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A Spectroscopic Study of Interaction of Auricyanide with *N*-Acetylcysteine

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Abstract Interaction of auricyanide, an important metabolite of anti-arthritic gold-based drug auranofin, was studied in vitro with a pharmacologically active ligand *N*-acetylcysteine with a view to understand reactivity of gold in vivo. Formation of reduction product aurocyanide occurred through mono- and di-*N*-acetylcysteine-substituted intermediates. The product and intermediates were identified and monitored spectrophotometrically and by electrospray ionization mass spectrometry. This study suggests successive substitution with *N*-acetylcysteine through trans effect. At equimolar concentrations of auricyanide and *N*-acetylcysteine, only mono-substituted mixed-ligand complex was formed. Substitution of the data obtained to various kinetic models suggested that the reaction orders are 0.6 in terms of *N*-acetylcysteine, 1.5 in terms of auricyanide, and 2 overall. The intermediates detected in this work may help to synthesize more effective and less toxic gold drugs.

Keywords Auricyanide · *N*-acetylcysteine · Electrospray ionization mass spectroscopy (ESI-MS) · Rheumatoid arthritis · UV-vis spectroscopy

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Introduction

Use of gold drugs in treatment of rheumatoid arthritis, tuberculosis, endocarditis, and syphilis, commonly known as chrysotherapy, is well documented [1–4]. In rheumatoid arthritis, the gold drugs have been considered as disease modifying. The first-generation gold drugs were injectables like Solganal (gold thioglucose, AuSTg) and Myochrysine (gold sodium thiomalate, AuSTm), whereas the second-generation drug auranofin, (2,3,4,6-tetra-O-acetyl-β-1-*D*-thioglucopyranosato-S-(triethylphosphine) gold(I), abbreviated here as $Et_3PAuSATg$, is administered orally. With the advent of more effective and less toxic anti-inflammatory drugs, their use has become very limited. However, there have been new indications in cancer and parasitic diseases [5–7]. In cancer, gold drugs act through apoptosis, which is a preferred approach to treat the disease. Recently, in a screening study, auranofin showed about ten times activity against *Entamoeba histolytica* [8], which presents a great revival of gold drugs in treatment of parasites. The pharmacological properties and cellular activities of these drugs mainly depend upon the ligands attached to gold. Radiotracer studies of $[Au^{35}STg]_n$, $[Au^{35}STm]_n$, and $[Et_3{}^{32}PAu^{35}SATg]$ in vivo have shown that ligand exchange reactions occur with endogenous ligands [9–11]. Thus, the gold drugs may be considered as pro-drugs, since they metabolize in vivo to active species [3, 4]. Gold drugs and their metabolites distribute throughout the body tissues and cells [3, 4]. Therefore, the fate of these drugs and metabolites in the body is difficult to determine.

Gold is found as Au(I) in the body [10–12]. Sulfur-containing compounds such as cysteine, cystine, methionine, peptides (such as glutathione), and thioethers are able to reduce Au(III) to Au(I) [13–15]. Lysosomes of the kidney, liver,

and phagocytic cells of rats treated with Au(I) or Au(III) contain predominantly Au(I) [16, 17]. Nonetheless, under biomimetic conditions, gold(I) can be oxidized to gold(III) in vivo [18, 19]. The enzyme myeloperoxidase, present in neutrophils and macrophages, generates hypochlorous acid from H_2O_2 and Cl^-, and likely causes the oxidation of gold(I) to gold(III) [19, 20].

It is well understood that cyanide ions are produced in polymorphonuclear leukocytes in the body by oxidation of SCN^-. Gold drugs and their metabolites react with this ion to form aurocyanide ($[Au(CN)_2]^-$) species, through intermediates such as [Et_3P-Au-CN], which are taken up by red cells. The levels of $[Au(CN)_2]^-$ are much higher in smokers than non-smokers due to uptake of HCN from tobacco smoke. Cyanide from the inhaled smoke alters the metabolism of gold [21, 22]. The formation of $[Au(CN)_2]^-$ by these types of scrambling reactions is of biological significance.

Previously, reduction of Au(III) to Au(I) by glutathione (GSH) has been reported, which showed that the reaction proceeds through two intermediates, $[Au(CN)_3(GS)]^{2-}$ and $[Au(CN)_2(GS)_2]^{3-}$. In the present study, we are reporting such reactions in vitro with *N*-acetylcysteine (abbreviated here as *N*-AcCySH instead of standard NAC in order to highlight the SH linkage). *N*-AcCySH, precursor of GSH, is an effective antioxidant present in biological system [23]. Primarily, *N*-AcCySH is used in clinical medicine as a mucolytic agent. In addition, it plays an important role in the treatment of acetaminophen overdose and rheumatoid arthritis. Intravenous administration of *N*-AcCySH has been reported to be useful in removal and/or redistribution of gold in the body for treatment of hematologic reactions to chrysotherapy [24]. Therefore, considering *N*-AcCySH, a simpler analog of GSH, and its importance in medicinal chemistry, it is relevant *per se* to the field of pharmacology to study its interaction with $[Au(CN)_4]^-$ ions.

Three analytical techniques including NMR [20], UV-visible spectrophotometry [9, 25], and ESI-MS [9] have been successfully employed to study ligand scrambling and exchange reactions of gold drugs in solution. All the three are complementary; the UV-visible spectroscopy is distinct because of its convenient application in studying kinetics and speciation of transition metal ions. In the present work, we used UV-visible spectroscopy in conjunction with ESI-MS technique in order to study the oxidation state of gold.

Materials and Methods

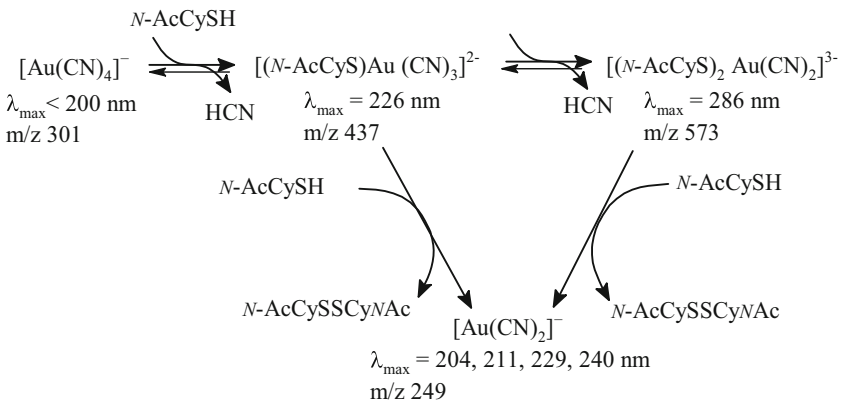
The chemicals used in this study were *N*-AcCySH (Acros Organics, USA), Nanopure® water (resistivity greater 18.0 MΩcm) generated by a Branstead Nanopure® system with reverse-osmosis water fed, $KAu(CN)_4$ (Alfa Aesar GmbH & Co, Germany), formic acid (E. Merck, Germany) and acetonitrile (E. Merck, Germany).

Preparation of Stock Solutions

Stock solution of auricyanide (50 mM) was prepared by dissolving $KAu(CN)_4$ (178.9 mg) in water (10 mL). Similarly, a stock solution of *N*-AcCySH (50 mM) was prepared by dissolving it (81.6 mg) in water (10 mL); this solution was stored at 4 °C to prevent oxidation of thiols to disulfides. These solutions were diluted as required for subsequent use.

Spectroscopic Study

Cary 100 Bio UV-vis spectrophotometer (Varian Inc., USA) was used to record spectra in 200–350 nm range at 25 °C. The measurements were recorded repetitively by scanning spectra for a set of time intervals. The reactions were studied by varying concentrations of *N*-AcCySH and $[Au(CN)_4]^{-1}$ in millimolar range. Scanning kinetics mode of the instrument was used to record the spectra at selected time intervals. Absorbances at 200 nm ($[Au(CN)_4]^-$), 226 nm ($[Au(CN)_3(SR)]_n$), 250, 286 nm ($[Au(CN)_2(SR)_2]_n$), 204, 211, 230, and 240 nm ($[Au(CN)_2]^-$) were used to monitor the reactions; these absorbances have been reliably used for these species previously for study with GSH [9]. The



Scheme 1 Interaction of $[Au(CN)_4]^-$ with *N*-AcCySH

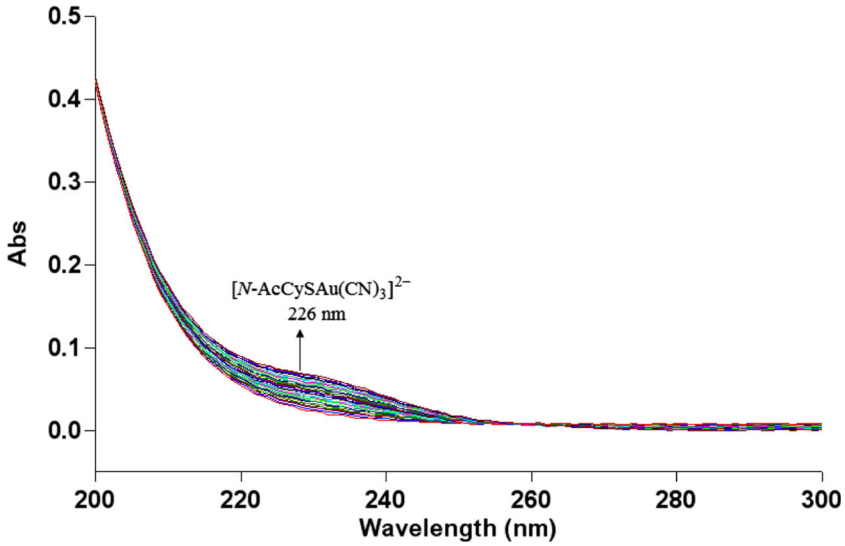


Fig. 1 UV-vis spectra of 25 μM $[Au(CN)_4]^-$ with 25 μM *N*-AcCySH in water, one cycle of 30 s for 15 min

absorbances at 226 and 286 nm were recorded at 25 °C in a cyclic mode (one cycle of 30 s for 15 min). In order to study the effect of concentration of the thiols on the formation of these intermediates, this experiment was repeated by fixing the concentration of auricyanide at 25 μM and increasing the concentration of the *N*-AcCySH to 50, 100, 200 and 250 μM. Similarly, in order to investigate the effect of concentration on the formation of these intermediates, the above experiment was repeated by fixing the concentration of the *N*-AcCySH at 25 μM and increasing the concentration of auricyanide to 50, 100, 200 and 250 μM. Reaction rate constants and order of reaction were determined by fitting the data into various kinetic models.

ESI-Mass Spectrometry

ESI-Mass spectra along with UV-vis spectra were used to characterize the intermediate species in the reaction mixtures. ESI-mass spectra were recorded on a Hewlett Packard (Agilent) LC-MS (Series 1100 LC-MSD with a UV-vis diode array detector) set in the negative ion mode with 3500 V capillary voltage; 40 V fragmentation voltage; ionization temperature 250 °C, and N_2 flow 10 L min^{-1} at 25 psi. The samples (20.0 μL) of 1.0–10.0 mM reaction mixtures were injected into the ESI chamber with a flow rate of 0.400 mL min^{-1}. The two-component mobile phase used was A: formic acid (0.1% in water) and B: formic acid (0.1% in acetonitrile). Unless otherwise indicated, the mobile phase composition was 50:50 *v*/*v*. Maximum pressure was set at 400 bar. Spray parameters were fixed for all the analyses. Using the autosampler, the delay time between mixing and the first spectral accumulation was set at 3 min.

Results and Discussion

Auricyanide is thought to react with *N*-AcCySH according to Scheme 1. This is a reduction reaction, which proceeds through two intermediates $[(N\text{-AcCyS})Au(CN)_3]^{2-}$ and $(N\text{-AcCyS})_2Au(CN)_2]^{3-}$ to produce aurocyanide

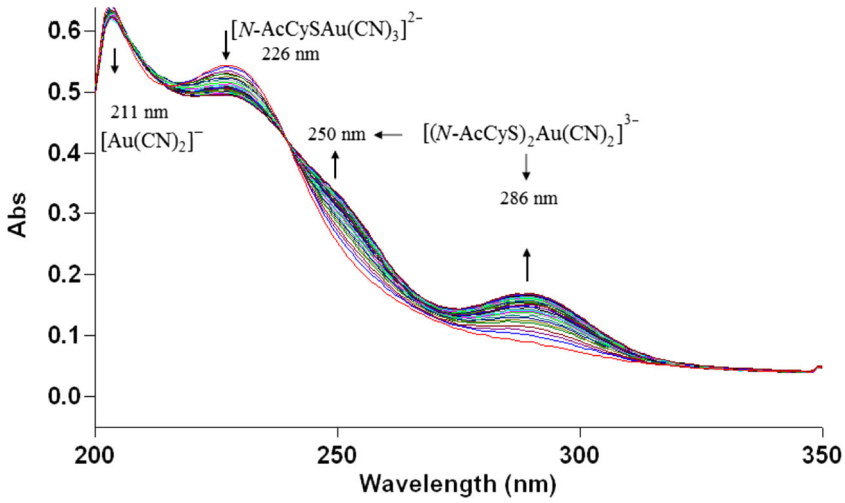


Fig. 2 UV-vis spectra of 25 μM $[Au(CN)_4]^-$ with 25 μM *N*-AcCySH in NH_4HCO_3 buffer, one cycle of 30 s for 15 min

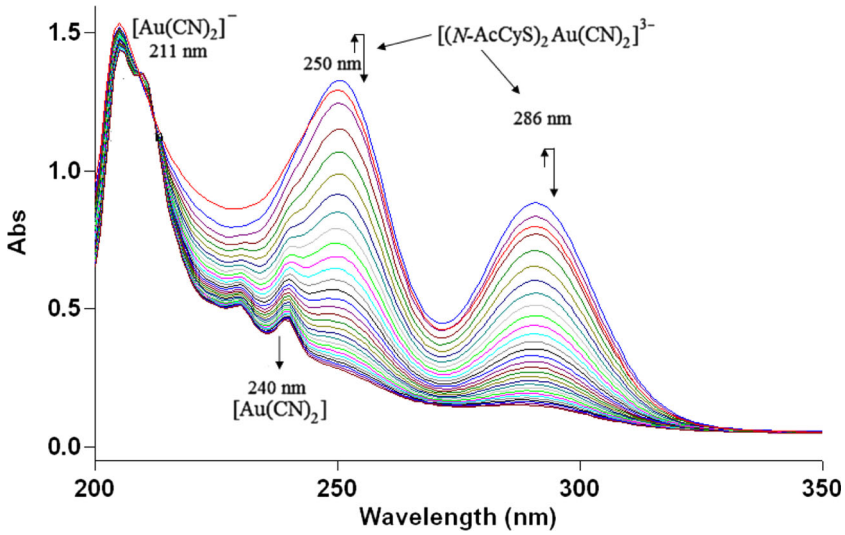


Fig. 3 UV-vis spectra of 25 μM $[Au(CN)_4]^-$ with 250 μM *N*-AcCySH in NH_4HCO_3 buffer, one cycle of 30 s for 15 min

$[Au(CN)_2]^-$; these species were characterized by their λ_{max} values at 226, 286 and 249 nm, respectively, according to the related literature values [9, 20, 25, 26]. They were further authenticated by their respective *m*/*z* values in ESI-MS spectra.

In neutral solution, the α-carboxylate and the thiol groups of *N*-AcCySH get completely and partially deprotonated, respectively, and produce an ion with 2– charge [27]. On interaction with $[Au(CN)_4]^-$, the *N*-AcCyS$^-$ ion is expected to produce mixed-ligand complexes as shown in Scheme 1. The reaction of *N*-AcCySH with $[Au(CN)_4]^-$ in micromolar quantities were monitored by using the UV-visible kinetics mode in water and in NH_4HCO_3 buffer (pH 7.8) by using λ_{max} at 204, 211, 226, 240, 250 and 286 nm as these bands were found to be present in a test run.

At equimolar concentrations of $[Au(CN)_4]^-$ and *N*-AcCySH, only one mixed-ligand complex $[(N\text{-AcCyS})Au(CN)_3]^{2-}$ showing absorbance at 226 nm appears to be predominantly formed in 0.5–15 min in water (Fig. 1). The bands due to aurocyanide and di-substituted intermediate were absent at *N*-AcCySH concentrations 2–10 times greater than $[Au(CN)_4]^-$. The reaction appears to be slow in water with continuous increase in intensity of 226-nm band. This may be attributed to the negative charges on *N*-AcCyS$^-$ and $[Au(CN)_4]^-$, which make ligand exchange a difficult process due to similar charges on the reacting species. Thus in water, only mono-substituted intermediate, $[(N\text{-AcCyS})Au(CN)_3]^{2-}$, is formed at all concentrations of *N*-AcCySH while the concentration of $[Au(CN)_4]^-$ is constant. Similar results were obtained on increasing the concentration of $[Au(CN)_4]^-$ from 1 to 10 times that of *N*-AcCySH.

In NH_4HCO_3 buffer (pH 7.85), at equimolar concentrations of $[Au(CN)_4]^-$ and *N*-AcCySH, intense absorbances at 204, 211 nm due to $[Au(CN)_2]^-$, 226 nm due to $[(N\text{-AcCyS})Au(CN)_3]^{2-}$, and 250, 286 nm due to $[(N\text{-AcCyS})_2\ Au(CN)_2]^{3-}$ were observed (Fig. 2). Similar pattern was observed at 1:2 ratio of $[Au(CN)_4]^-$ and *N*-AcCySH. In both the cases, the isosbestic

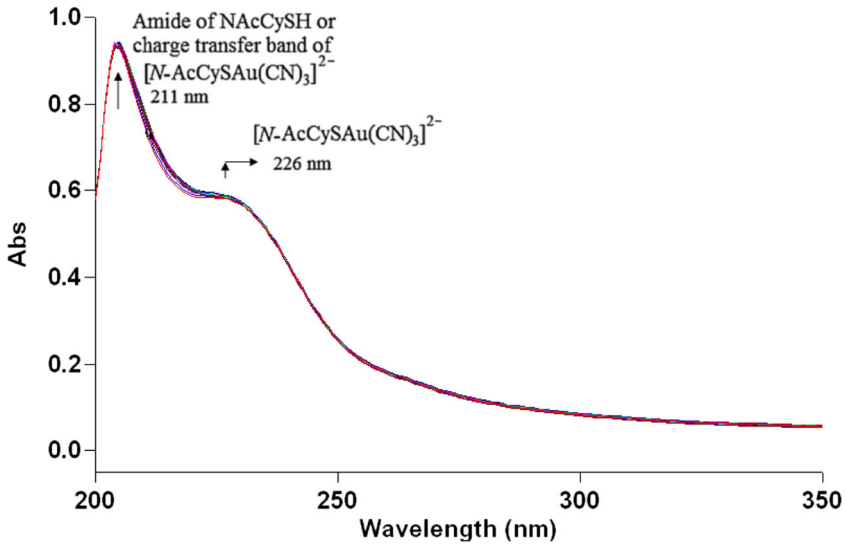


Fig. 4 UV-vis spectra of 100 μM $[Au(CN)_4]^-$ with 25 μM *N*-AcCySH in NH_4HCO_3 buffer, one cycle of 30 s for 15 min

point at 237 nm exists, which indicates the conversion of [(*N*-AcCyS)Au(CN)$_3$]$^{2-}$ to [(*N*-AcCyS)$_2$ Au(CN)$_2$]$^{3-}$. At concentrations 4–10 times of *N*-AcCySH, the band at 226 nm diminished with a simultaneous increase in absorbance at 211 nm. The absorbances at 250 and 286 nm first increased and then decreased after 2–3 min with simultaneous increase at 211 nm indicating the conversion of [(*N*-AcCyS)$_2$Au(CN)$_2$]$^{3-}$ to [Au(CN)$_2$]$^-$ quite rapidly. This was also evidenced by disappearance of 229 and 240 nm bands.

The pH and ionic strength used in the reaction had a profound effect on its rate. The reaction in pure water was the slowest. However, in buffers, a high ionic strength would increase the rate, probably due to charge stabilization of the already heavily negative system. Therefore, in NH_4HCO_3 buffer (pH 7.85), formation of two intermediates was observed at 226 and 286 nm at lower concentrations of *N*-AcCySH. An increase in the concentration of *N*-AcCySH favors the formation of the second intermediate with a decrease of absorbance of the first intermediate, thus driving the reaction to the forward direction. An isosbestic point at 237 nm confirmed the formation of [*N*-AcCyS)$_2$Au(CN)$_2$]$^{2-}$ from [(*N*-AcCyS)Au(CN)$_3$]$^{2-}$ (Fig. 3).

On the other hand, an increase in concentration of [Au(CN)$_4$]$^-$, while keeping the concentration of *N*-AcCySH constant, leads to formation of [Au(CN)$_2$]$^-$ quite rapidly through [(*N*-AcCyS)Au(CN)$_3$]$^{2-}$ (Fig. 4). The spectrum shows the completeness of reaction at mixing time. Formation of disulfide of *N*-AcCySH may be anticipated due to oxidation by air, which pulls [(*N*-AcCyS)Au(CN)$_3$]$^{2-}$ towards the reactants in the equilibrium. This can result in a larger amount of disulfide and a lower amount of reduced gold. The other possibility may be the formation of the disulfide and then a cyanide ligand reforming [Au(CN)$_4$]$^-$ in the presence of free thiol as shown in Scheme 1.

The existence of the species under investigation was authenticated by ESI-MS technique. The representative spectrum is shown in Fig. 5. The peaks at *m*/*z* 249 ([Au(CN)$_2$]$^-$), 301 ([Au(CN)$_4$]$^-$), 437 ([(*N*-AcCyS)Au(CN)$_3$]$^{2-}$), and 573 ([(*N*-AcCyS)$_2$Au(CN)$_2$]$^{3-}$) amu were observed; their intensities changed according to their abundance with time.

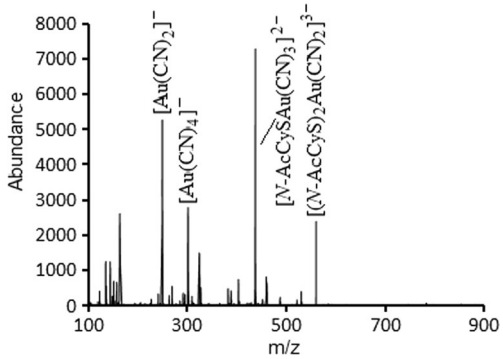


Fig. 5 Typical ESI-MS spectrum of the reaction mixture showing relevant peaks

Table 1 Kinetics data of the reactions

	N-AcCySH (μM)	Reaction order (*n*)		Rate constant (mol$^{(n-1)}$s^{-1})
[Au(CN)$_4$]$^-$ at 25 μM	25		0.64	− 4.1
	50	SE	0.06	24.6×10^{-2}
	100	r^2	0.97	5.1×10^{-2}
	200	F	110.18	3
	250	SS_{reg}	0.28	7.6×10^{-3}
	[Au(CN)$_4$]$^-$ (μM)			
N-AcCySH at 25 μM	25		1.50	-20.4×10^{-2}
	50	SE	0.14	55.0×10^{-2}
	100	r^2	0.98	11.29×10^{-2}
	200	F	119.08	3
	250	SS_{reg}	1.52	3.8×10^{-2}

The rate constants and orders of reaction determined from these data are given in Table 1. The orders of reactions were 0.6 in terms of *N*-AcCySH, 1.5 in terms of [Au(CN)$_4$]$^-$, and 2 overall. This suggests that an increase in concentration of [Au(CN)$_4$]$^-$ causes shifting of equilibrium more towards right as compared to that with *N*-AcCySH, which results in reduction of Au(III) to Au(I) ending up in the formation of [Au(CN)$_2$]$^-$. This observation is in line with the finding that lysosomes treated with Au(I) or Au(III) contain predominantly Au(I) [15, 16].

It is important to note that the mono- and di-substituted intermediates reported with GSH [8] and *N*-AcCySH (this work) have never been isolated. It is hypothesized that if these can be synthesized, they can prove to be active metabolites and provide for more effective and less toxic gold drugs.

Conclusions

It was clear from this study that [Au(CN)$_4$]$^-$ is liable to ligand exchange reactions with *N*-AcCySH. At low thiol:gold ratios, only one cyanide ligand was exchanged forming [(*N*-AcCyS)Au(CN)$_3$]$^{2-}$. At higher ratios (thiols:gold ≥ 2), reaction occurred through two ligand exchanges leading to ultimate reduction of [Au(CN)$_4$]$^-$ to [Au(CN)$_2$]$^-$ and the disulfide. The successive substitution with *N*-AcCyS$^-$ appears to take place through trans effect. This ligand being negatively charged is expected to react slower than ligands like O-MeCySH having positive charge, which may be verified through a comparative study. The product and intermediates were successfully identified and monitored by spectrophotometry and ESI-MS. The kinetic study suggested that the reaction orders were 0.6 in terms of *N*-AcCySH, 1.5 in terms of [Au(CN)$_4$]$^-$, and 2 overall. The reactions are faster in pH 7.8 buffer as compared with those in water. The intermediates detected in this work may help to understand the in vivo

biochemistry, design, and synthesize more effective and less toxic gold drugs.

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Compliance with Ethical Standards No animals, humans, or their materials were used in this work.

Conflict of Interest The authors declare that they have no conflict of interest.

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