

Insights on the Mechanism of Thioredoxin Reductase Inhibition by Gold N-Heterocyclic Carbene Compounds Using a Synthetic Selenododecapeptide: an ESI-MS investigation

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Abstract

Gold-based drugs typically behave as strong inhibitors of the enzyme thioredoxin reductase (TrxR), possibly as the consequence of direct gold(I) coordination to its active site selenocysteine. To gain a deeper insight into the molecular basis of enzyme inhibition and prove gold-selenocysteine coordination, the reactions of three parent gold(I) NHC compounds with a synthetic dodecameric selenopeptide, exactly reproducing the C-terminal portion of TrxR, were investigated by ESI-MS. Formation of 1:1 gold-peptide adducts, though in highly different amounts, was demonstrated in all cases. In these adducts the same $[\text{AuNHC}]^+$ moiety is always associated to the intact peptide. Afterward, tandem MS experiments, conducted on a specific gold-peptide complex, pointed out that gold is coordinated to the selenolate group. The relatively large strength of the gold-selenolate coordinative bond well accounts for potent enzyme inhibition typically afforded by these gold(I) compounds. In a selected case, the time course of enzyme inhibition was explored. Interestingly, enzyme inhibition turned out to show up quickly and reach its maximum just a few minutes after mixing. Overall, the present results offer some clear insight into the process of thioredoxin reductase inhibition by gold-based drugs.

INTRODUCTION

Thioredoxin reductase (TrxR) is an important and ubiquitous enzyme critically involved in the regulation of intracellular redox metabolism. The crystal structures of a few enzyme isoforms are now available.¹ The human enzyme typically consists of two 55 KDa subunits, each bearing two distinct active sites located far apart within the protein structure; remarkably, the C-terminal active site - comprising a 12 amino acid extension - contains a functional selenocysteine residue adjacent to a cysteine,² allowing redox cycling.

Figure 1. Crystal structure of human thioredoxin reductase (PDB 2CFY).

A number of recent reports suggest that thioredoxin reductase constitutes an important “druggable target” for the development of new anticancer agents.³ Indeed, modulation of TrxR activity through selective inhibitors may contrast effectively cancer cell proliferation. Owing to the presence of a functional selenolate group, it was suggested that thioredoxin reductase might represent a primary target for experimental gold compounds possessing a pronounced thiophilic character. Later on, a few studies documented potent inhibition of TrxR activity by a variety of cytotoxic gold compounds with IC₅₀ values typically falling in the low micromolar (and even nanomolar) range.^{3a,4} It was hypothesised that such strong inhibition of TrxR activity might result into a profound alteration of the mitochondrial membrane potential, ultimately leading to apoptotic cell death through activation of the mitochondrial pathway.

The precise molecular mechanism of TrxR inhibition by gold compounds has not been elucidated yet, in a conclusive way. However, there are several indications that enzyme inhibition may arise from direct coordination of gold(I) to the functional selenocysteine.⁵ This view is corroborated by the established coordination chemistry concept that “soft” gold(I) ions manifest high affinity for “soft” donors such as the selenolate group. A previous study of ours concerning

the interactions of two gold compounds with a short tetrapeptide sequence containing a selenocysteine provided indeed results fully consistent with this hypothesis.⁶

Gold carbene N-heterocyclic carbene (NHC) compounds have attracted a great deal of interest during the last few years for their potential bio-medical applications.⁷ Three structurally related gold NHC complexes (**1**, **2** and **3**) were prepared and characterised, recently, in one of our laboratories, showing outstanding anti-proliferative and anti-cancer properties.⁸ These compounds are synoptically depicted in Chart 1. They are linear di-coordinated gold(I) complexes. On one side, they invariantly bear the same carbene ligand (L1) *i.e.* 1,3-diethylbenzimidazol-2-ylidene. On the opposite side the gold(I) center is bonded to a second ligand (L2), which can be, in turn, chloride (**1**) or a second NHC ligand (identical to the first one) (**2**), or triphenylphosphine (**3**) (as specified in Chart 2).

Chart 1. The gold compounds tested.

Chart 2. Differentiation of L1 and L2 ligands.

These compounds were previously reported to cause pronounced inhibition of TrxR, with IC₅₀ values of 0.36 μ M, 4.89 μ M, and 0.66 μ M respectively.⁸ TrxR inhibition is believed to be at the basis of their remarkable antiproliferative effects *in vitro*.⁸ In line with the above arguments, TrxR inhibition is thought to occur through direct binding of the gold(I) center to the active site selenocysteine following ligand substitution.

To prove this mechanistic hypothesis we have prepared the C-terminal dodecapeptide of TrxR, corresponding to the sequence Ac-SGGDILQSGCUG-NH₂, and analysed its interactions with the three investigational gold carbenes, mainly by ESI-MS analysis. For comparison purposes the synthetic peptide was

also reacted with *Auranofin* (**4**), an established gold(I) containing drug in clinical use since 1985 for the treatment of rheumatoid arthritis.⁹ ESI-MS analysis support our working hypothesis. In addition, the time dependence of TrxR inhibition by these compounds has been investigated to better elucidate the time course of the enzyme inhibition process.

MATERIALS AND METHODS

Preparation of the seleno-dodecapeptide and ESI-MS characterisation.

The seleno-dodecapeptide was prepared through classical solid phase peptide synthesis, in accordance with the method previously described.⁶ Fmoc-Sec(Mob)-OH was prepared starting from Selenocystine (Sigma-Aldrich), in accordance with Moroder¹⁰ and Hondal,¹¹ with a bidimensional orthogonal protection scheme suitable for solid-phase peptide synthesis. The peptide Ac-SGGDILQSGCUG-NH₂ was synthesized starting from Rink Amide MBHA resin (500 mg; 0.4 mmol/g). All the reactions were performed on a semi-automatic synthesizer (MultiSynTech, Germany) following the standard SPPS method and using the orthogonal Fmoc/*t*-Bu protection scheme. All the amino acids, according to the reported sequence, were introduced following the HBTU/NMM coupling strategy via active esters. The coupling reactions were performed by using an excess of protected amino acids and of the activating agent HBTU (2.5 eq.) and of NMM (5 eq.) in DMF, vortexing for 40 min at room temperature. After each coupling, the resin was washed with DMF and DCM. Coupling reactions efficiency was monitored by the Kaiser test.¹²

Deprotections were performed with 20% piperidine in DMF and after deprotection of the first amino acid of the sequence, the NH₂ terminal group was acetylated with a solution of acetic anhydride (20 eq.) and NMM (20 eq.). The resin-containing mixture was then swollen at room temperature for 30 min. The reaction was repeated once again with fresh solutions. Afterward, the resin was washed with DMF, DCM, and dried under vacuum.

Formation of the S-Se bridge was obtained by changing the original method for Mob removal,¹³ while cleaving the peptide from the resin (Scheme 1) using 92.5% trifluoroacetic acid (TFA), triisopropylsilane (TIS) 2.5%, thioanisole 2.5%, and H₂O 2.5%, as cleavage cocktail with 1.0 equivalent of 2,2'-Dithiobis(5-nitropyridine) (DTNP) *per* selenium.

Scheme 1. Supposed mechanism of DTNP mediated S-Se bond formation.

On the ground of literature data, the rationale for this reaction is that the highly acidic solution of DTNP in TFA activates DTNP by protonation of the pyridine nitrogen atom. This creates a very good electron sink and enhances the electrophilicity of the disulfide bond of DTNP. The highly nucleophilic selenium atom attacks the DTNP to form a Sec(5-Npys) residue. Since there is a nearby Cys(Mob) residue, the sulphur atom of this amino acid can attack the reactive selenylsulfide bond, with concomitant loss of the Mob group and the formation of the desired cyclic peptide (Scheme 2). At the end of the reaction, the cleavage mixture was filtered and the resin was washed with TFA. The filtrate was evaporated under nitrogen flow. The crude product was precipitated with cold diethyl ether, collected by centrifugation, dissolved in H₂O and lyophilized. The crude peptide was then pre-purified by solid-phase extraction (SPE) with a RP-8 LiChroprep silica from Merck and using H₂O/CH₃CN as eluents. The purification of the peptide was performed by semi-preparative RP-HPLC on a Phenomenex C₁₈ 180 Å (250 × 10 mm, 5 µm) column; eluents: A 0.1% TFA in H₂O; B 0.1% TFA in CH₃CN; flow 4 ml/min; gradient 20% to 60% of B in 20 min. Characterization of the product was performed by HPLC-ESI-MS analysis carried out by a Phenomenex Aqua C₁₈ column (5 µm, 250 × 2.0 mm) (flow rate: 1 ml/min) on a Thermo Finnigan Surveyor HPLC-MS, using the previous solvent systems.

ESI-MS Experiments

The aqueous solution of the dodecapeptide Ac-SGGDILQSGCUG-NH₂, (1×10⁻⁴

M) was treated by 5.0 equivalents of dithiothreitol (DTT) to reduce the –S–Se– bond, and then allowed to react with 1.0 equivalent of the different gold complexes for 30 min at room temperature. The reaction products were analysed through ESI mass spectrometry, in negative ion mode.

ESI-MS spectra were recorded by direct injection at a 5 $\mu\text{l min}^{-1}$ flow rate in a LCQ Advantage 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 4.0 kV, the capillary voltage was 1.7 V and the capillary temperature was kept at 270 °C. Sheath gas was set at 25 (arbitrary units) whereas auxiliary gas was kept at 0 (arbitrary units). All ESI spectra were elaborated using Xcalibur software (Thermo). Under these conditions the dodecapeptide Ac-SGGDILQSGCUG-NH₂ gave a peak centred at 1181.1 m/z , perfectly matching expectations.

ESI-MS spectra in positive ion mode were recorded with the solution containing the dodecapeptide Ac-SGGDILQSGCUG-NH₂, incubated with **1**, in order to completely assess the nature of the adduct **5** (Chart 3), by direct introduction at 20 $\mu\text{l/min}$ flow rate in an Orbitrap high-resolution mass spectrometer (Thermo, San Jose, CA, USA). The working conditions were the following: spray voltage 3.1 kV, capillary voltage 22 V, capillary temperature 215°C and tube lens voltage 245 V. The sheath and the auxiliary gases were set, respectively, at 17 (arbitrary units) and 1 (arbitrary units). For spectra acquisition a nominal resolution (at m/z 400) of 100,000 and Xcalibur 2.0 software (Thermo) were used. For CID experiment on reduced dodecapeptide we used a normalized collision energy of 23 and an isolation width of 6.5 m/z while for dodecapeptide reacted with **1** the normalized collision energy was set to 24 and an isolation width was 10 m/z .

Time-dependence of TrxR inhibition by complex 1

To determine the time-dependent inhibition of TrxR, commercially available rat liver TrxR (from Sigma-Aldrich) were used and diluted with distilled water to

achieve a concentration of 2.0 U/mL. The compounds were freshly dissolved as stock solutions in DMF. To each 25 μ L aliquots of the enzyme solution each 25 μ L of potassium phosphate buffer pH 7.0 containing the compound (0.5 μ M) or vehicle (DMF) without compounds (control probe) were added and the resulting solutions (final concentration of DMF: max. 0.5% V/V) were incubated with moderate shaking for 0, 5, 10, 20, and 75 minutes at 37 $^{\circ}$ C in a 96-well plate. To each well 225 μ L of reaction mixture (1000 μ L reaction mixture consisted of 500 μ L potassium phosphate buffer pH 7.0, 80 μ L 100 mM EDTA solution pH 7.5, 20 μ L BSA solution 0.05%, 100 μ L of 20 mM NADPH solution, and 300 μ L of distilled water) were added and the reaction started by addition of 25 μ L of 20 mM DTNB solution in ethanol. After proper mixing, the formation of 5-TNB was monitored with a micro-plate reader (Perkin Elmer VictortmX4) at 405 nm in 10 s intervals for 6 minutes. The increase in 5-TNB concentrations over time followed a linear trend ($r^2 \geq 0.99$) and the enzymatic activities were calculated as the slopes (increase in absorbance per second) thereof. For each tested compound the non-interference with the assay components was confirmed by a negative control experiment using an enzyme free solution. The IC₅₀ values were calculated as the concentration of compound decreasing the enzymatic activity of the untreated control by 50% and are given as the means and error of 2-3 independent experiments.

RESULTS AND DISCUSSION

ESI-MS spectra of gold-peptide complexes

The synthetic peptide Ac-SGGDILQSGCUG-NH₂ was treated with the various gold compounds **1**, **2**, and **3**, at 1:1 molar ratio, in a 1:1 H₂O/CH₃CN solution. Incubation was allowed for 30 minutes at room temperature; then solutions were analysed by ESI-MS. The resulting ESI-MS spectra are shown in Figure 2a-c. For comparison purposes the mass spectrum of the same peptide reacted with **4** was also recorded (see Figure 2d).

Figure 2. ESI-MS spectra of the synthetic reduced dodecapeptide Ac-SGGDILQSGCUG-NH₂ incubated with **1** (a), **2** (b), **3** (c) and **4** (d), acquired in negative ion mode.

From inspection of the reported ESI-MS spectra it emerges that stable adducts are formed between the dodecapeptide Ac-SGGDILQSGCUG-NH₂ and the three gold NHC compounds. However, the degree of peptide metallation and adduct formation is greatly different in the three examined cases as it can be judged from direct comparison of peak intensities. Indeed, **1** causes extensive peptide metallation, while **2** induces moderate metallation. At variance, **3** is found to produce only a very tiny and nearly negligible amount of metallated peptide. Notably, in all cases, the same molecular fragment, corresponding to Au-NHC⁺, is found associated to the peptide, as highlighted in Figure 3. Evidence for the formation of a 2:1 metal/peptide adduct, in a very little amount, was obtained only in the case of **1**. Appreciable adduct formation was also observed when reacting the dodecapeptide with **4** as shown in figure 2d.

Based on the above results, it is inferred that the peptide (most likely through its selenolate group) is able to displace - at least partially - the L2 ligand from gold(I) coordination. However, this ligand substitution reaction appears to be progressively more difficult and less efficient upon passing from chloride to carbene to phosphine. Notably, this order does not reflect the relative strengths of gold ligand binding energies previously determined.⁸

Figure 3. Isotopic pattern of peptide Ac-SGGDILQSGCUG-NH₂/**1** complex (acquired in negative ion mode).

ESI-MS and MS² analysis of peptide-1 complex

To further prove direct binding of the gold center to the selenolic group of the dodecapeptide (please repeat the sequence), a more detailed spectral analysis

was carried out on the peptide-**1** complex and additional MS² experiments performed on this derivative. Notably, the ESI-MS spectrum of this adduct reveals four principal peaks $[M+H]^+$, respectively centred at: *i*) 1185.41 m/z , corresponding to the free reduced peptide; *ii*) 1555.48 m/z , corresponding to species **5** in Chart 3; and *iii*) 1751.44 and *iv*) 1925.55 m/z , corresponding to species **6** and **7**, as depicted in Chart 3.

Figure 4. High-resolution ESI-MS spectra of the reduced dodecapeptide Ac-SGGDILQSGCUG-NH₂ incubated with **1**.

Chart 3. Principal adduct of **1**.

Afterward, the peak centered at 1555.48 m/z , and tentatively assigned to species **5**, was analysed in tandem mass spectrometry. Inspection of the corresponding peaks (Figure 5) revealed the presence of a number of fragments some of which are of paramount importance for elucidating the interaction of the dodecapeptide with this Au⁺ species (see Chart 4). While fragment *b9*, that does not contain C or U, is devoid of Au-NHC groups, the fragment *b10*, that bears the Cys residue, was found associated to the AuNHC group (through the sulphur atom). However, this same fragment was also found in the free form (i.e., without AuNHC). In contrast, fragment *b11*, which contains both C and U residues, showed *always* the presence of a bound AuNHC group. These results strongly support the view that binding of gold occurs preferentially at the selenolic group of selenocysteine and, only secondarily, at the thiol group of cysteine. Overall, these results provide a reliable and solid molecular basis for the mechanism of the TrxR inhibition by gold compounds, though analysed on a simplified dodecapeptide model.

Chart 4. Principal fragments derived from the adduct **5**.

Figure 5. Principal fragments of the synthetic dodecapeptide incubated with **1**: (a) *b9* and (b) *b10* corresponding only to the fragmented peptide. (c) *b10* and (d) *b11* corresponding to the peptide bonded to a residue of the gold-drug.

Time-dependence of TrxR inhibition by complex 1

Finally, the influence of a selected gold(I) NHC complex, namely **1**, on thioredoxin reductase activity was investigated at different time intervals working at a concentration slightly above the IC₅₀ value. Interestingly, soon after mixing relevant inhibition of the enzyme activity was observed that reached its maximum after about 5 minutes (corresponding to an inhibition of enzyme activity of ~ 50%). A slight progressive decrease in enzyme inhibition was then observed after 10, 20, and 75 minutes incubation (see Figure 6). These results clearly demonstrate that this gold(I) NHC complex manifests a strong and fast interaction with the target enzyme TrxR, significantly inhibiting its activity soon after mixing.⁸

Figure 6. Time-dependent inhibition of TrxR.

CONCLUSIONS

In conclusion, we have shown herein that three novel gold NHC complexes, endowed with interesting biological and pharmacological profiles, are able to react with the synthetic seleno-dodecapeptide Ac-SGGDILQSGCUG-NH₂ exactly reproducing the C-terminal portion of TrxR and containing one functionally relevant redox site. Such reactions result into formation of variable amounts of monometallated adducts where a gold NHC fragment is directly anchored to the selenolate group. Direct gold(I) coordination to the selenium atom was strongly supported by a tandem MS experiments. The ability of the three gold carbenes to bind firmly the selenopeptide and blocking the selenolate group, nicely accounts,

at least qualitatively, for profound inhibition of thioredoxin reductase activity induced by the same gold complexes. In the case of complex **1** the time dependency of enzyme inhibition was explored showing that substantial enzyme inhibition is achieved soon after mixing. Further support is thus offered to the hypothesis that thioredoxin reductase is a realistic target for cytotoxic gold compounds and details are given on the possible molecular mechanism of enzyme inhibition and its time course.

Acknowledgements

Authors acknowledge Beneficentia Stiftung (Vaduz, Liechtenstein), Ente Cassa di Risparmio di Firenze and Regione Toscana (NANO-TREAT project) for financial support. E.M. thanks Ministero dell'Istruzione, dell'Università e della Ricerca (PRIN 2008). COST action CM1105 is also gratefully acknowledged.

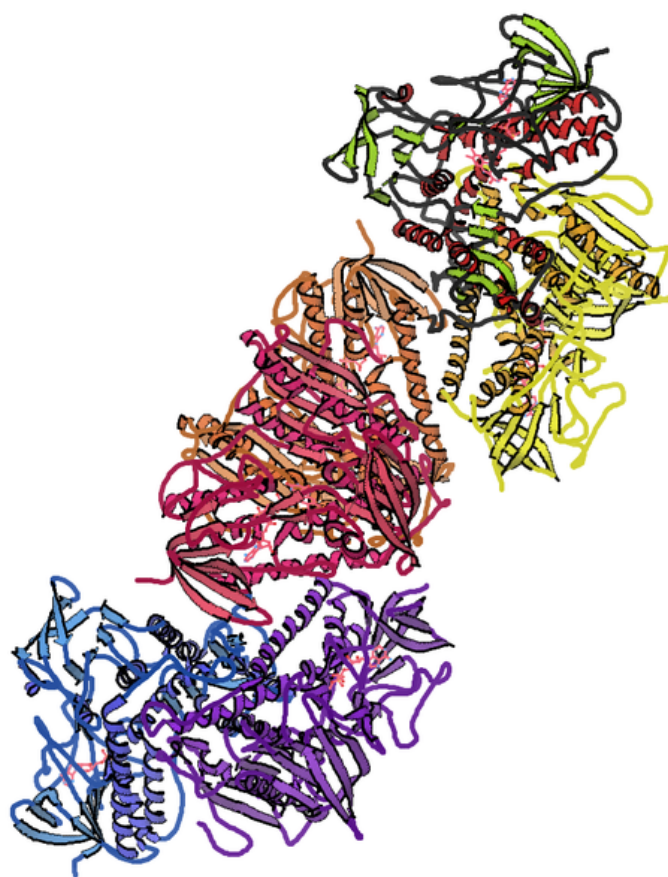


Figure 1. Crystal structure of the human thioredoxin reductase (PDB 2CFY).

Chart 1. The gold compounds tested.

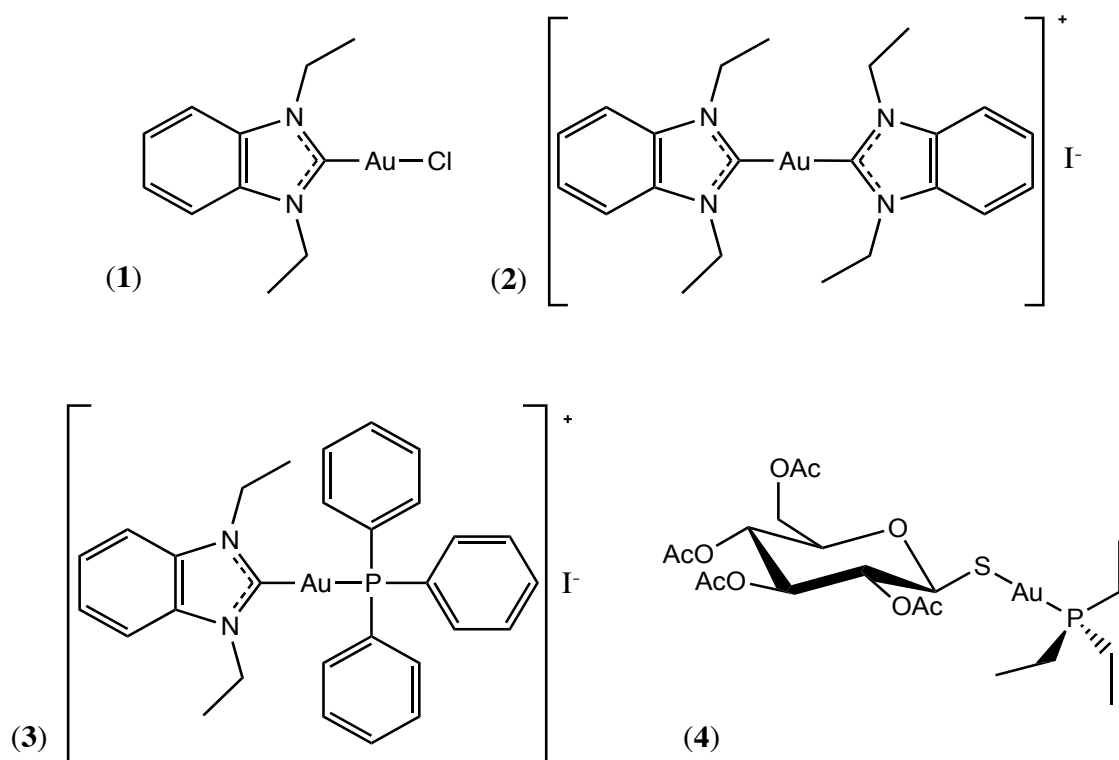
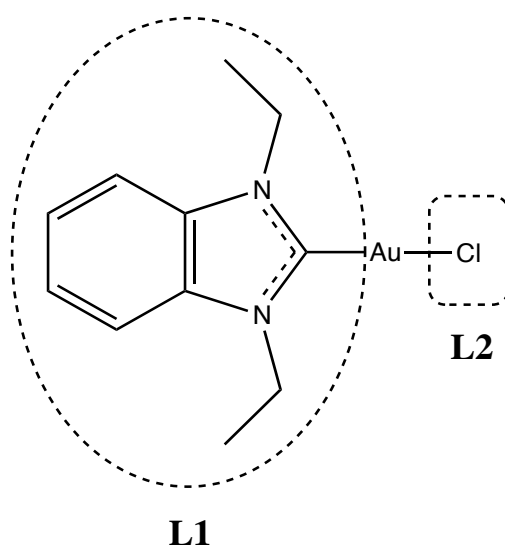
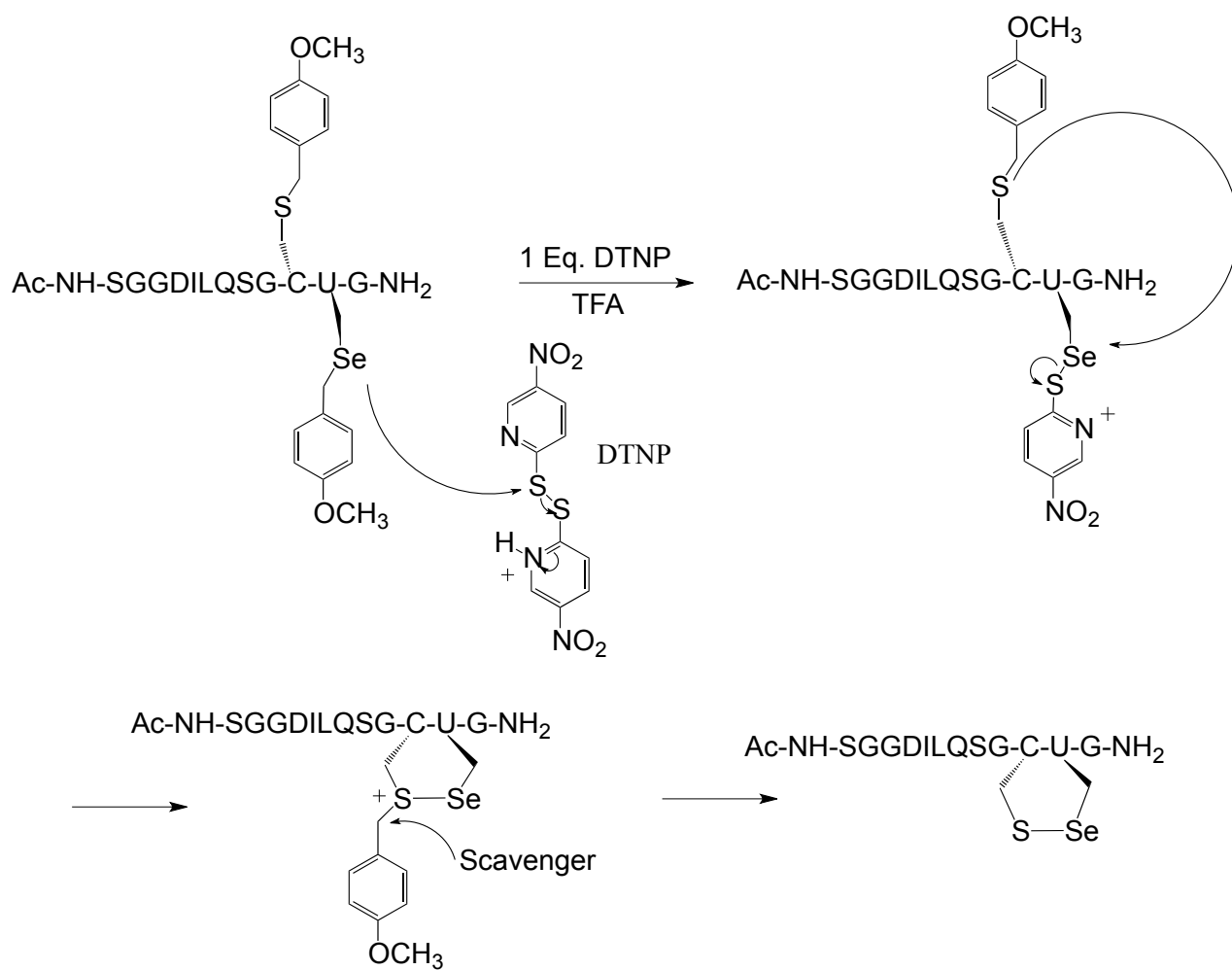


Chart 2. Differentiation of L1 and L2 ligands.



Scheme 1. Supposed mechanism of DTNP mediated S-Se bond formation.



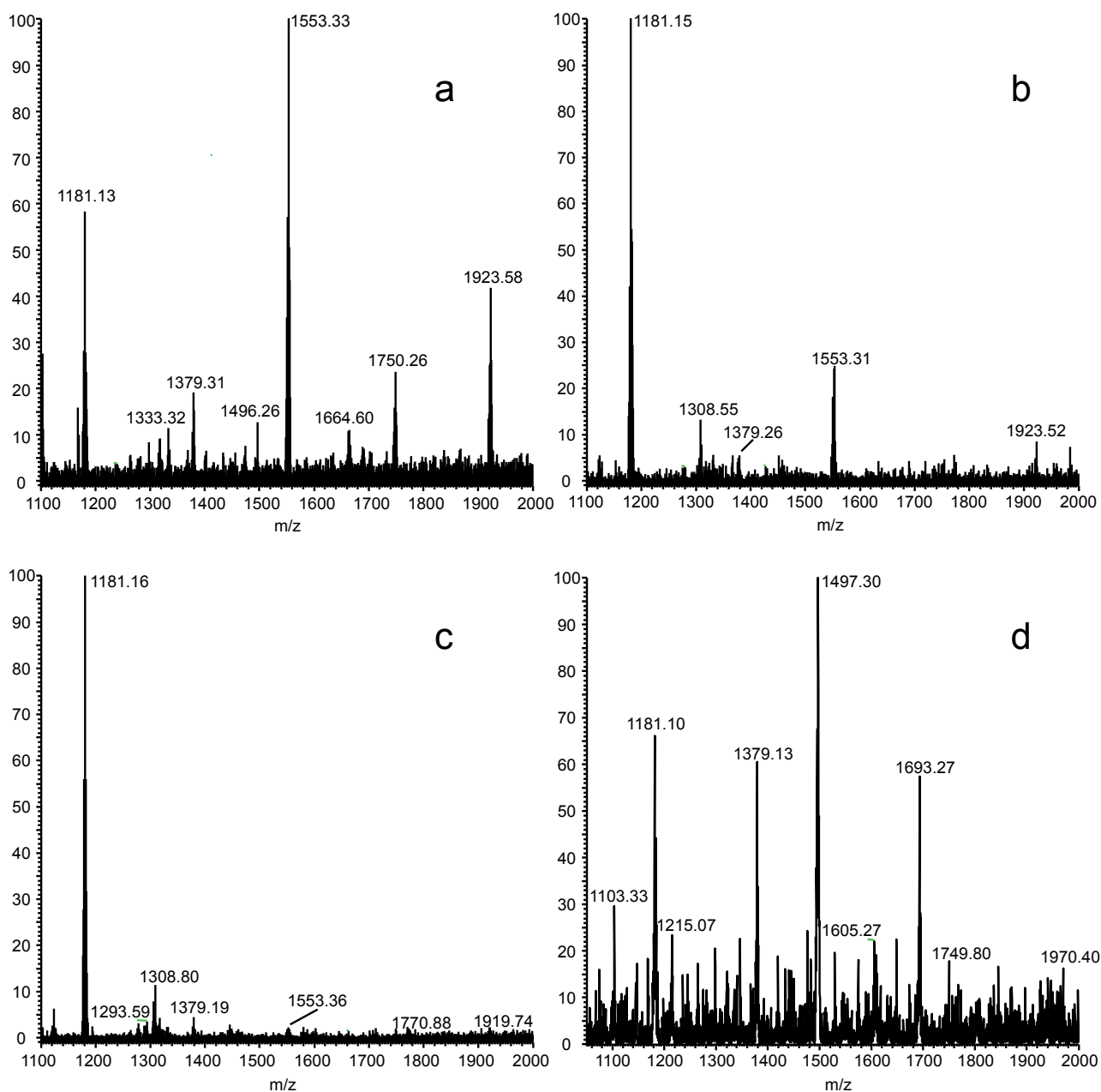


Figure 2. ESI-MS spectra of the synthetic reduced dodecapeptide Ac-SGGDILQSGCUG-NH₂ incubated with **1** (a), **2** (b), **3** (c) and **4** (d), acquired in negative ion mode.

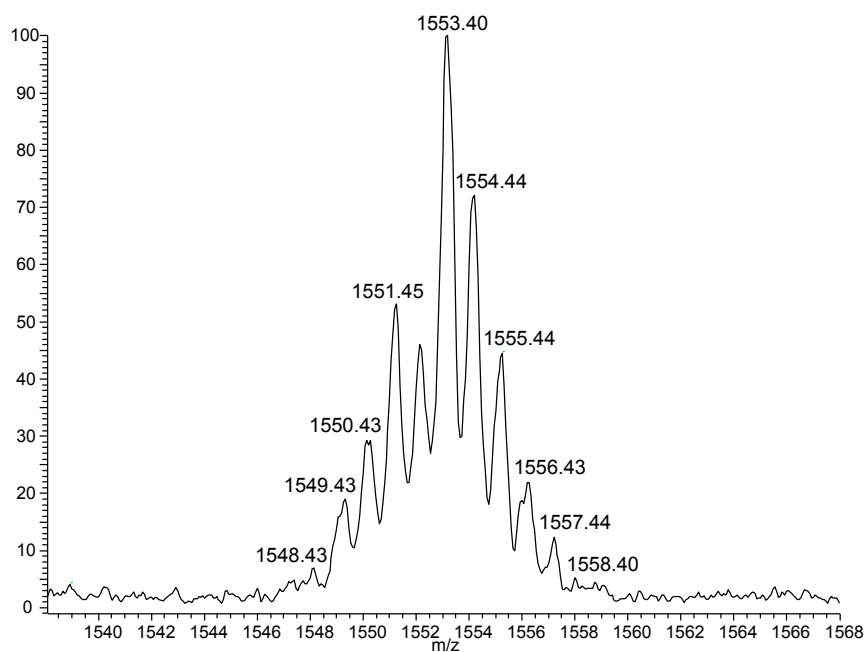


Figure 3. Isotopic pattern of peptide Ac-SGGDILQSGCUG-NH₂/1 complex (acquired in negative ion mode).

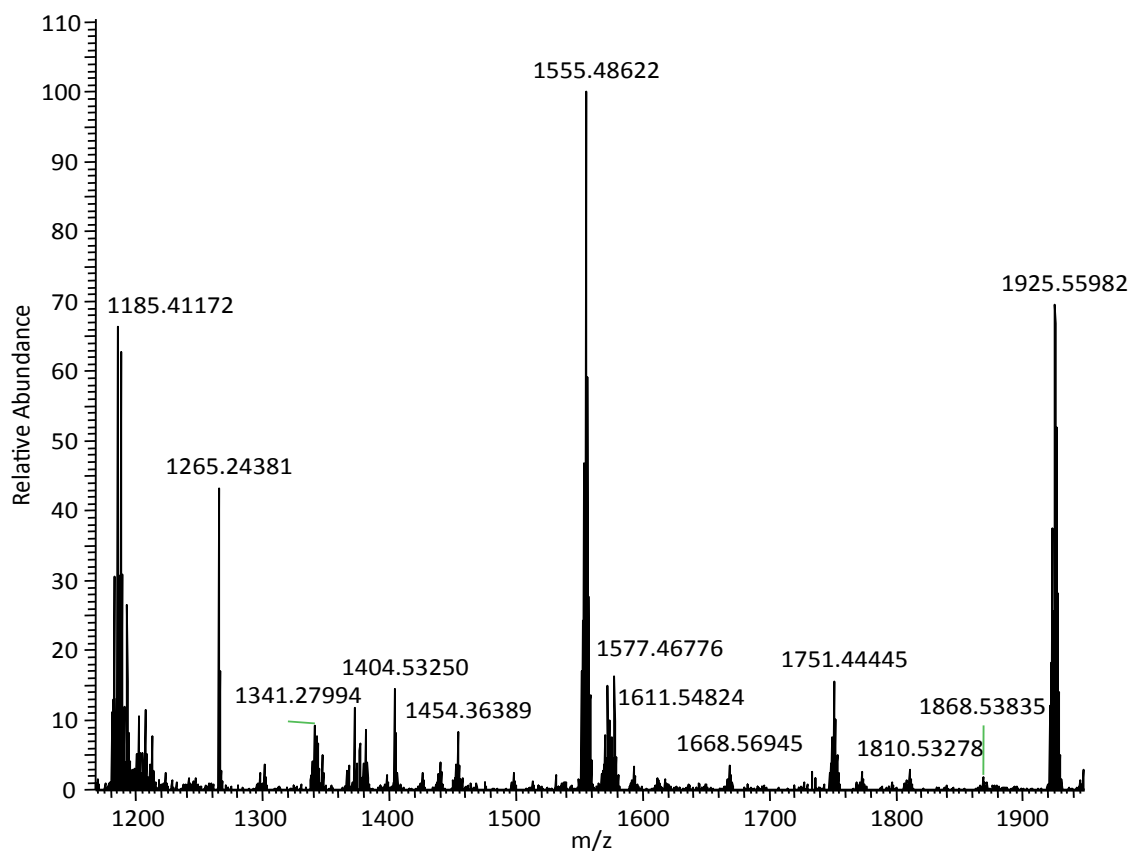


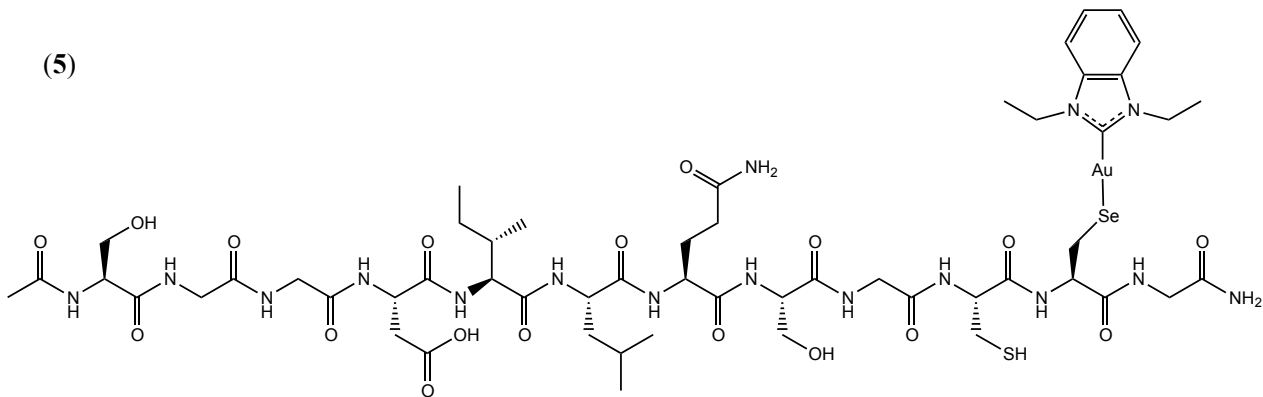
Figure 4. High-resolution ESI-MS spectra of the reduced dodecapeptide Ac-SGGDILQSGCUG-NH₂ incubated with **1**.

(5)

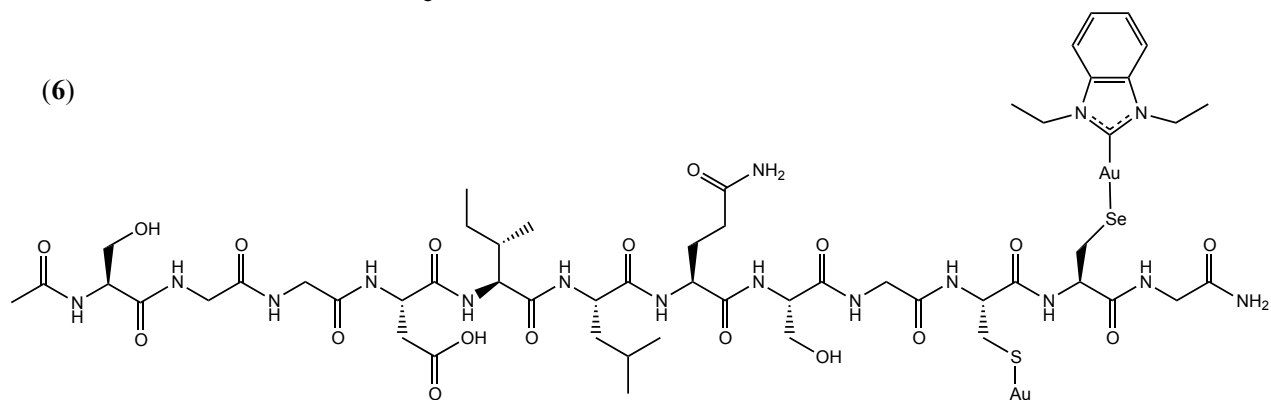
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(5)



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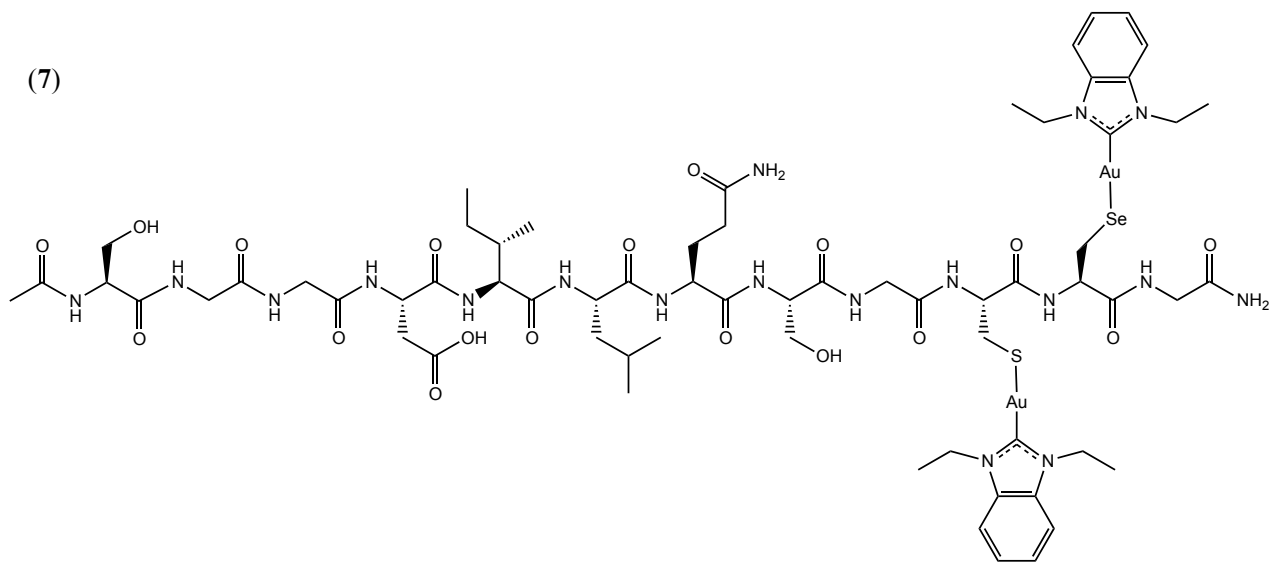
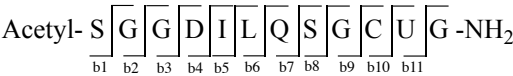
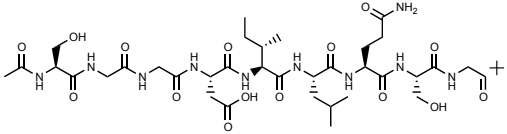


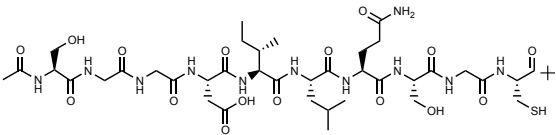
Chart 4. Principal fragments derived from the adduct **5**.



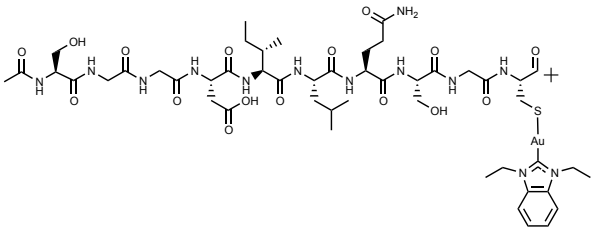
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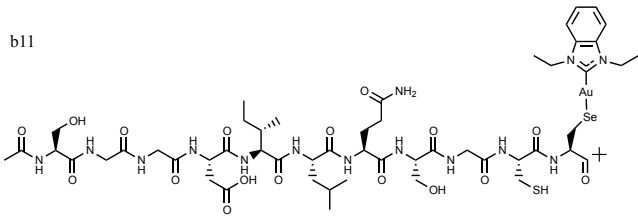
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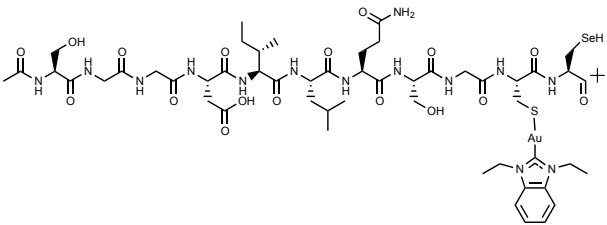
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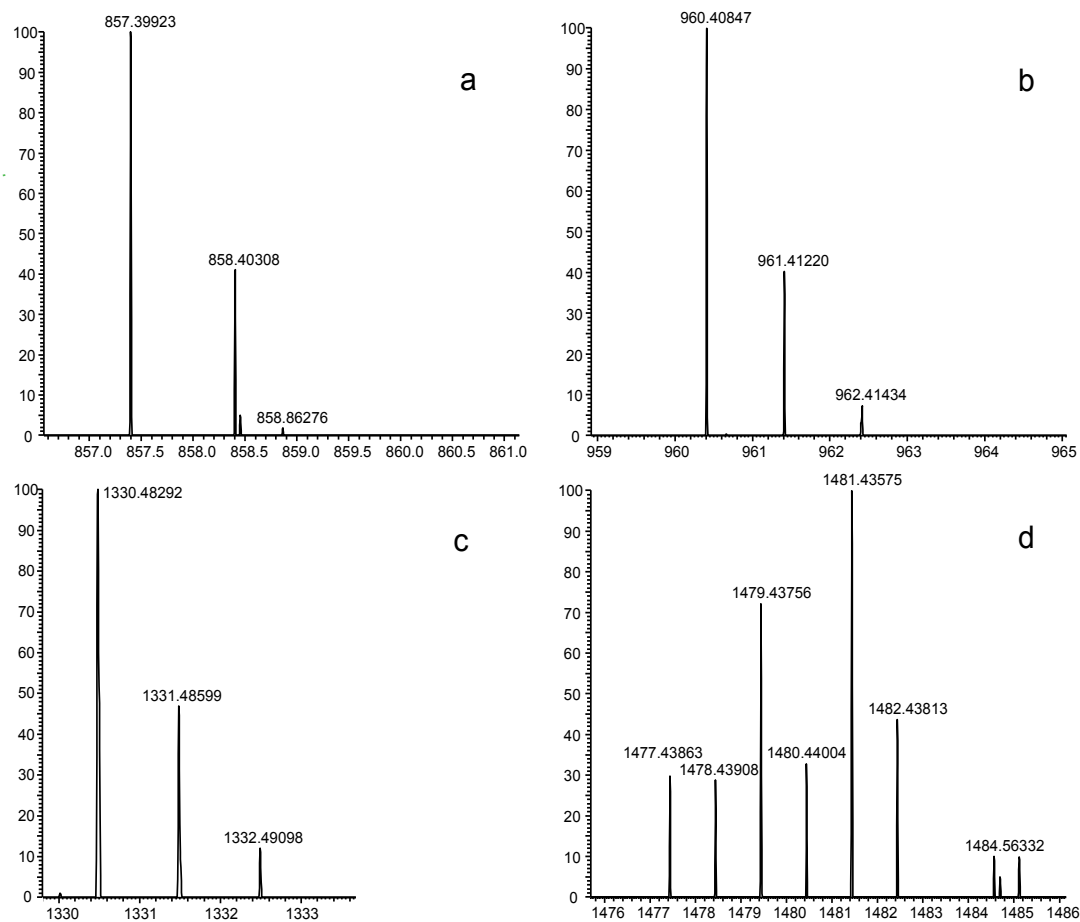


Figure 5. Principal fragments of the synthetic dodecapeptide Ac-SGGDILQSGCUG-NH₂ incubated with 1: (a) *b9* and (b) *b10* corresponding only to the fragmented peptide. (c) *b10* and (d) *b11* corresponding to the peptide bonded to a residue of the gold-drug.

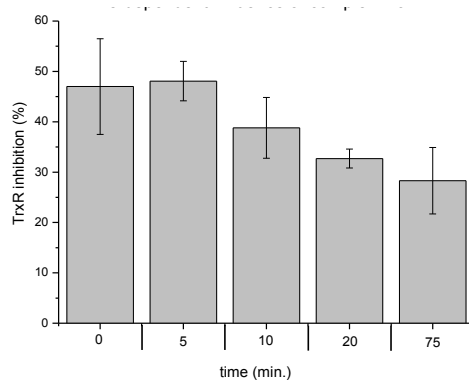


Figure 6. Time-dependent inhibition of TrxR treated with complex 1 (0.5 μM); results are expressed as mean (± error) of repeated experiments.

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