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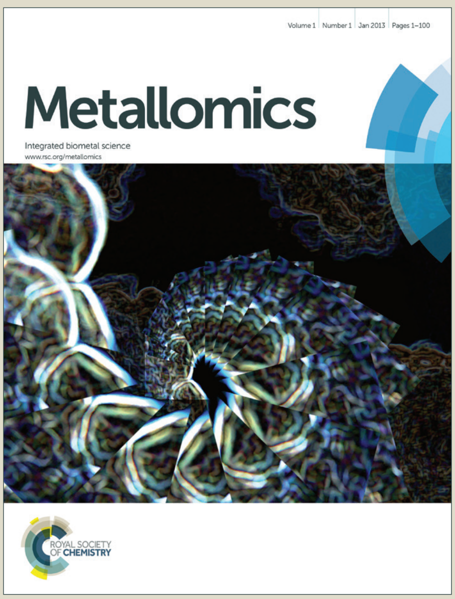

**Antimicrobial effects of copper(II) *bis*(thiosemicarbazonato) complexes provide new insight to their biochemical mode of action**

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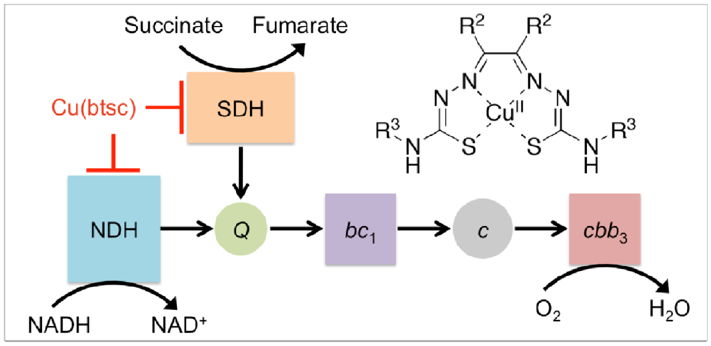
# ABSTRACT




The copper(II) complexes of *bis*-thiosemicarbazones (Cu(btsc)) such as Cu(atsm) and Cu(gtsm) are neutral, lipophilic compounds that show promise as therapeutics for the treatment of certain neurological diseases and cancers. Although the effects of these compounds have been described at the cellular level, there is almost no information about their biochemical mode of action. In this work, we showed that Cu(atsm) and Cu(gtsm) displayed antimicrobial activities against the human obligate pathogen *Neisseria gonorrhoeae* that were more than 100 times more potent than $Cu(NO_3)_2$ salt alone. Treatment with Cu(btsc) also produced phenotypes that were consistent with copper poisoning, but the levels of intracellular copper were undetectable by ICP MS. We observed that Cu(btsc) interacted with proteins in the cell membrane. Systematic measurements of $O_2$ uptake further demonstrated that treatment with both Cu(atsm) and Cu(gtsm) led to dose-dependent inhibition of respiratory electron transfer processes *via* succinate and NADH dehydrogenases. These dehydrogenases were not inhibited by a non-btsc source of $Cu^{II}$. The results led us to conclude that the biochemical mechanism of Cu(btsc) action is likely more complex than the present, simplistic model of copper release into the cytoplasm.

## INTRODUCTION

In the diagnosis and treatment of a variety of cancers and neurodegenerative disorders, the use of a class of neutral, lipophilic *bis*(thiosemicarbazonato)copper(II) complexes (Cu(btsc); Scheme 1) such as diacetyl- and glyoxal-*bis*[*N*(4)-methylthiosemicarbazonato]copper(II) (Cu(atsm) and Cu(gtsm), respectively) is emerging as a promising strategy. There is already significant progress in the application of Cu(btsc) in nuclear medicine. The radioactive $^{64}$Cu isotope of Cu(atsm) is currently in human clinical trials as an imaging agent of hypoxia in head and neck cancer.(*1*) More recent studies in animal models have also demonstrated the therapeutic potential of Cu(atsm) and Cu(gtsm) for Parkinson's(*2*) and Alzheimer's(*3*) diseases, respectively. In addition, experiments using tissue cultures have shown that Cu(gtsm) also inhibited the growth of brain tumor(*4*) and prostate cancer(*5*) cells.

Although the pharmacological effects of Cu(btsc) have been investigated in some detail (reviewed recently by Paterson and Donnelly(*6*)), a biochemical understanding of the interactions between these compounds and cells remains to be elucidated. Pioneering early studies suggested that Cu(btsc) compounds oxidised intracellular thiols(*7-9*), leading to global defects in metabolism, including inhibition of oxidative phosphorylation(*10*) and DNA synthesis(*11*). Incidentally, severe oxidative stress is also a hallmark of copper (Cu) poisoning (reviewed by Valko and colleagues(*12*)). Thus, the present model for the mechanism of Cu(btsc) action is generally linked to the ability of these complexes to increase retention of intracellular Cu ions.(*13*) Whether the intact Cu(btsc) unit itself exerts a toxic effect is not known.

We are interested in the potential of Cu and Cu-containing compounds to fight bacterial infections. To date, the use of Cu as an antimicrobial agent has been met with relative success in environmental settings. The effects associated with physical contact with metallic Cu surfaces have proven to be efficient in stemming bacterial cross-contamination in several hospital trials.(*14, 15*) By comparison, development of Cu-based drugs has been impeded by a general lack of selective toxicity against the infecting pathogens over host tissue. Their wide use in animal and clinical experiments suggests that Cu(atsm) and Cu(gtsm) are relatively well tolerated by healthy tissues. Although present efforts are concentrated on the development Cu(btsc) as future therapeutics in neurological diseases and cancers, it ought to be possible to extend the work to treat bacterial infections. Data on the antimicrobial activity of Cu(btsc) is still limited, but a recent study suggested that they could be effective against *Mycobacterium tuberculosis*.(*16*)

Here we show that Cu(atsm) and Cu(gtsm) are highly toxic to *Neisseria gonorrhoeae* (the gonococcus), which causes the sexually transmitted infection gonorrhoea. Using this bacterium as a model system, we further provide insight into the biochemical action of Cu(btsc) in cells and identify that their primary mode of action is *via* inhibition of respiratory electron transport.

## RESULTS AND DISCUSSION

**Antimicrobial activity of Cu(btsc).** The antimicrobial effects of Cu(btsc) were characterised in this work using the obligate human pathogen *N. gonorrhoeae* strain 1291 as a model system. This bacterium tolerates excess Cu by expressing a Cu efflux pump encoded by a *copA* gene, and a mutant strain with a deletion in *copA* (1291*copA*) is susceptible to killing by Cu salts.(*17*) Exposure to 50 µM $Cu(NO_3)_2$ for 20–24 h led to a 6-log reduction in the number of 1291*copA* colony forming units (CFU; Figure 1a). By contrast, the wild type strain (1291wt) remained fully viable up to 100 µM $Cu(NO_3)_2$.

Compared to $Cu(NO_3)_2$, Cu(btsc) was several magnitudes more toxic. Viability of

1291*copA* decreased a million fold after exposure to only 100 nM of Cu(atsm) or 30 nM of Cu(gtsm) (Figure 1a). Both complexes were also effective against wild type *N. gonorrhoeae* at sub-micromolar concentrations. A reduction in the survival of 1291wt was detected in the presence of as little as 500 nM of Cu(atsm) or 50 nM of Cu(gtsm) (Figure 1a). These concentrations are well below those tolerated by various human and animal tissues.(*2, 5, 16*) Notably, Cu(atsm) and Cu(gtsm) were more toxic than their respective copper-free ligands (Supplementary Figure 1), confirming that the Cu centre was required for toxicity.

Cu(gtsm) was approximately 10 times more effective at killing *N. gonorrhoeae* strains than was Cu(atsm) (Figure 1a). This difference arises presumably due to the different degrees to which the compounds can increase bio-available levels of $Cu^{I}$, which is cytotoxic (Scheme 1). Neutral Cu(btsc) complexes are thermodynamically and kinetically stable with respect to dissociation, but they are susceptible to reduction of the metal ion.(*13*) Due to a low $Cu^{II}/Cu^{I}$ reduction potential ($E_{1/2} = [(E_p^{red} + E_p^{ox})/2] = -440$ mV *vs.* AgCl/Ag), Cu(gtsm) is reduced readily by intracellular reductants such as glutathione. The resulting $[Cu^{I}G]^{-}$ species is unable to compete effectively with $Cu^{I}$ sinks such as protein thiols, and thus the $Cu^{I}$ ion is released (Scheme 1). By comparison, reduction of Cu(atsm) occurs at a more negative potential ($E_{1/2}$ = -600 mV) and hence Cu(atsm) is thought to retain its $Cu^{II}$ centre more efficiently. As a result of this subtle difference in the chemistry of these Cu(btsc) complexes, cells treated with Cu(gtsm) frequently accumulate higher levels of intracellular bio-available $Cu^{I}$ compared to those exposed to Cu(atsm) (Scheme 1).(*3, 5, 18, 19*)

**Cu(btsc) treatment leads to a small increase in cytoplasmic Cu levels.** Excess intracellular Cu in *N. gonorrhoeae* appears to exert a toxic effect by arresting heme biosynthesis at the step catalysed by coproporphyrinogen III decarboxylase (HemN;

Supplementary Figure 2).(*20*) This occurs presumably due to poisoning of the [4Fe-4S] cluster by bio-available $Cu^{I}$ that accumulates in the cytoplasm. Consequently, there is a decrease in total intracellular heme levels, accompanied by a build-up of the biosynthetic intermediate coproporphyrin III (Cop III). The latter is detected by a fluorescent emission at 620 nm following excitation at 410 nm.(*20*)

Growth of 1291*copA* in the presence of 10 μM $Cu(NO_3)_2$ led to a 10-fold gain in Cop III fluorescence (Figure 1b(i)). An increase in Cop III fluorescence was also detected in cells that were exposed to 30 nM Cu(gtsm) (Figure 1b(ii)) or 80 nM Cu(atsm) (Figure 1b(iii)). This suggests that *N. gonorrhoeae* was able to metabolise both Cu(btsc) complexes to raise the levels of cytoplasmic $Cu^{I}$ pools, although these concentrations of Cu were undetectable by ICP MS (Supplementary Figure 3). The amounts of $Cu(NO_3)_2$, Cu(atsm), and Cu(gtsm) used in these experiments led to a comparable inhibition in the growth rates of 1291*copA* (Figure 1c). However, cells exposed to Cu(gtsm) and Cu(atsm) produced less than 50% of the amount of Cop III generated by those treated with $Cu(NO_3)_2$ (Figure 1b). These results suggested that Cu(btsc) might exert additional toxic effects other than those caused by the trapping of bio-available $Cu^{I}$ in the cytoplasm.

**Cu(btsc) inhibits aerobic respiration by intact cells.** The lipophilicity of Cu(atsm) and Cu(gtsm) has been shown to result in significant interactions with and retention in the cell membranes of glial and neuronal cell lines.(*21*) Similarly, addition of Cu(btsc) to a suspension of *N. gonorrhoeae* 1291wt followed by immediate sedimentation of cells led to trapping of the characteristic orange-red colour of Cu(btsc) in the bacterial pellet (Supplementary Figure 4). This association with the cell membrane could disrupt the action of membrane proteins, such as those involved in aerobic electron transfer.

The sensitivity of the pathway for aerobic electron transport to inhibition by Cu(btsc) was examined by measuring the rates of $O_2$ consumption by intact 1291wt cells following incubation with Cu(atsm) or Cu(gtsm) (50 μM each). *N. gonorrhoeae* is able to efficiently use *L*-lactate, pyruvate, and glucose as carbon sources in aerobic metabolism (Scheme 2)(*22, 23*), but pre-exposure to Cu(gtsm) for 3 min led to a partial decrease in the rates of lactate respiration and a complete inhibition of pyruvate- and glucose-dependent respiration (Figure 2a). The reduction in rate was dose-dependent (Figure 2b) and immediate; it was observed at approximately 1 min after addition of Cu(gtsm) into the reaction (Figure 2c). By comparison, pre-treatment with Cu(atsm) induced only a minor, albeit detectable effect on the rates of aerobic respiration, regardless of the electron donor (Figure 2a). Extending the incubation time for up to 1 h did not lead to any further loss in respiration rates (Supplementary Figure 5). Due to poor solubility in the assay buffers, higher concentrations of Cu(atsm) were not tested.

**Cu(btsc) impairs electron transport to $O_2$.** The data in Figure 2 indicated that transfer of electrons from lactate, glucose, and pyruvate to $O_2$ may be impaired by Cu(gtsm). The core electron transport chain in *N. gonorrhoeae* is organised into four complexes that resemble those present in mitochondria (shaded box in Scheme 2). Reduction of $O_2$ to $H_2O$ is catalysed by a terminal oxidase (cytochrome *cbb*$_3$). Upstream of this oxidase is a cytochrome *bc*$_1$ complex (Complex III-like), which uses ubiquinol as an electron donor. The quinone (*Q*) pool accepts electrons from succinate and NADH dehydrogenases (SDH (Complex II-like) and Nuo (Complex I-like), respectively). In *N. gonorrhoeae*, additional primary dehydrogenases deliver electrons to the *Q* pool. These include a sodium-motive NADH dehydrogenase (Nqr) and an FMN-dependent *L*-lactate-ubiquinone reductase (*L*-LDH) (Scheme 2).(*24*) There is a previous suggestion that Complex I in the mitochondria (homologue of Nuo in *N. gonorrhoeae*) may participate in Cu(btsc) homeostasis.(*25*) However, to our knowledge, interactions between

Cu(btsc) and cytochrome oxidase, cytochrome $bc_1$, SDH, and *L*-LDH have not been reported in any system.

***(1) Activities of cytochrome oxidase and lactate dehydrogenase are not affected.*** As shown clearly in Figure 2, lactate respiration by intact cells was susceptible to inhibition by Cu(gtsm). Thus, transport of electrons from *L*-LDH to the terminal oxidase may be impaired. To test this proposal, the components of the core respiratory chain in *N. gonorrhoeae* (shaded box in Scheme 2) were isolated from bacterial milieu by preparing cell-free membrane vesicles of 1291wt. The impact of Cu(btsc) was subsequently examined by measuring the rates of $O_2$ consumption following treatment with Cu(gtsm) or Cu(atsm) (50 μM each). By using an excess of ascorbate-reduced TMPD to drive steady-state reduction of *c*-type cytochromes and act as electron donors to cytochrome $cbb_3$ (Scheme 2), it was found that the activity of the terminal oxidase was not affected by Cu(gtsm) or Cu(atsm) (Figure 3a). Similarly, by using *L*-lactate as an electron donor to *L*-LDH (Scheme 2), no change was detected in the rate of $O_2$ reduction (Figure 3b). Taken together, these results suggested that the entire chain of electron transfer from *L*-lactate to $O_2$ *via* *L*-LDH, the *Q* pool, cytochrome $bc_1$, and cytochrome $cbb_3$ (Scheme 2) was not affected by Cu(btsc).

***(2) Cu(btsc) inhibits electron transfer* via *succinate dehydrogenase.*** Oxidation of *L*-lactate by *L*-LDH generates pyruvate, a central metabolite in the pathway for electron transport and energy production in *N. gonorrhoeae* (Scheme 2). Thus, the site of Cu(gtsm) inhibition in Figure 2 may occur within the branch of electron transfer fed by pyruvate. This molecule is also produced by the catabolism of glucose *via* the Entner-Doudoroff pathway (Scheme 2). It is subsequently oxidised by a cytoplasmic, $NAD^+$-dependent pyruvate dehydrogenase complex (PDH), generating NADH and acetyl-CoA. The latter is oxidised further to succinate in the TCA cycle and electrons from succinate finally enter the *Q* pool *via* SDH (Scheme 2). By measuring the rates of succinate-dependent consumption of $O_2$, it was observed that pre-treatment with 0.5–50 μM Cu(gtsm) led to a dose-dependent reduction in the rates of electron transfer *via* SDH (Figure 4; Scheme 2). This result is consistent with the inability of intact *N. gonorrhoeae* cells to oxidise lactate, glucose, and pyruvate in the presence of Cu(gtsm) (Figure 2).

A comparable loss in the rates of $O_2$ reduction by succinate was also detected after exposure to Cu(atsm) (Figure 4). This result was unexpected, as the effects of Cu(atsm) on aerobic respiration by intact cells were negligible (Figure 2a and Supplementary Figure 5). This apparent discrepancy may be related to the orientation of SDH and lipophilicity of Cu(atsm). Like other respiratory dehydrogenases, SDH is located on the cytoplasmic face of the cell membrane.(*26*) It may be readily accessible by Cu(btsc) in isolated membrane vesicles but not in intact cells. Cu(atsm) is more lipophilic than Cu(gtsm), and thus it may cross intact cell membranes less efficiently.(*21*) Under our experimental conditions, prolonged incubation with Cu(atsm) did not result in an enhanced inhibition of aerobic respiration by intact cells (Figure 2a and Supplementary Figure 5). By comparison, a short exposure to Cu(gtsm) was sufficient to elicit a strong inhibitory effect (Figure 2).

***(3) Cu(atsm) suppresses electron transfer* via *NADH dehydrogenases.*** The rates of NADH-dependent consumption of $O_2$ were also suppressed in a dose-dependent manner by 10–100 μM Cu(atsm) (blue traces in Figure 5a). This result indicated that the pathway of electron transfer *via* NADH dehydrogenases (NDHs) was also a likely site of Cu(atsm) toxicity in *N. gonorrhoeae.* As mentioned earlier, the genome of *N. gonorrhoeae* encodes for two putative NDHs, namely Nuo ($H^+$-translocating, Complex I-like) and Nqr ($Na^+$-translocating). To identify the specific enzyme that was inactivated by Cu(atsm), NADH oxidation was monitored in cell-free membrane vesicles of mutant strains, in which the gene encoding for the NADH-binding subunit of either one of the NDHs was deleted.

High turnover of Nqr was detected in the 1291*nuoF* mutant strain (Nuo-inactivated) and a relatively lower turnover for Nuo was observed in 1291*nqrF* (Nqr-inactivated) (black traces in Supplementary Figure 6). Both Nuo and Nqr were inhibited by Cu(atsm), but the impact appeared more pronounced for Nqr (blue traces in Supplementary Figure 6).

***(4) NADH dehydrogenases mediate redox cycling of Cu(gtsm).*** The rates of electron transfer from NADH to $O_2$ were reduced slightly by low doses of Cu(gtsm), but this effect was reversed by higher concentrations of Cu(gtsm) and the rates of $O_2$ consumption became apparently stimulated (red traces in Figure 5a and Supplementary Figure 6). This phenomenon was investigated further by blocking the transport of electrons from NADH to $O_2$ at the step catalysed by cytochrome $bc_1$ using 10 μM myxothiazol (Figure 5c). This treatment efficiently suppressed the rates of NADH respiration in the absence of Cu(btsc) (grey traces in Figure 5b). However, consumption of $O_2$ was resumed immediately upon addition of Cu(gtsm) (red traces in Figure 5b). NADH was apparently able to mediate one-electron transfer to Cu(gtsm). The reduction product, $Cu^I$ (presumably as $[Cu^IG]^-$), is air-sensitive and was subsequently re-oxidised by $O_2$ (Figure 5c).

By contrast to Cu(gtsm), redox cycling of Cu(atsm) was not detected in our system (blue traces in Figure 5b). Reduction of Cu(atsm) occurs at a more negative reduction potential ($E_{1/2}$ = -600 mV) than that of Cu(gtsm) ($E_{1/2}$ = -440 mV), and thus it may not be favourable under our experimental conditions. NADH-dependent reduction of Cu(btsc) has been demonstrated previously in mammalian systems for Cu(atsm) and a related analog $Cu^{II}P$ ($Cu^{II}$-pyruvaldehyde-*bis*(*N*4-methylthiosemicarbazone)). However, this occurs exclusively in hypoxic or xenocybrid mitochondria, where the reducing capacity of the system is high.(*25, 27, 28*)

**How do Cu(btsc) compounds exert their toxicity?** $Cu^{II}$ ions are known to damage components of the respiratory chain due to undesirable oxidation of crucial thiols.(*29, 30*) Yet, recent work has demonstrated that the $Cu^{II}$ ion trapped in both Cu(atsm) and Cu(gtsm) does not readily dissociate, even in the presence of an external high-affinity $Cu^{II}$ scavenger such as EDTA.(*13*) Thus, indirect toxicity associated with the generation of "free" $Cu^{II}$ ions in the solvent (Scheme 3a) and/or direct exchange of $Cu^{II}$ with protein ligands (Scheme 3b) is probably not the dominant mode of action of Cu(btsc) under our experimental conditions. Nevertheless, the $Cu^{II}$ centre in Cu(btsc) was required for the inhibition of electron transfer *via* SDH and NDH, as the copper-free $H_2$btsc ligands were not inhibitory (Figure 6b). Conversely, in control experiments where the $Cu^{II}$ ion was complexed to a non-btsc ligand, ethyleneglycol tetraacetate (Cu(EGTA)), only minor losses were detected in the rates of electron transfer *via* SDH and NDH (Figure 6a). These results indicated that an intact Cu(btsc) unit was necessary to elicit an inhibitory effect.

Although the $Cu^{II}$ ion in Cu(btsc) is inert with respect to exchange and dissociation, it is susceptible to reduction to $Cu^{I}$ in the presence of intracellular reductants (Scheme 3c).(*13*) The fate of the immediate reduction product, $[Cu^{I}(btsc)]^{-}$, is complex. The btsc ligands displays a high affinity for $Cu^{I}$ ($K_d$ in the pM range) and thus release of "free" $Cu^{I}$ to the solvent (Scheme 3d) is not likely.(*13*) However, direct transfer of the $Cu^{I}$ ion can still occur between $[Cu^{I}btsc]^{-}$ and high-affinity $Cu^{I}$ sinks (Scheme 3e) such as thiols or iron-sulfur clusters(*31, 32*). This ability of Cu(btsc) compounds to act as a source of toxic $Cu^{I}$ ions has in fact been identified as a major mechanism of their biological action (Scheme 1). However, it has already been demonstrated that "free" $Cu^{I}$ ions do not affect SDH from *Escherichia coli* or the isozyme fumarate reductase.(*31, 33*)

Furthermore, although the above model is true for Cu(gtsm), excess glutathione, ascorbate, or protein thiols are unable to effect reduction of Cu(atsm) *in vitro*, even in the presence of high-affinity $Cu^{I}$ scavengers.(*13*) Thus, our observation that Cu(atsm) was able to suppress electron transfer *via* SDH (Figure 4) and NDH (Figure 5a) suggests that the mechanism of inhibition likely involves direct binding of this compound to the dehydrogenases. This situation is plausible, as the non-redox active Zn(atsm) analogue was comparably efficient as an NDH inhibitor under the same conditions (data not shown), although a detailed follow-up study is required to confirm this model. Similarly, Cu(gtsm) may exert a direct inhibitory effect on SDH (Figure 4) and NDH (Figure 5a). However, the situation for Cu(gtsm) is more complex as this compound can also generate "free" $Cu^{I}$.(*13*) As shown in Figure 5a, a low concentration of Cu(gtsm) led to an initial decrease in the rates of NADH respiration. However, as the concentration increased, Cu(gtsm) led to an enhancement of NADH oxidation (Figures 5a and 5b), presumably due to direct reduction of $Cu^{II}$.

The resulting $Cu^{I}$ species from the reduction of Cu(gtsm) is also air-sensitive (Scheme 3f) and is able to rapidly reduce molecular $O_2$. This model is supported by the observation that in the presence of myxothiazol, addition of Cu(gtsm) leads to stimulation of $O_2$ uptake only (Figure 5b). Such redox cycling of Cu(gtsm) may generate toxic reactive oxygen species as the superoxide anion $O_2^-$ (*cf.* Figure 5c). This resembles the situation for Nuo in *E. coli*, where blockage of electron transfer and subsequent over-reduction of the *Q* pool led to production of $O_2^-$.(*34*)

**Concluding Remarks.** Gonorrhea remains a major public health concern worldwide. Vaccines are not available and treatment has become particularly challenging due to the appearance of multidrug-resistant strains.(*35*) Our work here clearly demonstrated the efficacy of Cu(btsc) as a potential antimicrobial agent against *N. gonorrhoeae*. Sub-micromolar concentrations of Cu(atsm) and Cu(gtsm) well below the tolerability limit by human tissues and animal models were sufficient to reduce viability of *N. gonorrhoeae* by a million fold (Figure 1a).

More importantly, our results also established that electron transfer processes from succinate and NADH dehydrogenases are *bona fide* sites of Cu(btsc) action (Figures 4 and 5, respectively). These dehydrogenases are flavoenzymes that contain iron-sulfur clusters and use ubiquinone as the electron acceptor. Although we cannot be precise about the mechanism, our results indicate that it is likely more complex than the existing model of Cu(btsc) reduction, $Cu^I$ release, and subsequent redox damage (Scheme 1). The observed interaction between Cu(btsc) and these respiratory enzymes on the cell membrane may also suggest that the lipophilicity of Cu(btsc) play a key role. This property may help the compounds access hydrophobic pockets that would otherwise be inaccessible by "free" $Cu^I$ and $Cu^{II}$ ions. Further investigations in mammalian mitochondria models will be important in the development of Cu(atsm), Cu(gtsm), and related compounds for anti-cancer strategies.

## EXPERIMENTAL

**Materials.** Cu(atsm), Cu(gtsm), $H_2A$, and $H_2G$ were synthesised following published procedures.(36) All other chemicals were from Sigma-Aldrich unless specified otherwise.

**Preparation of Cu stocks.** Master stocks of $Cu(NO_3)_2$ (1 M) were prepared in deionised water. The $[Cu^{II}(egta)]^{2-}$ complex (10 mM) was prepared by titration of $Cu(NO)_3$ into a solution of the copper-free $H_4$egta ligand in water, as monitored by the solution absorbances at 610 nm. Master stocks of Cu(atsm) and Cu(gtsm) (10 mM each) were prepared in neat DMSO. All other concentrations were prepared by serial dilutions of the master stocks in deionised water or DMSO.

**Bacteria culture conditions.** *N. gonorrhoeae* strains were propagated on solid GC base (Oxoid) supplemented with 1% (v/v) IsoVitaleX enrichment (Becton Dickinson).

Where required, kanamycin was used at a concentration of 100 µg $mL^{-1}$. Visible lawns were obtained after overnight incubation at 37 $^{o}$C in the presence of 5% (v/v) $CO_2$ and used as pre-cultures for further experiments. Liquid cultures were prepared in Chemically Defined Medium (CDM)(*37*) containing 0.5% (v/v) glucose as the sole carbon source. Cultures (50 mL) were grown in conical flasks (250 mL) at 37 $^{o}$C and aerated by vigorous shaking at 200 rpm.

**Killing assays.** Killing of gonococci by $Cu(NO_3)_2$ (0–100 µM), Cu(atsm) (0–1 µM) and Cu(gtsm) (0–0.1 µM) were examined on solid GC media. Pre-cultures were resuspended in phosphate buffered saline (PBS) to an $OD_{600}$ of 0.4 (ca. 1 x $10^8$ CFU $mL^{-1}$). Serial dilutions (1:10) were prepared in the same buffer and 5 µL of each dilution was spotted on fresh solid GC medium containing a Cu donor at the specified concentration. The final concentration of DMSO in cultures was < 0.05% (v/v). Each medium was prepared immediately before use. Surviving CFU were counted after 24 h of growth at 37 $^{o}$C and 5% (v/v) $CO_2$. The results were plotted as a function of Cu concentrations.

**Growth of bacteria for biochemical analyses.** To measure the rates of aerobic respiration, gonococci were grown in aerated liquid cultures without any added Cu. To measure production of Cop III, bacteria were grown in aerated liquid cultures containing $Cu(NO_3)_2$ (10 µM), Cu(atsm) (80 nM) and Cu(gtsm) (30 nM). After 5 h ($OD_{600}$ ~ 0.9–1.0), bacteria were collected by centrifugation and rinsed with 3 x 25 mL of ice-cold Wash Buffer (50 mM Na-Hepes, 10 mM $MgCl_2$, pH 7.4).

**Cell fractionation.** To obtain whole, intact gonococci, centrifuged pellet from a liquid culture were resuspended in Wash Buffer to an $OD_{600}$ of ~ 100 and kept on ice until further use. To obtain cell-free extracts, centrifuged bacterial pellet was resuspended in ice-cold Wash Buffer (10 mL) and disrupted by passage through a French Press cell (3 x 15000 psi). Unbroken cells and insoluble cell debris were removed by centrifugation

(5000*g*, 20 min) and the supernatant was kept on ice until further use. To obtain cell-free membrane vesicles, the cell-free supernatant was filtered through a 0.22 μm filtration unit (MilliPore) and centrifuged at high speed (150000*g*, 2 h, 4 $^{o}$C). The membrane-rich pellet was homogenised in ice-cold Wash Buffer to a final protein concentration of ca. 200 mg mL$^{-1}$ and stored on ice until further analyses. All analyses were performed on the same day of preparations.

**Measurement of intracellular Cop III levels.** Tetrapyrroles were extracted from centrifuged bacterial pellet into in 500 μL of 80/20/1 (v/v) EtOH/DMSO/acetic acid. Insoluble debris was removed by centrifugation (15000*g*, 5 min) and the supernatant was analysed for Cop III fluorescence. All samples were prepared in a glass cuvette with a path length of 1 cm. Fluorescence emission spectra were recorded between 580 and 720 nm using an excitation wavelength of 405 nm, slit widths of 10 nm, and a scan speed of 500 nm min$^{-1}$. In our experimental conditions, the absorbance of Cu(btsc) was negligible ($Abs_{405}$ << 0.001).

**Measurement of $O_2$ consumption.** Consumption of $O_2$ by intact cells and cell-free membrane vesicles was measured at 30–35 $^{o}$C in potassium phosphate (20 mM, 150 mM NaCl, pH 7.4) or Na-Hepes (50 mM, pH 7.4) buffer, respectively. Where required, myxothiazol was added to the reaction buffer to a final concentration of 10 μM. Whole cells (50 μL, or a final optical density of ~2.5) and membranes (~10 mg) were added at t = 10 s. Where required, $Cu(NO_3)_2$, Cu(atsm), or Cu(gtsm) (0–100 μM each) were added at t = 20 s. Reduction of $O_2$ was initiated by addition of electron donors at t = 3 min. The following electron donors were used: sodium *L*-lactate (50 mM), sodium pyruvate (50 mM), D-(+)-glucose (0.5% (v/v)), sodium succinate (25 mM), β-NADH (1 mM), and TMPD/ascorbate (1 mM/5 mM). Rates of reactions were monitored for a further 3 min using an S1/Mini Clark-type oxygen electrode (Hansatech Instruments) in conjunction with an Oxytherm control unit.

**Measurement of protein concentrations.** Protein content was determined using QuantiPro BCA Assay Kit (Sigma).

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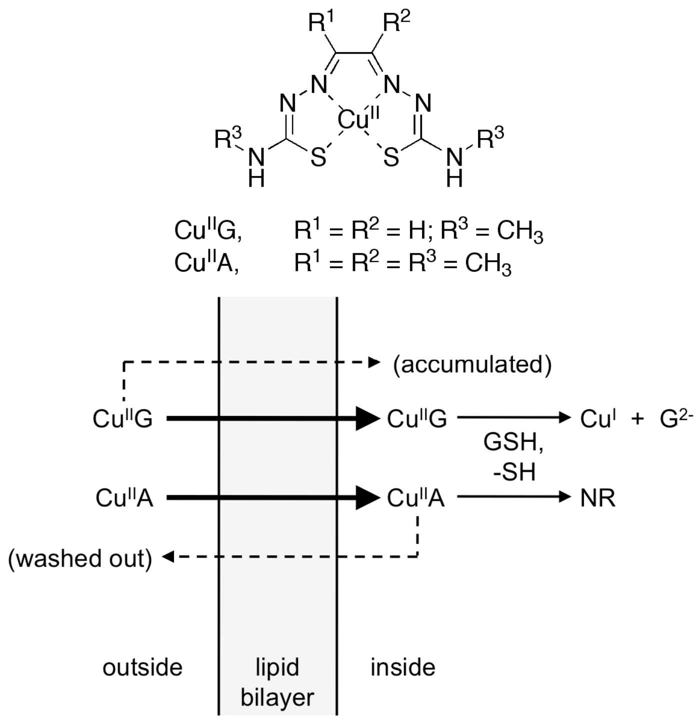
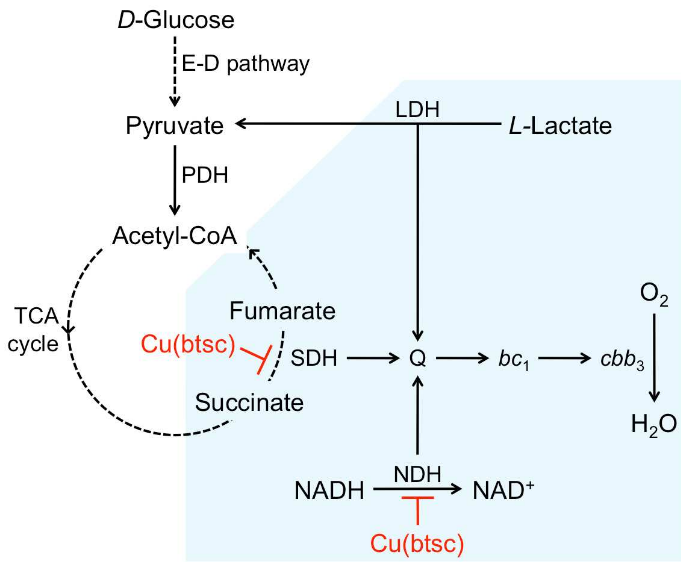
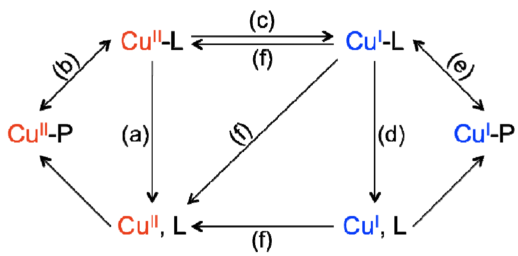
**SCHEMES AND FIGURES**




**Scheme 1.** Simplified model for the ability of Cu(btsc) to increase bio-available $Cu^I$. Upon entry into the cytoplasm, Cu(gtsm) is reduced by thiols to release $Cu^I$ ions while Cu(atsm) remains unaffected. -SH, protein thiols; GSH, glutathione; NR, no reaction.




**Scheme 2.** Organisation of the respiratory electron transfer chain in *N. gonorrhoeae*. Arrows indicate the direction of electron transfer. Dotted arrows indicate involvement of multiple enzymes (not shown). Processes catalysed by membrane-bound enzymes are shaded in blue. The identified sites of inhibition by Cu(btsc) are shown in red. E-D, Entner-Doudoroff; LDH, *L*-lactate dehydrogenase; NDH, NADH dehydrogenases; PDH, pyruvate dehydrogenase; SDH, succinate dehydrogenase; TCA, tricarboxylic (citric) acid.




**Scheme 3. Possible mechanisms of Cu(btsc) toxicity. a)** Dissociation of Cu(btsc) to release "free" $Cu^{II}$ ions. **b)** Direct exchange of $Cu^{II}$ ion between Cu(btsc) and protein sites. **c)** Reduction of Cu(btsc). **d)** Dissociation of $[Cu^{I}(btsc)]^{-}$ to release "free" $Cu^{I}$ ions. **e)** Direct exchange of $Cu^{I}$ ion between $[Cu^{I}(btsc)]^{-}$ and protein sites. **f)** Re-oxidation of $[Cu^{I}(btsc)]^{-}$ to Cu(btsc). L, btsc ligand; P, protein ligand.

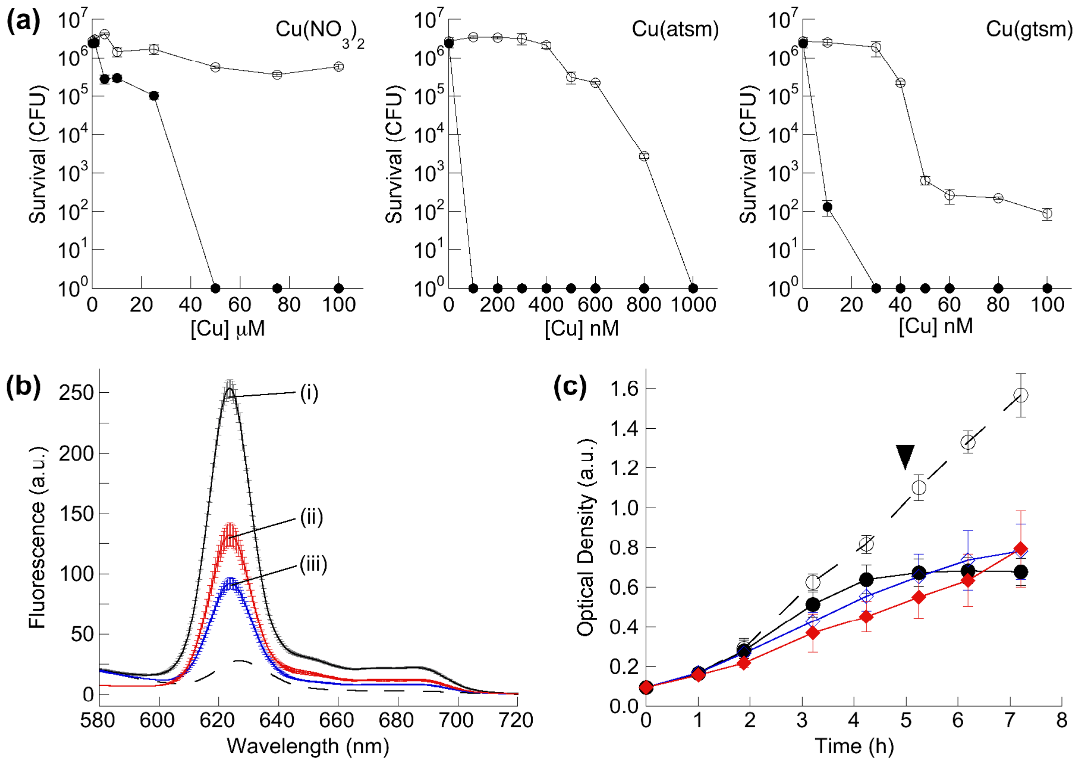



**Figure 1. Antimicrobial activity of Cu(btsc) against *N. gonorrhoeae*. a)** Bacterial killing. Survival of 1291wt (open circles ○) and 1291*copA* (closed circles ●) after exposure to increasing concentrations of $Cu(NO_3)_2$, Cu(atsm), or Cu(gtsm) for 20–24 h. **b)** Cop III production. Fluorescence emission spectra of porphyrin extracts of 1291*copA* cultures that were exposed to: (i) 10 µM $Cu(NO_3)_2$ (black trace), (ii) 30 nM Cu(gtsm) (red trace), (iii) 80 nM Cu(atsm) (blue trace). The emission spectrum of the untreated control is shown for comparison (dashed trace). **c)** Growth inhibition. Growth of 1291*copA* in the presence of 10 µM $Cu(NO_3)_2$ (closed circles ●, black trace), 30 nM Cu(gtsm) (closed diamonds ◆, red trace), or 80 nM Cu(atsm) (open diamonds ◇, blue trace). Growth of the untreated control is shown for comparison (open circles ○, dashed trace). Cells were harvested for Cop III analysis at the time indicated by the downward arrow. **a-c)** Each data point was generated from three independent experiments. Error bars represent ± standard deviation from the mean.

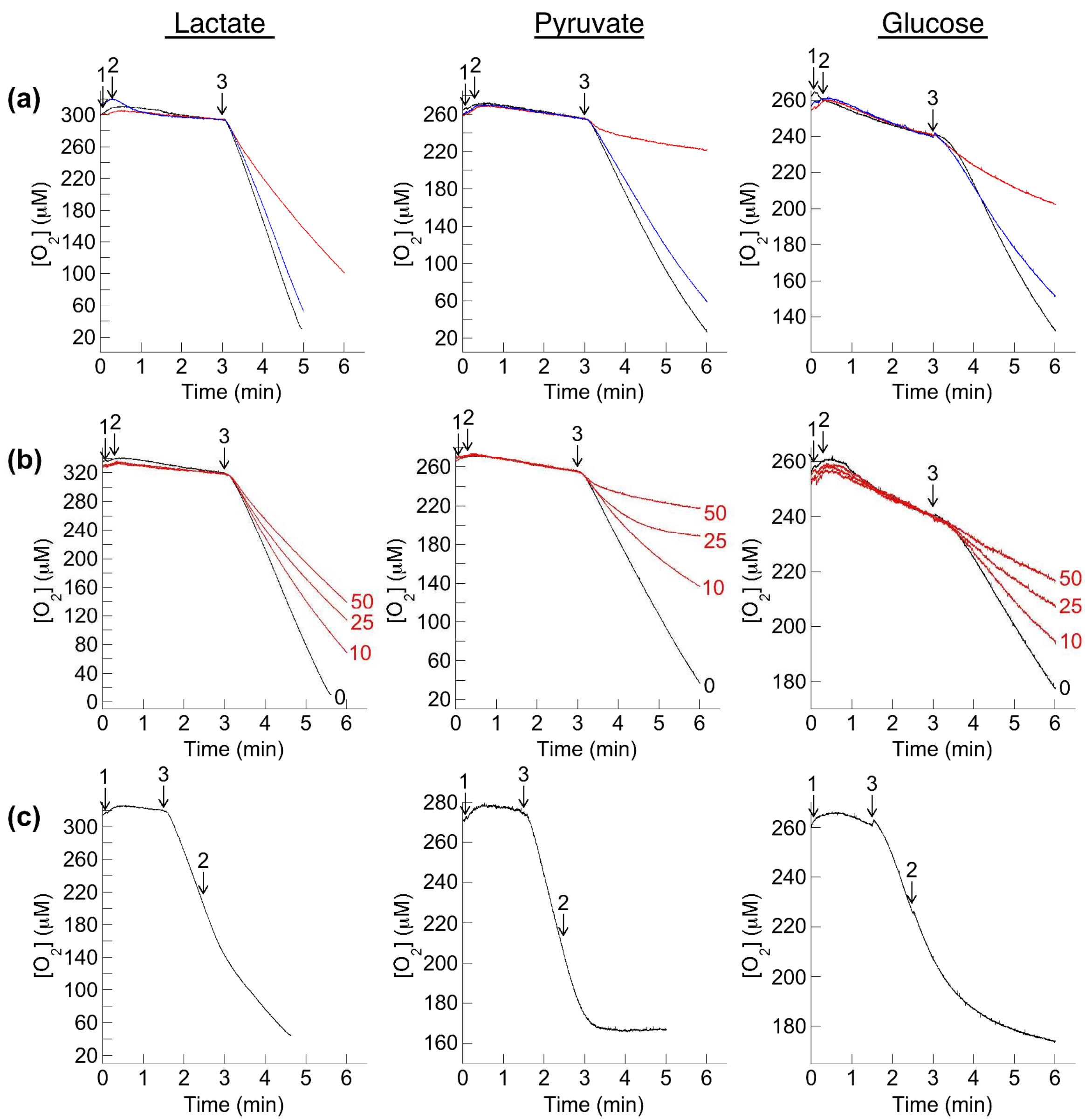



**Figure 2. Inhibition of aerobic respiration by Cu(gtsm).** Consumption of $O_2$ by intact 1291wt cells as driven by lactate, pyruvate, or glucose as indicated. **a)** Effects of Cu(atsm) and Cu(gtsm). Rates of $O_2$ consumption after pre-incubation with DMSO (black traces), or 50 µM each of Cu(gtsm) (red traces) or Cu(atsm) (blue traces). **b)** Dose-dependent inhibition by Cu(gtsm). Rates of respiration after pre-incubation with 0 (black traces) or 10–50 µM of Cu(gtsm) (red traces) as indicated. **c)** Immediate inhibition by Cu(gtsm).

Decrease in the rates of $O_2$ consumption upon addition of 50 μM Cu(gtsm). **a-c)** Addition of cells (1), DMSO or Cu compounds (2), and electron donors (3) are indicated by downward arrows. Representative results of at least three independent replicates are shown. These experiments have been repeated using 1291*copA* to produce identical results.

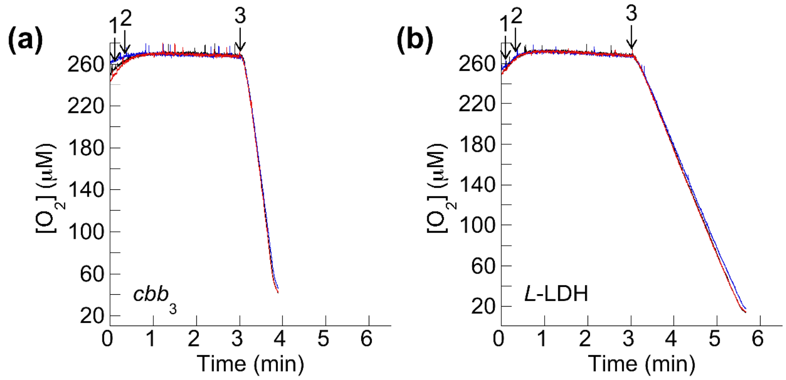



**Figure 3. Cu(btsc) does not affect activities of cytochrome *cbb*₃ and *L*-LDH.** Rates of electron transfer *via* cytochrome *cbb*$_3$ **(a)** and *L*-LDH **(b)**. Consumption of $O_2$ after pre-incubation with DMSO (black traces), or 50 μM each of Cu(gtsm) (red traces) or Cu(atsm) (blue traces). Addition of membrane vesicles (1), DMSO or Cu compounds (2), and electron donors (3) are indicated by downward arrows. Representative results of at least three independent replicates are shown.

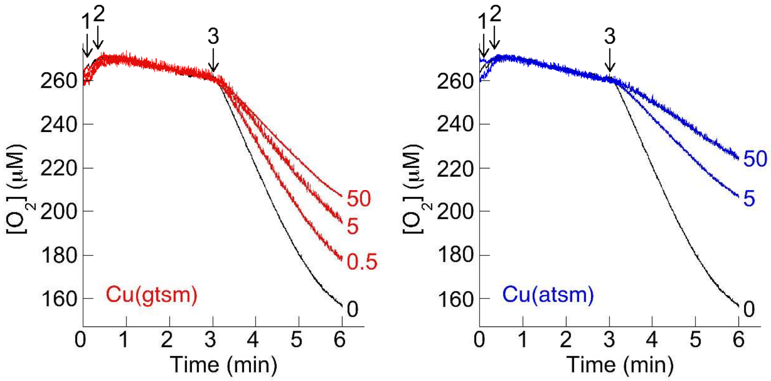



**Figure 4. Inhibition of electron transfer *via* succinate dehydrogenase.** Succinate-dependent consumption of $O_2$ by cell-free membrane vesicles after pre-incubation with 0–50 μM each of Cu(gtsm) (red traces) or Cu(atsm) (blue traces). Addition of membrane vesicles (1), DMSO or Cu compounds (2), and succinate (3) are indicated by downward arrows. Representative results of at least three independent replicates are shown.

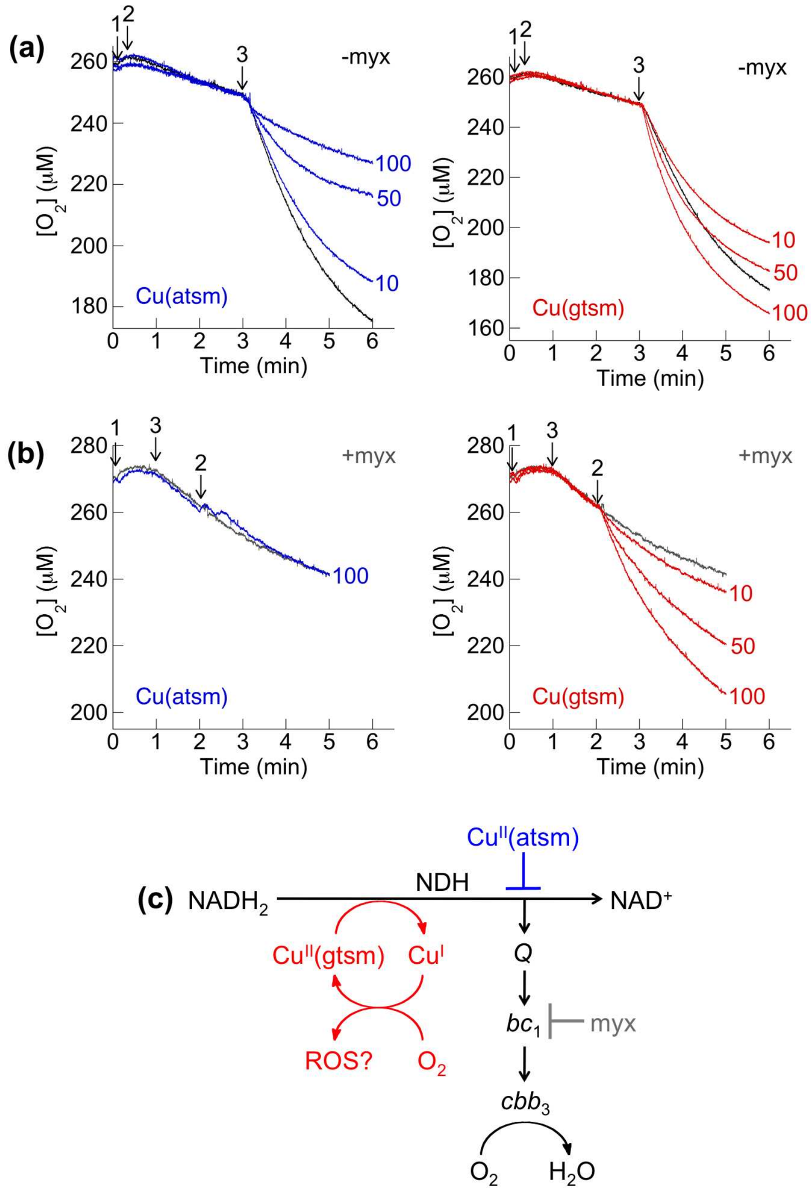



**Figure 5. Inhibition of NADH dehydrogenases by Cu(atsm) and NADH-dependent redox cycling of Cu(gtsm).** NADH-dependent consumption of $O_2$ by cell-free membrane vesicles. **a)** Effects of Cu(btsc) on the rates of NADH respiration. Rates of $O_2$ consumption after pre-incubation with 0 (black traces) or 10–100 μM each of Cu(atsm) (blue traces) or Cu(gtsm) (red traces). Measurements were performed in the absence of myxothiazol (-myx). **b)** NADH-dependent redox cycling of Cu(btsc). Rates of $O_2$ consumption after pre-incubation with 0 (grey traces), or 10–100 μM each of Cu(atsm) (blue traces) or

Cu(gtsm) (red traces). Measurements were performed in presence of 10 μM myxothiazol (+myx). **a-b)** Addition of membrane vesicles (1), DMSO or Cu compounds (2), and NADH (3) are indicated by downward arrows. Representative results of at least three independent replicates are shown. **c)** Schematic represantation of the results in panels a) and b). The effect of Cu(atsm) is shown in blue. Redox cycling of Cu(gtsm) was shown in red. The site of myxothiazol inhibition is shown in grey. myx, myxothiazol; NDH, NADH dehydrogenases.

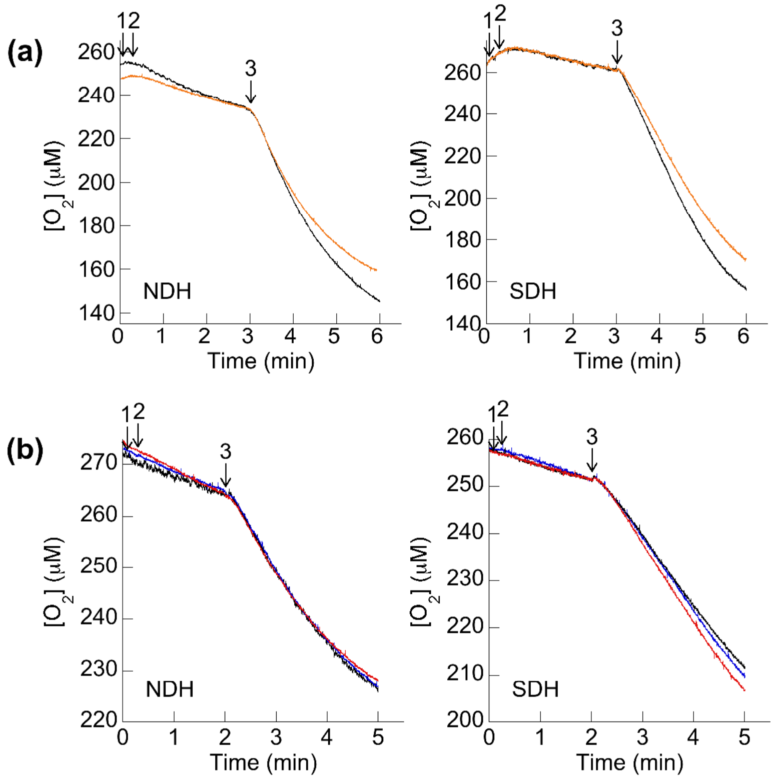
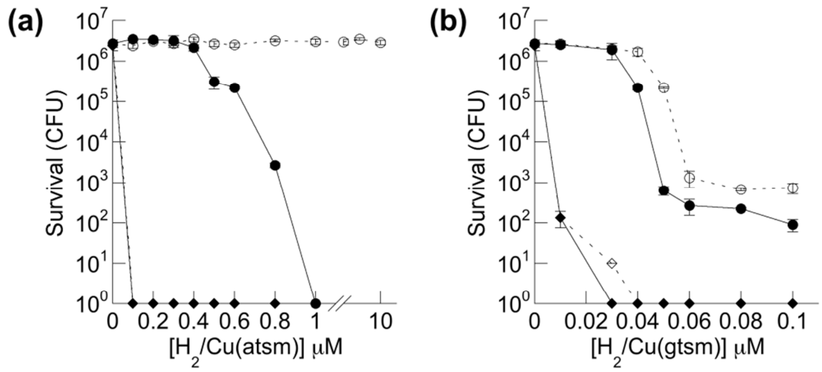
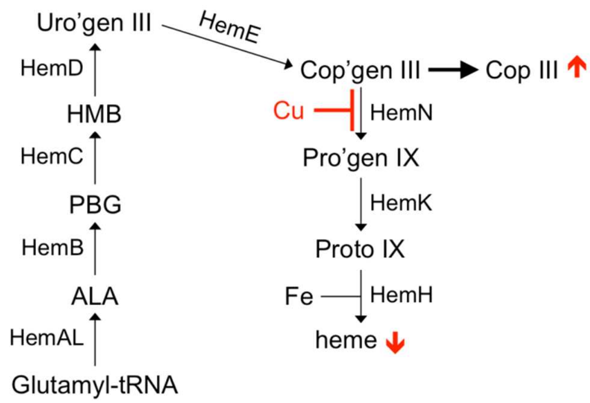



**Figure 6. The copper centre and btsc ligand were jointly required for inactivation of NDH and SDH.** Effects of: **a)** a non-btsc source of copper and **b)** copper-free $H_2$(btsc) ligands. **a-b)** Rates of electron transfer *via* NDH and SDH after pre-incubation with DMSO (black traces), Cu(EGTA) (orange traces), $H_2$G (red traces), or $H_2$A (blue traces) (50 μM each). Addition of membranes vesicles (1), DMSO, Cu(EGTA) or $H_2$(btsc) (2), and (3) electron donors are indicated by downward arrows. Representative results of at least three independent experiments are shown.

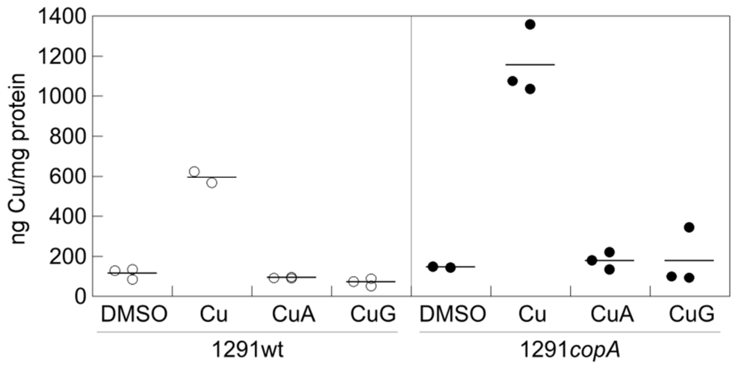
# SUPPORTING INFORMATION



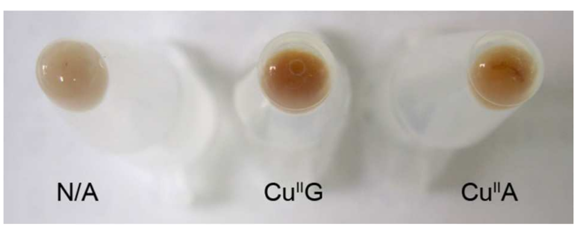

**Supporting Figure 1. Copper-free $H_2$btsc was less toxic than Cu(btsc).** Survival of 1291wt (circles ○ and ●) and 1291*copA* (diamonds ◇ and ◆) after exposure to increasing concentrations of: **a)** Cu(atsm) (closed symbols ● and ◆) and $H_2$atsm (open symbols ○ and ◇), or **b)** Cu(gtsm) (closed symbols ● and ◆) and $H_2$gtsm (open symbols ○ and ◇) for 20–24 h. Each data point was generated from three independent experiments. Error bars represent ± standard deviation from the mean. It is important to note that $H_2$atsm and $H_2$gtsm ligand are metallated by basal amounts of Cu from the growth media to generate the toxic species Cu(atsm) and Cu(gtsm). We have routinely detected between 1 and 10 nM of basal Cu in our media preparations. These concentrations are within the toxic range for Cu(atsm) and Cu(gtsm). Thus, the requirement for Cu is most apparent in panel a). The 1291wt strain was killed only by ~ 1 μM Cu(atsm). In this case, the $H_2$atsm ligand had no observable effect up to 10 μM.



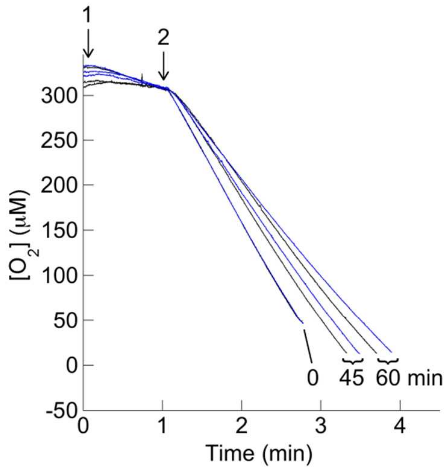

**Supporting Figure 2. Excess Cu arrests the heme biosynthesis pathway in *N. gonorrhoeae.*** Excess Cu blocks the step catalysed by HemN as indicated in red. As a result, there is a decrease in heme levels (red downwards arrow ↓) and a concomitant increase of Cop III levels (red upwards arrow ↑). ALA, aminolevulinic acid; PBG, porphobilinogen; HMB, hydroxymethylbilane; Uro'gen III, uroporphyrinogen III; Cop'gen, coproporphyrinogen III; Cop III, coproporphyrin III; Pro'gen IX, protoporphyrinogen IX; Proto IX, protoporphyrin IX.



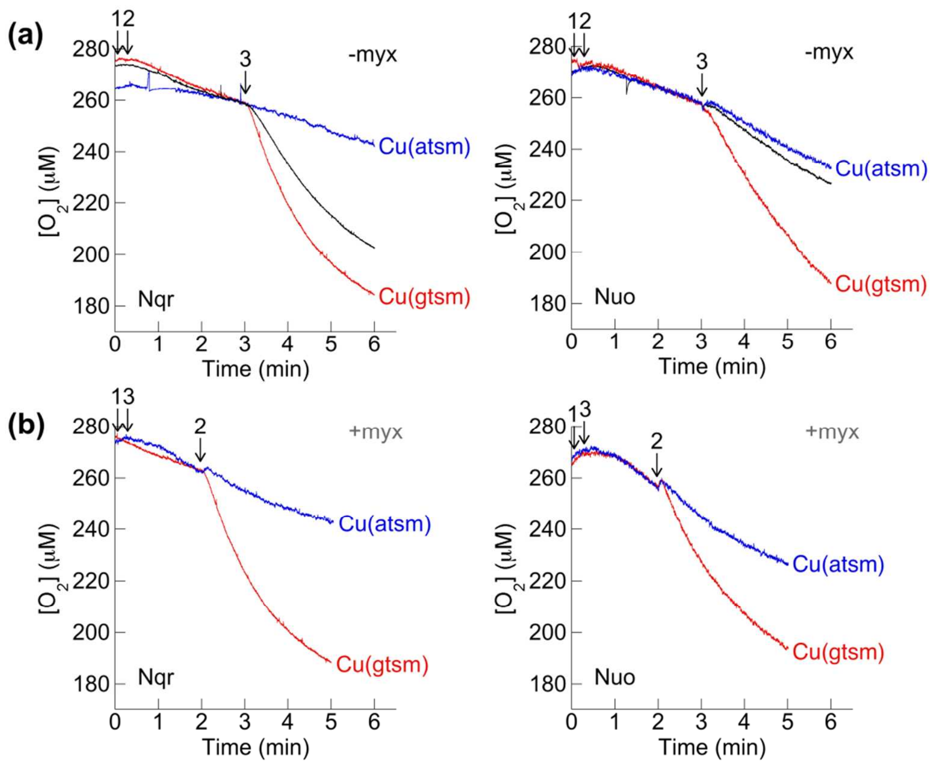

**Supporting Figure 3. Treatment with Cu(btsc) did not lead to a detectable increase in intracellular Cu levels.** Total amounts of intracellular copper as detected by ICP MS. *N. gonorrhoeae* strains 1291wt and 1291*copA* were treated with DMSO, $Cu(NO_3)_2$ (Cu), Cu(atsm) (CuA), or Cu(gtsm) (CuG) following the conditions shown on Figure 1c. At least two independent replicates were shown for each measurement.




**Supporting Figure 4. Association of Cu(btsc) with *N. gonorrhoeae* cell membranes.** Cu(gtsm) ($Cu^{II}G$) and Cu(atsm) ($Cu^{II}A$) (50 µM each) was added to a suspension of 1291wt in PBS to reflect the conditions used for the measurement of respiration in intact cells (see Materials and Methods). Cells were sedimented after 1 min and the supernatant was removed. Photos of centrifuged bacterial pellets were taken under ambient light conditions. The untreated control (N/A) was shown for comparison.




**Supporting Figure 5. Prolonged incubation with Cu(atsm) did not lead to an enhanced inhibition of aerobic respiration.** Pyruvate-driven consumption of $O_2$ by intact cells after pre-incubation without (black traces) or with 50 μM Cu(atsm) (blue traces) for 0, 45, and 60 min as indicated. Addition of cells (1) and pyruvate (2) are indicated by downward arrows. There was an overall decrease in the rates of pyruvate oxidation regardless of Cu(atsm) treatment, presumably due autolysis of *N. gonorrhoeae*.




**Supporting Figure 6. Inhibition of Nuo and Nqr by Cu(atsm) and NADH-dependent redox cycling of Cu(gtsm).** NADH-dependent consumption of $O_2$ by cell-free membrane vesicles of mutant strains containing only one active NADH dehydrogenase. Nqr activity was obtained from 1291*nuoF* mutant, while Nuo activity was measured using 1291*nqrF* mutant. **a)** Effects of Cu(btsc) on the rates of NADH respiration. Rates of $O_2$ consumption after pre-incubation with 0 (black traces) or 100 μM each of Cu(atsm) (blue traces) or Cu(gtsm) (red traces). Measurements were performed in the absence of myxothiazol (-myx). **b)** NADH-dependent redox cycling of Cu(btsc). Rates of $O_2$ consumption after pre-incubation with 100 μM each of Cu(atsm) (blue traces) or Cu(gtsm) (red traces). Measurements were performed in presence of 10 μM myxothiazol (+myx). **a-b)** Addition of membrane vesicles (1), DMSO or Cu compounds (2), and NADH (3) are indicated by downward arrows.