium-based metallodrugs look very promising. Remarkably, two ruthenium(III) complexes, that is, KP1019 (indazolium *trans*-[tetrachlorobis(1H-indazole)ruthenate(III)]) and NAMI-A (imidazolium *trans*-[tetrachloro(DMSO) (imidazole)ruthenate(III)]), were reported to exhibit outstanding anticancer and/or antimetastatic properties in vivo and are currently undergoing clinical trials.[2,3] In addition, a number of ruthenium(II) arene compounds were shown to possess very encouraging cytotoxic and antitumour properties in preclinical models[4] and are being intensely investigated.

As the mechanisms of action of ruthenium-based anticancer compounds are still largely unexplored and controversial,[5] it is of particular interest to develop specific methods to analyse in detail their reactivity toward potential biomolecular targets, in particular proteins. In fact, in contrast to classical platinum drugs that are known to target genomic DNA, the true targets of ruthenium drugs are not yet well defined; for instance, it was suggested that DMSO ruthenium(III) drugs might either directly interfere with specific proteins involved in signal transduction pathways or alter cell adhesion processes.[6]

In recent years, electrospray ionisation mass spectrometry (ESI–MS) has emerged as an extremely valuable and powerful method to monitor the formation of protein adducts of classical platinum drugs at the molecular level, and to identify the precise nature of the resulting metallic fragments attached to protein side chains.[7] However, to the best of our knowledge, only very few ESI–MS studies have dealt with protein adducts formed by nonplatinum anticancer drugs.[8] We report here an ESI–MS investigation of the reactions of three arene-capped ruthenium(II) compounds,[9] belonging to the RAPTA family, with two small proteins, namely horse heart cytochrome c (cyt c) and hen egg white lysozyme. Cyt c is an important protein crucially involved in apoptotic pathways,[10] whereas lysozyme is relevant in certain defence mechanisms.[11] Moreover, their size and overall properties render these proteins particularly suitable for ESI–MS studies.

Owing to the intrinsic high quality of the obtained ESI–MS spectra, a rapid and unambiguous assignment of the resulting metallodrug/protein adducts could be achieved as well as a straightforward identification of the nature of metal-containing molecular fragments, attached to these proteins. In addition, the presence of distinct metal binding sites on these proteins was revealed, favouring formation of different types of metallo-fragments.

The ruthenium compounds used in this investigation are shown . All of them share a common structural motif consisting of a ruthenium(II) centre bound to both an arene (cymene in this case) and to a 1,3,5-triaza-7-phosphaadamantane (pta) ligand. They only differ in the nature of the ligands located at the two remaining coordination positions. Notably, replacement of the two chloride groups (that are present in RAPTA-C) with bidentate ligands [either oxalate—to form Ru(η^6-cymene)(pta)(C_2O_4) (oxalo-RAPTA)—or cyclobutane dicarboxylate—to give Ru(η^6-cymene)(pta)($C_6H_6O_4$) (carbo-RAPTA)]—greatly reduces the rate of the aquation processes, thus modifying their overall solution behaviour, without adversely affecting cytotoxicity.[12] The three investigated complexes essentially manifest a similar cell-growth inhibition activity against a number of representative cancer cell lines (HT29 colon carcinoma, the A549 lung carcinoma, and the T47D and MCF7 breast carcinoma). The binding of a wide range of RAPTA derivatives to oligonucleotides was formerly studied but no direct correlation between oligonucleotide binding and cytotoxicity could be observed.[13] This finding might suggest that protein targets are of greater importance in producing the observed cytotoxic effects.



Adducts of RAPTA complexes with cyt c were prepared by incubating each RAPTA compound with horse heart cytochrome c, under the solution conditions described in the experimental section. The resulting ruthenium-protein adducts were analysed by visible absorption spectroscopy or ESI–MS. Even

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under aerobic conditions, cyt c, in the presence of RAPTA-C or carbo-RAPTA, undergoes partial reduction, with time, to the ferrous species, as may be inferred from the typical modifications of the Q bands, around 500–550 nm in the UV-Vis spectra. Figure 1 shows the progressive changes of the absorption spectra of cyt c, observed after addition of 1 equivalent RAPTA-C, over a period of 24 h. When the equilibrium is reached (after 24 h at 25 °C) about 30% of total cyt c is present in its reduced form. Similar spectral effects are observed for carbo-RAPTA but not for oxalo-RAPTA. Notably, this phenomenon was not observed for free cyt c, in the absence of ruthenium complexes, working under identical experimental conditions. Such behaviour is in line with spectrophotometric effects produced by other ruthenium compounds (unpublished results from our laboratory).

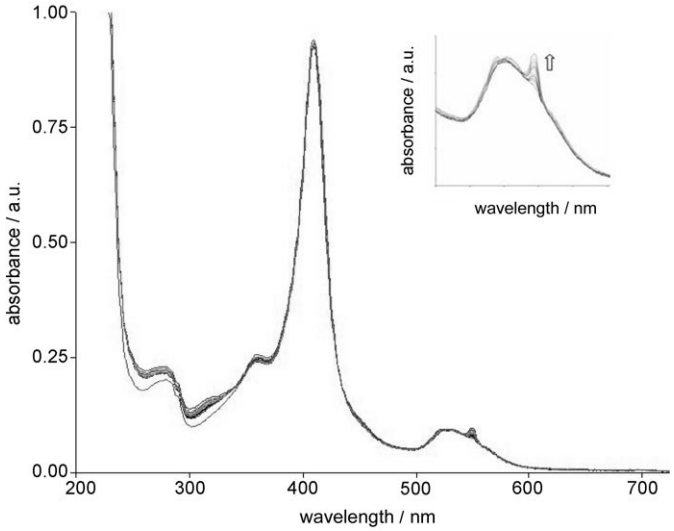

Figure 1. Absorption UV-visible spectra of cytochrome c a) before and b) after addition of 3 equivalents of RAPTA-C. Spectra were recorded at different times over 24 h at 25 °C. The inset shows the detail of the modification of the Q bands in the spectra with time due to the reduction of the iron center.

Deconvoluted ESI–MS spectra, recorded after 48 h incubation of cyt c with the three RAPTA compounds, are shown in Figure 2. The obtained ESI–MS spectra exhibit a very favourable signal to noise ratio; the presence of a limited number of well resolved peaks, with mass values higher than the native protein, renders their assignment to specific metallodrug/protein adducts rather straightforward. By comparing the intensity of the peak of the native protein with that of the main monoruthenated species it is evident that the reaction of cyt c with RAPTA-C results in extensive protein metallation; moderate protein ruthenation is observed in the case of carbo-RAPTA, whereas only a very low amount of ruthenated adducts are formed in the case of oxalo-RAPTA. These large differences in adduct formation are tentatively ascribed to intrinsic differences in the ability of the various RAPTA species to release their leaving groups and subsequently react with protein side chains. In fact, RAPTA-C is known to hydrolyse rather rapidly,

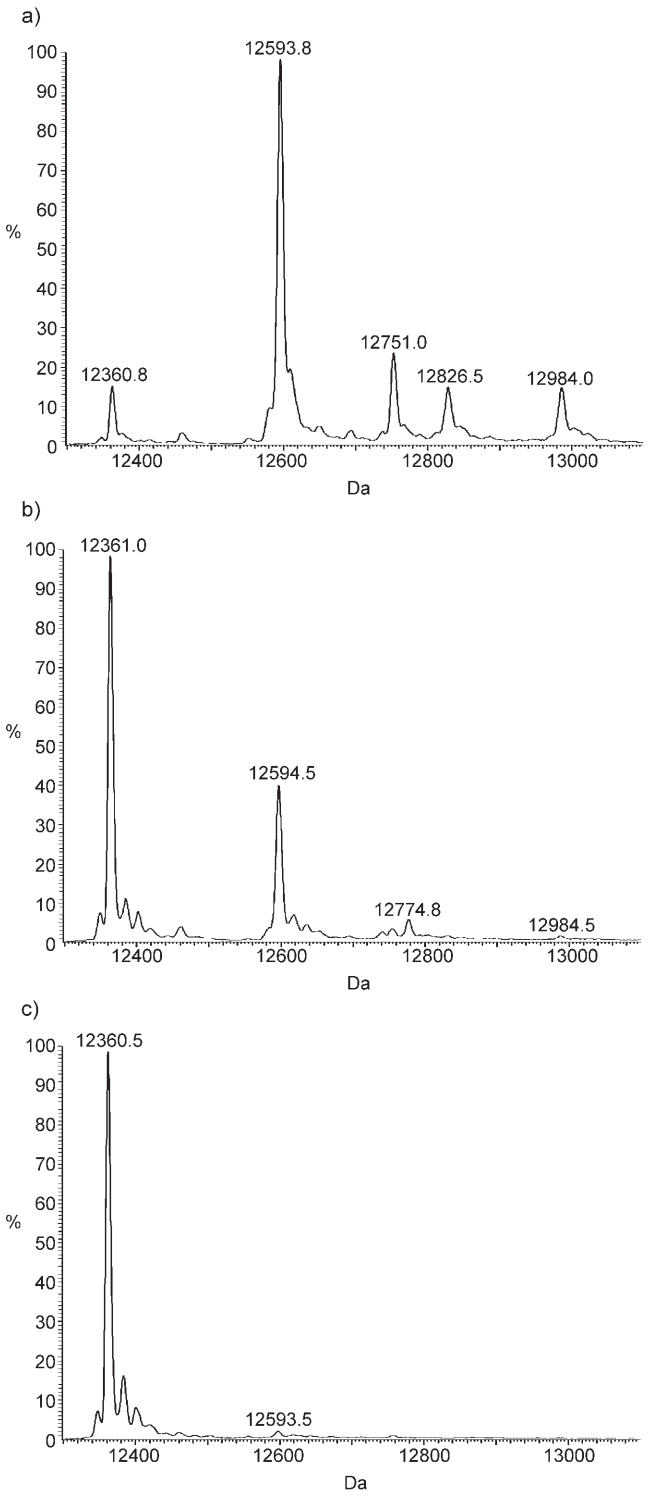

Figure 2. Deconvoluted ESI–MS spectra of adduct formed between cytochrome c and a) RAPTA-C, b) carbo-RAPTA and c) oxalo-RAPTA respectively, after 48 h incubation at 37 °C. The initial ruthenium/protein stoichiometry of each sample is 3:1.

whereas ligand substitution reactions are comparatively slower for carbo-RAPTA and much slower for oxalo-RAPTA.[12]

Notably, in the case of RAPTA-C, four distinct adducts with cyt c are formed; two of them are assigned to monoruthenat-

ed species whereas the other two are assigned to doubly ruthenated species. In more detail, the deconvoluted ESI–MS spectrum of the RAPTA-C derivative shows an intense peak at 12594 Da that corresponds to an adduct in which a single [(η^6-cymene)Ru] fragment is bound to the native holoprotein and another rather intense peak at 12751 Da, corresponds to the binding of a [(η^6-cymene)(pta)Ru] fragment.

Two additional intense peaks, of higher *m/z* value, are observed (see Figure 2a), at 12826 and 12984 Da, that correspond to 2:1 ruthenium–protein adducts; their masses exactly match the sum of cyt c plus two [(η^6-cymene)Ru] fragments or cyt c plus one [(η^6-cymene)Ru] and one [(η^6-cymene)(pta)Ru] fragment, respectively. The pattern of the observed ESI–MS peaks suggests that two distinct anchoring sites for ruthenium exist on cyt–c: the first one is able to accommodate a [(η^6-cymene)(Ru] moiety, favouring the release of the pta ligand, whereas the second one may bind either a [(η^6-cymene)(pta)Ru] or a [(η^6-cymene)(Ru] moiety.

Carbo-RAPTA exhibits a peak for the monometallated species at 12594 Da, analogous to RAPTA C, and an additional peak at ~12775 Da that may be tentatively assigned as a [Ru(pta)($C_6H_6O_4$)(OH)] adduct, that is, an adduct in which the η^6-cymene has been lost instead of the carboxylate ligand. In addition, because of the loss of the cymene ligand which uses three coordination sites on the ruthenium centre, a water or hydroxide ligand is also present in such a way to reduce coordinative unsaturation.[13] Loss of the η^6-cymene in the place of the carboxylate ligand is not an unexpected feature being in agreement with a previous study describing the binding of ruthenium(II) arene complexes to oligonucleotides.[13] In contrast, only one peak, of very low intensity, is observed at 12984 Da that may be ascribed to a bis-ruthenated species, in line with the lower reactivity of carbo-RAPTA.

In the case of oxalo-RAPTA a peak of very low relative intensity at 12593 Da is seen that corresponds to a monoruthenated, [(η^6-cymene)Ru], species.

Additional experiments were then carried out to monitor the binding process in more detail. In particular, protein metallation was investigated by collecting ESI–MS at increasing time intervals after mixing (see Supporting Information). From comparison of the relative intensities of the peak of the native protein with that of the major monoruthenated species a rough estimate of the binding kinetics could be obtained. In the case of the RAPTA-C/cyt c (3:1) system we observed that the percentage of ruthenation, three hours after mixing, is only 30% whereas, after 24 h, protein ruthenation is nearly complete.

Some of the above results (namely those concerning the RAPTA-C derivative) were subsequently confirmed by high resolution mass spectrometry measurements, carried out on an Orbitrap instrument. In Figure 3 observed and theoretical spectra of 8^+ charged state are shown for a) cyt c (I), b) cyt c + [(η^6-cymene)Ru] fragment (II) and c) cyt c + [(η^6-cymene)(pta)Ru] fragment (III) . Remarkably, the obtained experimental data perfectly match theoretical expectations, thus confirming our hypotheses on the chemical nature of protein bound fragments.

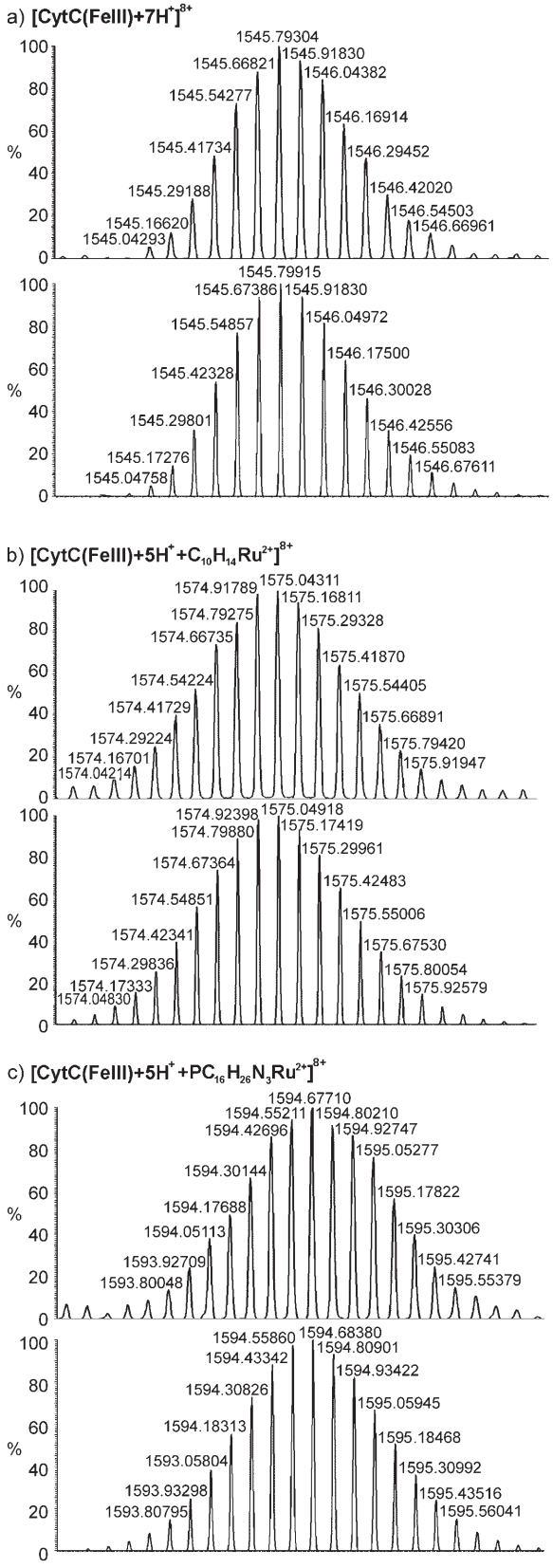

Figure 3. Comparison between the observed (upper) and theoretical (lower) spectra of 8^+ charge state of a) cyt c (I), b) cyt c + [(η^6-cymene)Ru] fragment (II), and c) cyt c + [(η^6-cymene)(pta)Ru] fragment (III). Data were recorded with an Orbitrap high-resolution mass spectrometer (Thermo, San Jose, CA).

A comparison of the protein binding behaviour of these three RAPTA complexes with that of related, clinically-proven, platinum drugs, such as cisplatin, carboplatin, and oxaliplatin, is worth making. In keeping with the RAPTA series, cisplatin shows more extensive binding to cyt c than the other derivatives.[14] Under essentially equivalent conditions cisplatin forms mono-, bis-, and tris-adducts whereas only mono- and bis-adducts are formed with RAPTA-C. Carboplatin and oxaliplatin produce only monoplatinated adducts closely resembling the reactivity pattern observed for the ruthenium analogues reported herein.

It is worth noting that incubation of [Ru(η^6-biphenyl)(en)Cl][PF_6] (where en = ethylenedianime) with cyt c (10:1 ratio) monitored by ESI–MS analysis showed the formation of only monoruthenated species,[15] which appear to be less abundant than those of RAPTA-C, possibly reflecting the difference in the sites that can undergo hydrolysis, that is, [Ru(η^6-biphenyl)(en)Cl][PF_6] has one chloride that can hydrolyse whereas RAPTA-C has two chloride ligands that can potentially be substituted by water.

For comparative purposes, these three RAPTA complexes were reacted with lysozyme and the resulting reaction products analysed by ESI–MS. The deconvoluted ESI–MS spectrum of the RAPTA-C adduct, after 48 h incubation at 37 °C, is shown in Figure 4. The spectrum is diagnostic of appreciable adduct formation. Two monoruthenated adducts are observed at 14538 Da for the [(η^6-cymene)Ru] fragment and 14694 Da for the [(η^6-cymene)(pta)Ru] adduct. Thus, analysis of the ESI–MS provides clear evidence for the presence of identical metallic fragments to those seen in the case of cyt c. Overall, the three RAPTA complexes show a markedly lower reactivity with lysozyme compared to cyt c. The structure of a Ru-η^6-cymene adduct of lysozyme was previously characterised by X-ray diffraction;[16] a [(η^6-cymene)$RuCl_2$] fragment was found to be coordinated to His 15. The sample preparation reported in that paper is somewhat different to that used herein, involving a chloride saturated solution; nevertheless, the X-ray data and the ESI–MS data are in reasonable agreement.

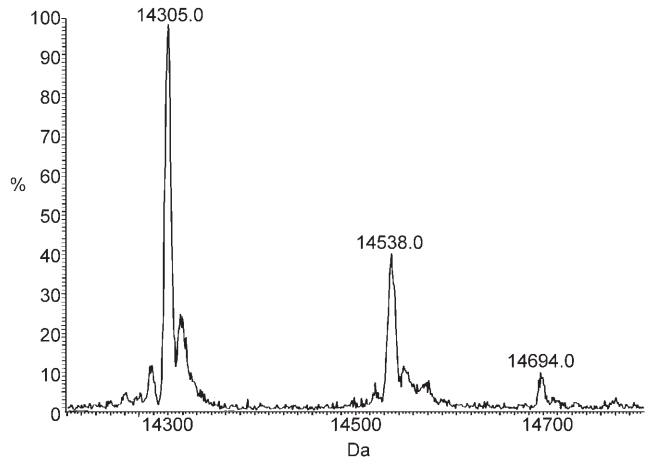

Figure 4. Deconvoluted ESI–MS spectra of lysozyme-adduct with the RAPTA-C complex after 48 h incubation at 37 °C. The initial ruthenium/protein stoichiometry is 3:1.

Previous studies had specifically addressed the interactions of a variety of ruthenium complexes and ruthenium anticancer drugs with proteins. For instance, extensive spectroscopic and crystallographic studies were reported on the reactions of Keppler-type anticancer ruthenium(III) complexes, in particular KP1019, with the major serum proteins serum albumin and serum transferrin. These studies clearly pointed out that preferential binding sites for Ru^{III} are histidine residues located on the protein surface.[17] Analogous studies were carried out on NAMI-A/serum protein adducts leading to similar results.[18]

Gray and co-workers had previously reported that a stable pentaammineruthenium(III)-histidine-33 complex is formed in the reaction between aquopentaammineruthenium(II) and horse heart ferricytochrome c.[19] HPLC analysis of the tryptic hydrolysate of the modified protein was employed to identify the pentaammineruthenium binding site. Spectroscopic measurements showed that the integrity of the native structure in the vicinity of the heme c group is maintained in the ruthenium-modified protein. We have repeated this investigational approach on our system. The cytochrome c RAPTA-C derivative was digested with trypsin and the resulting peptides analysed by direct mass (see Experimental Section for full details). A pattern of tryptic peptides fully consistent with that reported by Gray et al. was obtained. Remarkably, difference spectra of the free and ruthenium-bound digest patterns revealed a new parent ion carrying a double charge with an *m/z* value of 701 in the ruthenium-treated protein (see Figure b in the Supporting Information). Further analysis showed that the parent ion with *m/z* 701 had an isotope distribution characteristic of ruthenium. Notably, the molecular mass of this new fragment corresponds to the cyt c peptide ^{28}Thr-Gly-Pro-Asn-Leu-His-Gly-Leu-Phe-Gly-Arg^{38} bearing a Ru^{II}-η^6-(cymene) moiety. This observation strongly supports the idea that His 33 is a major interaction site for RAPTA-C.

In conclusion, our study has demonstrated the importance and the value of the ESI–MS method to gain specific and rapid information on the reactions of ruthenium metallodrugs with two representative small proteins. Formation of stable adducts could be unambiguously assessed and the nature of the protein bound metallic fragments fully elucidated. Preferential binding of ruthenium metallofragments to surface histidines has been shown. The reported experimental approach has the potential to be extended to larger proteins that are believed to be actual macromolecular targets for ruthenium metallodrugs, taking advantage of the rapid progresses of this analytical method.

Experimental Section

Materials. Horse heart cytochrome c and chicken egg white lysozyme were obtained from Sigma (Code C7752 and L7651, respectively).

RAPTA-C, carbo-RAPTA, and oxalo-RAPTA were prepared according to literature procedures.[20,12]

UV-visible absorption spectroscopy. The interaction of horse heart cytochrome c (10^{-5} M) with the ruthenium complexes, was monitored in H_2O MilliQ pH 5–6, at 25 °C, at different times over a

period of 24 h. The ruthenium/protein adducts were prepared in 1:1 ratio. Spectra were recorded on a Perkin–Elmer Lambda 20 Bio instrument.

ESI–MS analysis. Samples were prepared in H_2O MilliQ pH 5–6, with a protein concentration of 10^{-4} M, and a ruthenium to protein ratio of 3:1. The reaction mixtures were incubated for different time intervals (3, 6, 24 and 48 h) at 37 °C. Samples were extensively ultrafiltered using Centricon YM-3 (Amicon Bioseparations, Millipore Corporation) to remove the unbound complex. After a 100-fold dilution with MilliQ water, ESI–MS spectra were recorded by direct introduction at 3 μL min^{-1} flow rate in a LTQ linear ion trap (Thermo, San Jose, California), equipped with a conventional ESI source. The specific conditions used for these experiments were as follows: the spray voltage was 3.2 kV, the capillary voltage was 32 V, and the capillary temperature was kept at 353 K. Sheath gas was set at 15 (arbitrary units), the sweep gas and auxiliary gas were kept at 0 (arbitrary units). ESI spectra were acquired using Xcalibur 2.0 software (Thermo) and deconvolution was obtained using Bioworks 3.2 software (Thermo). The mass step size in deconvolution calculation was 0.25 Da and the spectrum range was 1100–2000 *m/z*.

Spectra of the same samples were also recorded on an Orbitrap high-resolution mass spectrometer (Thermo, San Jose, CA) and the obtained results were fully consistent (data not shown). The instrument was equipped with a conventional ESI source. The working conditions were the following: spray voltage was 2.3 kV, capillary voltage 20 V, and capillary temperature was kept at 403 K. Sheath gas was set at 16 (arbitrary units), the sweep gas and auxiliary gas were kept at 0 (arbitrary units). For acquisition, Xcalibur 2.0 software (Thermo) was used and monoisotopic and average deconvoluted masses were obtained by using integrated Xtract tool. For spectra acquisition a nominal resolution (at *m/z* 400) of 10 000 was used.

For tryptic digestion analysis 40 μL of cytochrome c (10^{-4} M), either free or reacted with RAPTA-C, were diluted in 10 mM ammonium bicarbonate and bovine trypsin was added in a ratio of 1:50 (*w/w*). The mixture was incubated for 16 h at 37 °C. Then, the trypsin digested samples were separated with an Ultimate3000 system (Dionex) coupled to the LTQ Orbitrap mass spectrometer (Thermo). In detail, 0.5 μL of each sample were loaded on a Vydac C4 column (150 mm × 300 μm, 5 μm pore size) by using 100% solution A (95% water, 5% acetonitrile, 0.01% formic acid) and eluted using a linear gradient up to 40% solution B (95% acetonitrile, 5% water, 0.01% formic acid) in 35 min.

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