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Inactivation of thiostrepton by copper(II)

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Summary. Among the various bivalent metal ions tested, only copper(II) was found to bind to thiostrepton (M_r 1650) in a stoichiometric ratio of 4:1. The binding of four copper ions to a thiostrepton molecule resulted in (a) irreversible loss in biological activity and (b) a change in the ultraviolet absorption spectrum of the antibiotic. Potentiometric titration of thiostrepton in the presence of copper(II) revealed dissociation of the antibiotic with a loss of 11 protons/molecule. Based on the preferential ability of copper(II) to bind to thiostrepton in the presence of some copper-complexing compounds containing similar ligand groups to the antibiotic, the possible co-ordinating atoms of the thiostrepton molecule involved in binding to the metal ion are discussed.

Key words: Thiostrepton – Copper(II) – Biological activity – Metal ion – Molar ratio

Introduction

The molecular stuctures of a considerable number of antimicrobial agents contain sites at which co-ordination with metal ions can occur. Binding of metal ions to these compounds at such sites may result in either abolishing (Weinberg 1957), stabilizing (Garbutt et al. 1961), or enhancing (Adler and Snoke 1962) their biological activity. Various mechanisms have been proposed to explain such altered activity of antimicrobial compounds on interaction with metal ions (Weinberg 1957). The study of metal interaction with antimicrobial compounds is thought to provide valuable information about the structure activity relationship of these agents, especially when metal co-ordination affects their biological activity. Hence, metal chelation of antimicrobial agents has been the subject of many investigations (Venkateswerlu 1981; Takahashi et al. 1987; Pearce and Friedman 1989).

Thiostrepton, a peptide antibiotic (Fig. 1) (Hensons and Schonberg 1983), is produced by *Streptomyces azureus*, a soil isolate (Pagano et al. 1956). The molecular structure of the antibiotic reveals that it contains many potential ligand atoms that can co-ordinate to metal ions. Complexes of thiostrepton with alkaline earth metals under anhydrous conditions in non-aqueous solvents has been reported in the literature (Donovick et al. 1961). However, such investigations are of little value in biological studies of the antibiotic. Therefore, the present work deals with the interaction of thiostrepton with various bivalent metal cations and the consequent effect on its biological activity.

Materials and methods

Materials. Thiostrepton standard (M_r 1650, 1080 U/mg) was a gift sample from The Squibb Institute, New Jersey, USA. The complex nitrogen sources used in the antibiotic assay medium were obtained from the following sources: Bacto peptone (Difco laboratories, USA), beef extract (Hi media, Bombay, India), yeast extract (Oxoid Co., England). Silica gel G for thin-layer chromatography (TLC) was purchased from Sarabhai Chemicals (Bombay, India). All the other chemicals were of analar grade and used directly.

Assays. Biological assay of thiostrepton against the Oxford strain of *Staphylococcus aureus* was performed by both the disc diffusion method (Levin et al. 1960) and the tube dilution method (Kelly et al. 1959) reported in the literature. A Beckman DU-50 recording spectrophotometer was used for the optical absorption studies. All the experiments were performed three times and the average values are reported.

Thiostrepton interaction with metal ions. To 100 µg thiostrepton (0.06 µmol) in 0.25-ml dimethylsulfoxide (Me_2SO) was added 1 ml of various metal ion solutions (taken either as sulfate or chloride) individually, containing varying molar ratios to the antibiotic, and thoroughly mixed. The metal ions employed were Cu(II), Co(II), Cd(II), Ni(II), Mg(II), Mn(II), Zn(II), Fe(II)/(III). The tubes were incubated in a water bath maintained at 70° C for 7 h until the reaction mixture in the tube containing copper(II) became pale yellow in colour. The tubes were then cooled to room tem-

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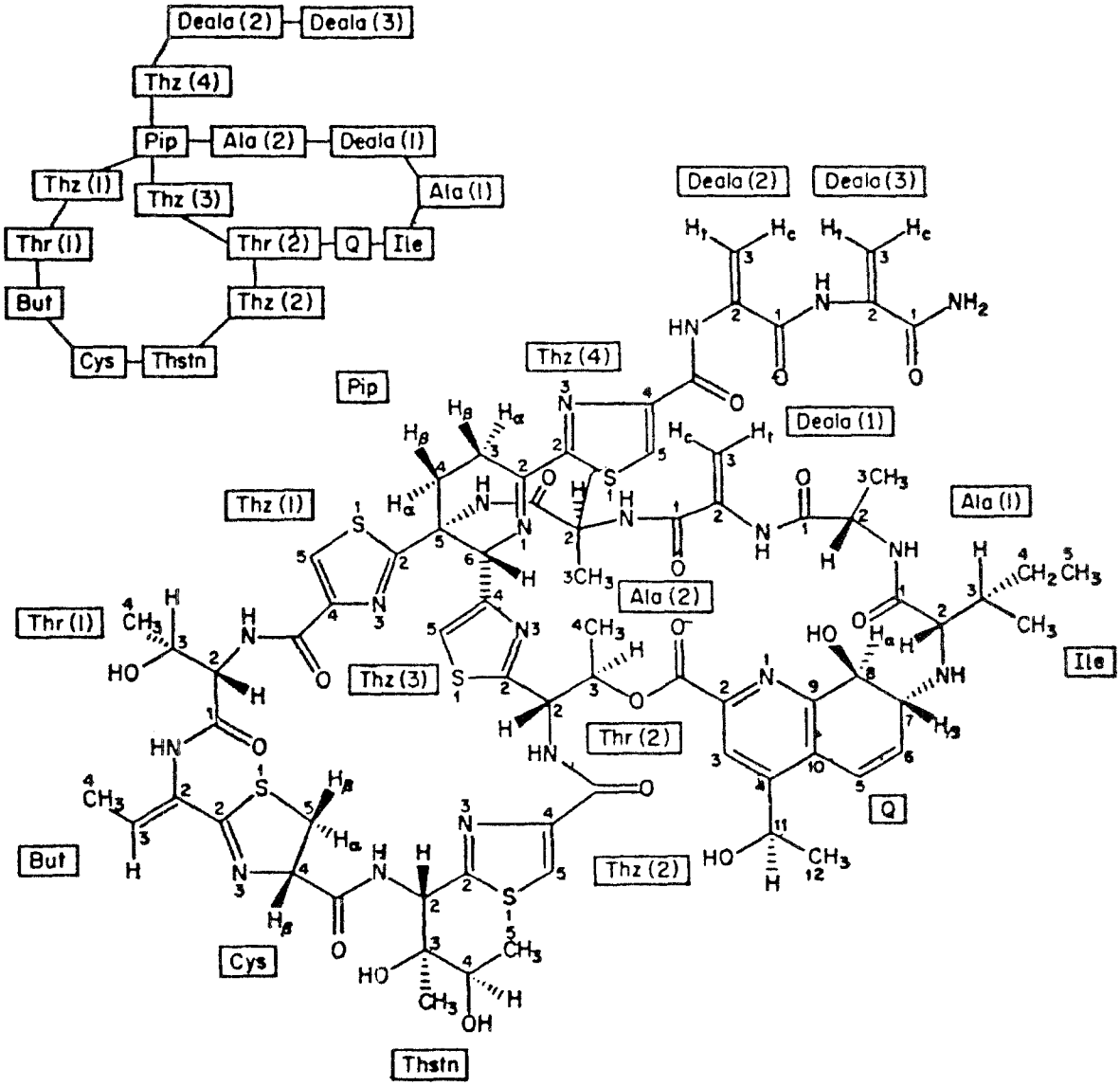

Fig. 1. Structure of thiostrepton. Thstn = Thiostreptine residue; Q = Quinaldic ocid precursor; Cys = Thiazoline residue; Thz = Thiazole ring; Pip = Piperidine ring

perature. Bioassay of the antibiotic in the reaction mixture was then carried out; it was always coupled to TLC using activated silica gel plates. Ultraviolet absorption spectra of thiostrepton in the reaction mixture were recorded using the respective metal salt solution at the same concentration as the corresponding blank.

Thin-layer chromatography. TLC plates (20 × 20 cm) containing silica gel G (250 μm-thick) were activated for 30 min at 100° C before use. An aliquot of the reaction mixture was then spotted on the TLC plate and chromatogram was run for 120 min using various solvent systems. The plates were then dried at 100° C for 30 min and the spots were located under short-wavelength ultraviolet light. For comparison, thiostrepton standard and the copper salt was also subjected to identical conditions and studied.

Time course of copper(II) interaction with thiostrepton. To determine the time course for completion of the interaction of copper(II) with thiostrepton, the reaction of the metal with thiostrepton (at 4:1 molar ratio) was carried out under the experimental conditions specified above. During the incubation, aliquots were withdrawn at regular intervals of 1 h and the minimal inhibitory concentration (MIC) determined by the tube dilution method. For comparison, thiostrepton in the absence of metal ion was incubated under identical conditions and bioassayed.

Determination of MIC value of thiostrepton in the presence of varying molar ratios of copper(II). The MIC value of thiostrepton from the reaction mixture of thiostrepton/copper(II) at varying molar ratios was determined, after incubation of the sample at 70° C for 7 h, by diluting an aliquot of the sample in 80% Me_2SO to appropriate concentrations.

Potentiometric titration of thiostrepton in the presence of copper(II). Acidified 50% dioxane solutions of thiostrepton in the presence of copper(II) at 1:4 molar ratios, and also in the absence of the metal, were individually treated with alkali (0.1 M KOH). The increase in pH after addition of alkali was noted. The acid blank was also titrated simultaneously for comparison. The blank titration contained 2.5 ml 0.04 M HNO_3, 5 ml 1 M KNO_3, 25 ml dioxane and 17.5 ml water; the ligand titration contained in addition 0.5 mM thiostrepton dissolved in the dioxane; the ligand titration in presence of the metal contained in addition to this 2 mM copper(II) dissolved in the water. The titration was carried out at a constant temperature of 30° C with the reaction mixture being continuously stirred.

Interaction of copper(II) with thiostrepton in the presence of some copper-complexing compounds. To a thiostrepton/copper(II) solution of 1:4 molar ratio in different tubes, 1 mg of various compounds was added individually and incubated at 70° C for 7 h. The reaction mixture was later bioassayed. Using appropriate blanks, the ultraviolet absorption spectrum of thiostrepton was recorded. The thiostrepton standard, in the presence of these compounds but without the metal ion, was subjected to identical conditions and simultaneously bioassayed for comparison.

Spectrophotometric method of determining copper complex formation by thiostrepton. The ability of thiostrepton to complex with copper(II) was determined spectroscopically by the molar ratio method (Srinivasa and Ramachandran 1980) as follows. To 0.25 ml thiostrepton solution in Me_2SO (0.06 µmol) was added 0.1, 0.2, 0.3, 0.4, 0.6, 0.8 and 1 ml of copper chloride solution (0.48 µmol copper) to obtain the molar ratios of 1:0.8, 1:1.2, 1:2.4, 1:3.2, 1:4.8, 1:6.4 and 1:8 of thiostrepton/copper(II). The total volume in all the tubes was made up to 1.25 ml with water. These tubes were designated as the test sample sets. To the control sample sets was added graded levels of copper(II) as above and 0.25 ml Me_2SO but without thiostrepton. Both sets were incubated at 70° C for 7 h. Then, the absorbance of the test sample was measured at 360 nm using the corresponding tubes of the control sample set as the blank to obtain the differential ΔA. These ΔA values were then plotted against the molar ratios of thiostrepton/copper(II). The maximal value of ΔA in the graph indicated the maximum molar ratio of the metal ion/ligand in the chelate formed.

Results and discussion

The striking observations of our results are that, among different bivalent metal cations tested, only copper(II) was found to complex with thiostrepton under the experimental conditions as monitored by biological activity (Table 1), ultraviolet absorption and formation of colour in the reaction mixture. Binding of copper to thiostrepton at 1:4 molar ratio resulted in total loss of biological activity of the antibiotic. The absorption spectrum of the copper-interacted thiostrepton revealed that it acquired an additional shoulder at 360 nm while retaining the 260-nm absorption peak and 280-nm shoulder of the native molecule (Fig. 2). The reaction mixture also became yellow, indicating complexation. The other metal ions affected neither the biological activity nor the absorption spectrum of thiostrepton, nor did they produce any colour in the reaction mixture

Table 1. Effect of different metal cations on biological activity of thiostrepton

Metal ion	Metal ion/ thiostrepton (mol/mol)	Activity (%)
None (control)	—	100
Copper(II)	4.0	0
Cobalt(II)	70.0	100
Cadmium(II)	45.0	100
Nickel(II)	50.0	100
Magnesium(II)	60.0	100
Manganese(II)	50.0	100
Zinc(II)	60.0	100
Iron(II)/(III)	50.0	100

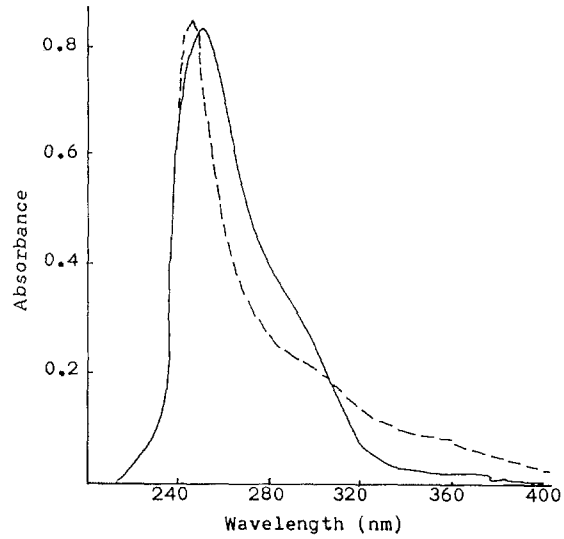

Fig. 2. Ultraviolet absorption of thiostrepton (——) and its copper(II) complex (- - - -) in 20% Me_2SO

when they were present in even larger molar ratios. Therefore, in all the subsequent investigations, only the interaction of copper(II) with thiostrepton was studied.

Preliminary studies revealed that for thiostrepton/copper(II) at 1:4 molar ratios, incubation for 72 h at 37° C was required for total loss in biological activity of the antibiotic. But when the temperature of the reaction mixture was elevated to 70° C, only 7 h of incubation time was required. Thus the binding of copper(II) to the antibiotic molecule during incubation appears to be a temperature-dependent process that is kinetically controlled. Thiostrepton in the absence of metal ion was, however, found to retain the same degree of antimicrobial activity, indicating that there is no decomposition of the antibiotic at this elevated temperature (Fig. 3).

The results presented in Table 2 show that a steady increase in the molar ratio of thiostrepton/copper(II) resulted in a gradual rise in the MIC value of the anti-

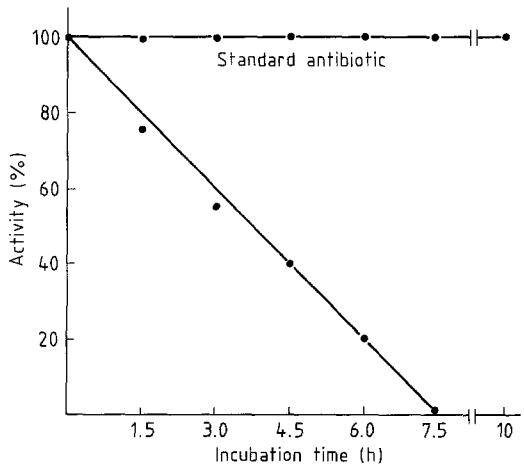

Fig. 3. Time course of copper(II) interaction with thiostrepton

Table 2. Effect of varying molar ratios of copper(II) on biological activity of thiostrepton

Copper(II)/ thiostrepton (mol/mol)	MIC (μg/ml)	Activity (%)
0 (Control)	0.02	100
0.4	0.05	97.5
0.8	0.14	93.0
1.2	0.33	83.5
1.6	0.53	73.5
2.0	0.84	53.0
2.4	1.11	45.5
2.7	1.41	29.5
3.0	1.68	16.0
3.5	1.92	4.0
4.0	0	0.0

biotic (indicating loss of biological activity): when incubated at 70° C for 7 h, and at 1:4 molar ratio, total loss of activity occurred. At this stage it was ascertained that further incubation up to 10 h did not abolish the MIC value of the antibiotic at lower molar ratios. Therefore, in all subsequent investigations, the thiostrepton/copper(II) ratio was maintained at 1:4 and the incubation at 70° C for 7 h only.

Since during the interaction of copper(II) with thiostrepton the antibiotic acquired a new shoulder at 360 nm which was absent in the native molecule (Fig. 2), this wavelength was selected in the spectroscopic method for determining the maximum number of metal ions that can bind to the antibiotic molecule. The results are shown in Fig. 4. This study also revealed a maximum of four copper(II) ions bind to one thiostrepton molecule.

The binding of four copper ions to one molecule of thiostrepton is evidently due to the availability of four different regions in the antibiotic molecule where the ligand atoms are present facilitating co-ordination. All the regions may not, however, be equally accessible for the four metal ions to bind simultaneously because of

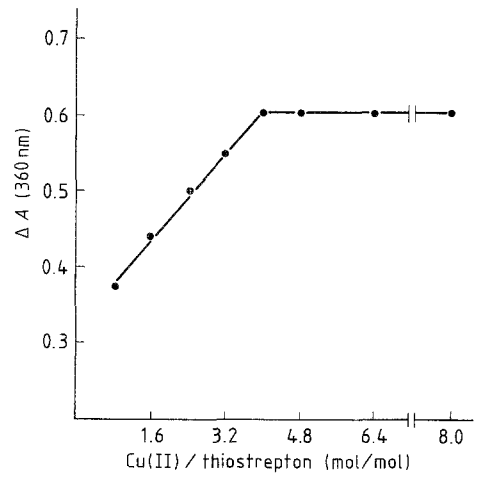

Fig. 4. Molar ratio method of determining the maximum binding capacity of thiostrepton to copper(II)

Table 3. TLC mobility of thiostrepton and its copper(II) complex in various solvent systems

Compound	R_f in solvent						
	1	2	3	4	5	6	7
Thiostrepton	0.79	0.7	0.81	0.56	1.0	0.9	0.7
Copper complex	0.71	0.68	0.64	0.71	0	0.81	0.6

Solvents: 1, acetic acid/methanol (1:7); 2, acetic acid/methanol/water (1:6:1); 3, butanol/acetic acid/water (4:1:1); 4, acetic acid/methanol/water (1:2.5:5.5); 5, acetone/methanol/water (5:2:7); 6, acetic acid/methanol/chloroform (1:3:4); 7, acetic acid/methanol/water (0.15:8:0.85)

unfavourable stereochemical orientations of some of the ligand atoms in these regions. But it is possible that binding of a copper(II) atom at one favourable region would induce a disturbed electronic structure and rearrangement of bond lengths in the entire ligand molecule (Chatterji and Nandi 1978). This would then bring the other ligand groupings to more favourable orientations with each other so that the subsequent metal ions can bind.

TLC of thiostrepton/copper(II) at 1:4 molar ratio in various solvent systems revealed the homogeneity of the sample by giving a single spot. The higher R_f value of the copper-bound thiostrepton as compared to the thiostrepton standard in more polar solvents and vice versa in less polar solvents (Table 3) indicates the hydrophilic nature of the complex.

Titration of thiostrepton with alkali in an acid solution of 50% dioxane revealed that the titration curves of the acid blank and thiostrepton are identical up to pH 9; above pH 9, the titration curve of thiostrepton was found to fall slightly below the acid blank (Fig. 5). This

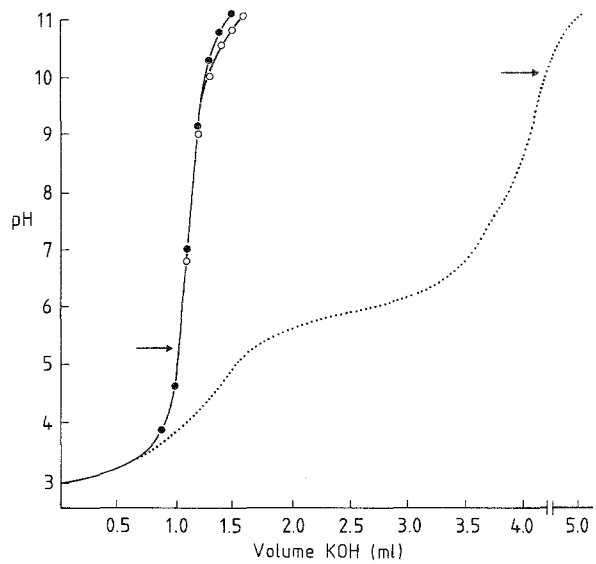

Fig. 5. Titration curves of (a) acid blank (●), (b) thiostrepton (○), (c) thiostrepton in presence of copper(II) at 1:4 molar ratios (····) in 50% dioxane solution. Arrows indicate the neutralization point

indicates that, up to pH 9, thiostrepton does not decompose; above this pH value, the lowering of the titration curve below that of acid blank probably indicates the release of proton from the amino group of the antibiotic. However, in the presence of copper(II), dissociation of thiostrepton with gradual release of protons starts at pH 3.5. The neutralization point on the titration curves of thiostrepton in the presence and absence of the metal (calculated from the plot of volume of base, V, versus $\Delta pH/\Delta V$ on a graph) indicated that, in the presence of copper, dissociation of the thiostrepton molecule releases 11 protons. Since there are 11 peptide nitrogens in the thiostrepton molecule, and since copper can deprotonate them (Sakurai et al. 1980), this suggests that these groups of the antibiotic are involved in binding to the metal and are being deprotonated in the presence of alkali.

In order to understand further the nature of the ligand groups of thiostrepton that are involved in binding to the metal, studies of copper interaction with thiostrepton were carried out in the presence of various compounds that can form complexes with the metal, such as amino acids (Albert 1952) and polymyxin (Srinivasa and Ramachandran 1980) etc. Biological activity and ultraviolet absorption of the antibiotic was then measured. It was ascertained that these compounds by themselves had no effect on the biological activity and the absorption spectra of thiostrepton. These results are presented in Table 4.

The studies revealed that, in the presence of copper-complexing compounds like amino acids, polymyxin etc., copper neither affected the biological activity, nor the absorption spectrum of thiostrepton. However, these compounds were unable to restore the biological activity of thiostrepton when they were added subsequently to the reaction mixture and incubated after copper interaction with the antibiotic was complete. This indicates an irreversible inactivation of thiostrepton by copper(II). In the presence of compounds like glucose and diamino compounds such as spermidine or monocarboxylic group compounds such as propionic acid, copper(II) was able to interact and inactivative the biological activity of thiostrepton. The formation of colour in the reaction mixture also confirmed the metal complexation to thiostrepton in the presence of these compounds.

Table 4. Effect of different compounds on copper(II) interaction with thiostrepton

Compound	Activity (%)
None (control)	100
Thiostrepton + copper(II) (1:4)	0
Thiostrepton + copper(II) (1:4) + L-methionine	100
L-lysine	100
L-ornithine	100
polymyxin	100
EDTA (pH 7.0)	100
spermidine	0
glucose	0
Sodium lactate	0
Sodium propionate	0

These results suggest that, along with peptide nitrogens, some of the amide carbonyls of thiostrepton are also required for the metal ion to bind. Additionally, some of the sulfur atoms of thiazole rings of the thiostrepton molecule may also be involved in binding to copper(II). This is based on the consideration that the bond between sulfur and copper involves not only the sulfur–metal σ bond, but also backbonding involving donation of electrons from a filled copper $d\pi$ orbital to an empty sulfur $d\pi$ orbital. It is this back donation of electrons from copper to sulfur that would ensure its co-ordination to the metal.

Therefore, in thiostrepton, it appears that the potential ligand atoms of the antibiotic, i.e. nitrogen atoms of amide groups, oxygen and sulfur atoms of some of the amide carbonyl and thiazole rings, are principally involved in binding to the metal. The involvement of these groupings in binding to copper(II) was revealed in circular dichroic, infra-red and nuclear magnetic resonance studies (results not presented here).

It is evident from the literature (Albert 1950; Albert and Rees 1956) that, among the bivalent metal cations, copper(II) has the highest avidity to chelate with many organic molecules. The non-oxidizable nature of copper(II) complexes (Calvin and Wilson 1945), the ability of copper(II) to deprotonate peptide nitrogens, and the stabilizing forces of copper chelates are possible reasons for the unique specificity of this metal to complex with thiostrepton when compared to the other metal ions tested.

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