Copper(II) binding by kanamycin A and hydrogen peroxide activation by resulting complexes

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Protonation and copper(II) coordination properties of kanamycin A were studied in solution by potentiometry, UV-Vis, circular dichroism (CD), EPR and cyclic voltammetry (CV). Only mononuclear complexes of stoichiometries ranging from CuH_2L to $CuH_{-2}L$ were found. Kanamycin A anchors Cu(II) ions with an $\{NH_2, O^-\}$ chelate of the C-ring of its molecule. At pH higher than 6 the amino and hydroxyl groups of the A-ring of kanamycin A also participate in binding. The resulting structure, similar to that of complexes of other unsubstituted aminoglycosides studied previously, involves Cu(II) coordination by donors of terminal aminosugar rings, rather than those of the central unit. The results of cyclic voltammetry investigations, kinetic studies of H_2O_2 disproportionation and ROS detection experiments, further supported the mechanism of oxidative reactivity of cupric complexes of aminoglycosides, proposed by us recently [M. Jeżowska-Bojczuk, W. Leśniak, W. Bal, H. Kozłowski, K. Gatner, A. Jezierski, J. Sobczak, S. Mangani and W. Meyer-Klaucke; *Chem. Res. Toxicol.*, 2001, **14**, 1353–1362], which involves Cu(I) and Cu(III) redox states and both metal-bound and free ROS.

Introduction

Kanamycins belong to aminoglycosides, a very important class of antibiotics, active against Gram-negative and some Gram-positive bacteria. The mechanism of their action has been studied very extensively since the discovery of their first representative—streptomycin. The current view of their mode of action includes several toxic effects, such as ribosomal blockage, misreading in translation, cellular membrane damage and irreversible uptake of the antibiotic.[1–7] Interactions between ribonucleic acid molecules and aminoglycosides underlie many of the above effects.[8] The strength of these interactions is determined by electrostatics between negatively charged backbone phosphates and amino groups localized on the aminosugar or aminocyclitol rings of aminoglycoside molecules, which are positively charged at physiological pH.

The results of recent studies introduce aminoglycosides as a novel family of stimulators of reactive oxygen species (ROS) formation.[9,10] Such reactions may be involved in aminoglycoside-induced oto- and nephrotoxicity, which are major severe side-effects, limiting clinical use of these antibiotics.[11] The enhancement of ROS formation following metal ion complexation is, apart from structural studies, the main subject of the present paper.

Kanamycin A, discovered by Umezawa and coworkers,[12] is active against mycobacteria and has gained wide usage as antituberculous agent.[13] It is the main component of kanamycin complex (at 98%) and its toxicity is much lower than that of kanamycin B.[14] Its molecule (Fig. 1) consists of the central 2-deoxystreptamine ring, to which sugar moieties of 3-D-glucosamine (kanosamine) and 6-D-glucosamine are attached by glycosidic bonds. The crystal structure of kanamycin A shows that all three rings in the molecule assume the chair conformation.[15] Its four amino functions, together with the neighboring hydroxyl groups, offer several potential binding sites for metal ions, such as copper(II), which was previously demonstrated to form stable complexes with related aminoglycosides.[16–19]

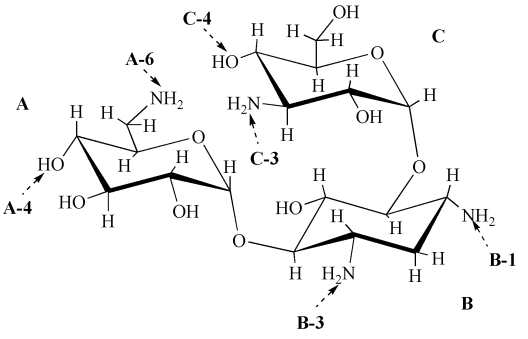

Fig. 1 The molecule of kanamycin A in its deprotonated form. A, B and C represent the component rings of the molecule. Arrows indicate the sites of deprotonation and/or metal chelation by the antibiotic.

Aminosugars bind Cu(II) effectively. The anchoring of the cupric ion to their molecules is accomplished through amine functions. The stability and stoichiometry of the resulting complexes depend, however, on the formation of chelate rings using appropriate hydroxyl groups of the ligand.[20–27] Monodentate complexes missing such groups cannot withstand hydrolysis at neutral pH.[26,27] Kanamycin B[16] and tobramycin,[17] aminoglycosidic antibiotics of the kanamycin group studied by us previously, are much more complicated ligands. They bind copper(II) ions with donor atoms of the two terminal aminosugar rings, while the central deoxystreptamine ring does not participate in the metal ion complexation. In contrast

it does in the case of amikacin,[18] a semisynthetic derivative of kanamycin A, where amidation of one of the deoxystreptamine nitrogens with 4-amino-2-hydroxybutyric acid alters its donor properties.

Several mutually incompatible binding modes of Cu(II) coordination were proposed previously for kanamycin A. Yamabe, on the basis of IR and polarographic studies, suggested the binding through both nitrogens of the deoxystreptamine ring.[28] The alternative binding mode, proposed by Hanessian *et al.* on the basis of ^{13}C NMR,[29] and CD and reactivity studies,[30] involves C ring donors: C3 amine nitrogen and a neighboring hydroxyl oxygen (C4), but neglects deprotonation of this oxygen. Grapsas *et al.*[31] used ^{1}H NMR results to suggest an octahedral complex structure with a donor set similar to that of Hanessian *et al.*, but they also proposed another coordination mode, using C2 hydroxyl instead of C4 to complete the 5-membered chelate ring. Their third proposal, based upon computer modeling, suggests the C-ring oxygen to take part in the chelate ring, although it would require a twisted boat conformation for the C ring. This structure is the least favorable of all three, due to a high potential energy. The coordination mode previously proposed by Nagabhushan *et al.*,[32] which uses the non-vicinal B1-amino and C2-hydroxyl groups for Cu(II) chelation, is very unlikely in the absence of definitive evidence, due to the formation of a thermodynamically unfavorable 8-membered chelate ring. Sreedhara *et al.*[33] essentially reproduced the structure of Hanessian *et al.*[30] and Grapsas *et al.*,[31] but allowed for deprotonation of the coordinated hydroxyl (which we demonstrated earlier for aminosugars, their derivatives and many aminoglycosides at neutral pH[16–27,34]). In none of the kanamycin A papers mentioned above were the complexation phenomena studied systematically.

We have recently studied elements of the mechanism of oxidative reactivity of three antibiotics related to aminoglycosides: kasugamycin,[35] lincomycin[36] and amikacin,[37] using cyclic voltammetry, spectroscopic detection of reactive intermediates and oxygen evolution measurements. The results suggest that the disproportionation of hydrogen peroxide to dioxygen and water is the main reaction pathway, and that both Cu(I)/Cu(II) and Cu(II)/Cu(III) redox pairs participate in the process. The production of hydroxyl radicals was postulated to be a side reaction, which, however, may contribute substantially to oxidative damage of biomolecules exerted by metal complexes of aminoglycosides, studied also by others.[9–11,33,38–40] In this paper we present the results of mechanistic studies on the cupric complexes of kanamycin A, correlating reactivity with complex structure.

Materials and methods

Materials

Kanamycin A, $CuCl_2$, KNO_3, K_2SO_4, Nitro Blue Tetrazolium (NBT; 2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-[3,3′-dimethoxy-4,4′-diphenylene]ditetrazolium chloride), *N*,*N*-dimethyl-*p*-nitrosoaniline (NDMA), 5,5-dimethylpyrroline-*N*-oxide (DMPO), H_2O_2, Chelex-100 resin, sodium and potassium phosphates and all other simple chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Potentiometry

Potentiometric titrations of kanamycin A and its complexes with Cu(II) in the presence of 0.1 M KNO_3 were performed at 25 °C using pH-metric titrations over the pH range 3–11.5 (Molspin automatic titrator, Molspin Ltd, Newcastle upon Tyne, UK) with 0.1 M NaOH as titrant. Changes in pH were monitored with a combined glass–Ag/AgCl electrode (ATI Russell pH Ltd, Fife, Scotland) calibrated daily in hydrogen ion concentrations by HNO_3 titrations.[41] Sample volumes of 2 ml, kanamycin A concentration of 1 mM and Cu(II):kanamycin A molar ratios of 1:1, 1:2 and 1:4 were used. These data were analyzed using the SUPERQUAD program.[42] Standard deviations computed by SUPERQUAD refer to random errors only.

Electronic absorption (UV-Vis)

The electronic absorption spectra were recorded at 25 °C on a Beckman DU-650 (Beckman, Palo Alto, CA) spectrophotometer over the spectral range of 200–900 nm, in 1 cm cuvettes. *N*,*N*-dimethyl-*p*-nitrosoaniline (NDMA),[43] a scavenger used commonly in studies of hydroxyl radicals and similarly reactive species, and Nitro Blue Tetrazolium (NBT),[44] a superoxide anion radical scavenger, were used in separate experiments at 37 °C as reporter molecules in tests of ROS formation by Cu(II) complexes with kanamycin A, at concentrations of 25 μM and 0.1 mM, respectively. These reaction mixtures also contained sodium phosphate buffer (50 mM), kanamycin A and Cu(II) (each 50 μM), and H_2O_2 (0.5 mM). In preliminary studies, the course of reaction was monitored by periodically recording the whole spectra, in order to find out whether any absorbing reaction products would interfere with the detection of radicals. After eliminating this possibility, the reactions were monitored at the characteristic wavelength of NDMA (440 nm). Each experiment was repeated five times. The preliminary NBT studies revealed no reactivity, and thus were not continued.

CD spectroscopy

The spectra were recorded at 25 °C on a Jasco J-715 spectropolarimeter (JASCO, Japan Spectroscopic Co., Hiroshima, Japan), over the range of 200–800 nm, using 1 cm cuvettes. Samples contained 1:1 Cu(II)-to-kanamycin A ratios at complex concentrations varying from 2 to 5 mM. The spectra are expressed in terms of $\Delta\varepsilon = \varepsilon_l - \varepsilon_r$, where ε_l and ε_r are molar absorption coefficients for left and right circularly polarized light, respectively.

EPR

The spectra of the Cu(II) complex with kanamycin A were recorded at 77 K on a Bruker ESP 300E spectrometer (Karlsruhe, Germany) at the X-band frequency (9.3 GHz). Ethane-1,2-diol:water (1:2) was used as solvent in low-temperature experiments to obtain homogeneity of frozen samples. The concentrations of Cu(II) and kanamycin A in the samples were both 5 mM.

In spin-trapping studies of free radical formation with DMPO, EPR spectra were recorded at room temperature on an Radiopan X-band spectrometer, model SE/X 2543 (Poznań, Poland) immediately after mixing of reaction components. The instrumental parameters were: field setting of 334 mT, scan range of 10 mT, microwave power of 4 mW and modulation amplitude of 0.1 mT. The concentration of DMPO was 0.1 M. Other components of reaction mixtures were present at concentrations identical to those used in the spectrophotometric measurements of oxygen radical formation (sodium phosphate buffer, 50 mM; kanamycin A, 0 or 50 μM; Cu(II), 50 μM; H_2O_2, 0.5 mM). Ethanol at 1.5 M was used as a specific scavenger for hydroxyl radicals in the system containing the complex and H_2O_2.

Cyclic voltammetry

CV measurements were done using an Autolab PG-12 potentiostat–galvanostat (Eco Chemie BV, Utrecht, Netherlands) with GPES 4.8 software, at voltage sweep rates between 10 and 1000 mV s^{-1}. The working electrode was a planar Pt electrode (1.6 mm diameter). The auxiliary electrode was platinum

wire and the reference electrode was Ag/AgCl (3 M KCl) fitted with a Vycor tip. The supporting electrolyte was 100 mM K_2SO_4. Measurements were performed at 25 °C, under nitrogen. The concentrations of components were 1 mM and the Cu(II)-to-kanamycin A ratio was kept constant at 1:1, wherever appropriate. The pH of the samples containing kanamycin A was adjusted with small amounts of concentrated KOH or H_2SO_4. The samples of Cu(II) alone were set to pH 6.5 to avoid precipitation of $Cu(OH)_2$. All potentials were given relative to NHE.

Dioxygen generation measurements

The generation of dioxygen in solutions containing combinations of Cu(II), kanamycin A and H_2O_2 was studied over the temperature range 23–37 °C and at pH = 6.1–11.2 (0.2 M phosphate buffer), for equimolar samples of Cu(II) and kanamycin A (concentrations from 0.25 to 2.5 mM) with varied concentrations of H_2O_2 (from 25 mM to 100 mM). The reactions were carried out at constant volume in a thermostated glass-reactor equipped with a magnetic stirring bar and connected to an automatic gas absorption or desorption measuring apparatus controlled by a computer. All reactions were performed with non-degassed solutions, while degassing was shown previously to have no influence on H_2O_2 dismutation catalyzed by manganese– and iron–porphyrin complexes.[45] A water solution of the Cu(II) complex with kanamycin A, adjusted to the appropriate pH, was added to the reactor under air atmosphere. Next, varied amounts of aqueous H_2O_2 were introduced in one portion, immediately after which the stirring was started and the progress of dioxygen evolution was measured. The molar amounts of dioxygen evolved were calculated from the volume, atmospheric pressure and temperature data using the ideal gas equation.

In a separate set of experiments, the effect of the presence of NDMA on dioxygen evolution was investigated. In these experiments NDMA was added at final concentrations between 0 and 25 mM to solutions containing 1 mM Cu(II) and kanamycin A, and 50 mM H_2O_2, at pH = 7.4 (0.2 M phosphate buffer) and temperature 37 °C.

Results and discussion

Protonation constants of kanamycin A

The protonation constants calculated on the basis of potentiometric titrations are presented in Table 1. These four constants correspond to the four amino groups present in the antibiotic molecule. Two of them are located at the central 2-deoxystreptamine ring B, and one each at the two aminosugar rings, A and C (Fig. 1). One pK_a value is very low, 6.19, while the other three are more typical for aminosugars, 7.4–9[20–27] (Table 1). The assignments provided in Table 1 are based on our potentiometric and NMR characterization of protonation equilibria in amikacin[18] and on potentiometric studies of other aminoglycosides, kanamycin B,[16] tobramycin[17] and geneticin,[19] as well as their structural components.[21,25]

The lowest protonation constant was thus assigned to one of the ring B amino groups. Low values of the first deprotonation constant were found for all other aminoglycosides studied to date. These values were 6.0 for geneticin,[19] which carries the +4 charge at low pH, the same as kanamycin A, and *ca.* 5.7 for kanamycin B[16] and tobramycin,[17] which have the +5 charge. On the other hand, amikacin,[18] also a +4 ion, has a much higher value of the lowest pK_a, 6.8. Amikacin is the only derivative with one, rather than two, amino groups at the deoxystreptamine (B ring). Therefore, the probable reason for the lowering of the first pK_a in aminoglycosides is a local electrostatic interaction between the neighboring NH_3^+ groups of ring B, rather than the overall charge of the whole molecule.

The next two deprotonations are separated by only *ca.* 0.7 log units. The statistics of deprotonation of bifunctional molecules indicate that these two processes are actually parallel (the "ideal" statistical log value is 0.6).[46] Therefore, these macroconstant values, which correspond to the amino groups of ring C and B, cannot be assigned individually. This assignment is based on the analogy with amikacin and geneticin, in which the highest pK_a value was assigned to the amine group of ring A.

Table 1 Stability constants of kanamycin A and its copper(II) complexes, 25 °C, I = 0.1 M (KNO_3)

Species	log β	pK	Location of protonating group
LH_4	30.800(1)	6.19	ring B
LH_3	24.607(6)	7.42	ring B or C
LH_2	17.188(5)	8.16	ring B or C
LH	9.030(4)	9.03	ring A
CuH_2L	22.41(1)	—	
CuHL	15.51(1)	6.9	
CuL	8.83(1)	6.68	
$CuH_{-1}L$	0.55(2)	8.28	
$CuH_{-2}L$	−8.58(2)	9.13	

Copper(II) complexes of kanamycin A

Combined results of spectroscopic (UV-Vis, CD, EPR) and potentiometric studies indicated that in this system only monomeric equimolar complexes are formed at the millimolar concentration range. This is typical for aminoglycosides.[16–19] Potentiometry detected five CuH_nL complexes (n = +2 to −2) in the pH range between 4 and 11. Table 2 presents their spectroscopic data and Fig. 2a shows the species distribution calculated for the concentration used in spectroscopic experiments, along with parameters of absorption spectra. As can be seen clearly, the electronic absorption and CD spectra provide evidence for just three coordination species (I, II, and III, Fig. 2b), corresponding to stoichiometric forms as follows: I - CuH_2L; II - $CuHL + CuL + CuH_{-1}L$; III - $CuH_{-2}L$.

The first complex species detected by potentiometry and spectroscopy at low pH is CuH_2L. A CuH_3L complex, with {NH_2, OH} coordination, detected by EPR for geneticin,[19] is not present here, apparently due to the higher basicity of kanamycin A. Among the aminoglycosides studied by us, geneticin is the closest analogue of kanamycin A. The only differences between these two molecules are the position of the amino group in ring A (at A2 for geneticin, and at A6 for kanamycin A) and the methylation of the amino group of ring

Table 2 Spectroscopic characterization of kanamycin A and its copper (II) complexes

	UV-Vis[a]		CD[a]		EPR[b]	
Species	λ	ε	λ	$\Delta\varepsilon$	$A_\parallel$	$g_\parallel$
CuH_2L	646	36	705[c]	+0.03	163	2.29
	252	1187	286[d,e]	−0.50		
$CuHL + CuL + CuH_{-1}L$	600	71	585[c]	−0.14		
	255	2612	343[e]	−0.02	190	2.23
			269[d]	+2.03		
$CuH_{-2}L$	618	68	588[c]	−0.11	188	2.24
	270	2228	267[d]	+1.93		

[a] λ units are nm, ε and $\Delta\varepsilon$ units are M^{-1} cm^{-1}. [b] $A_\parallel$ units are Gauss. [c] d–d electronic transitions of Cu(II) in a tetragonal complex. [d] $NH_2 \rightarrow$ Cu(II) charge transfer band. [e] $O^- \rightarrow$ Cu(II) charge transfer band.

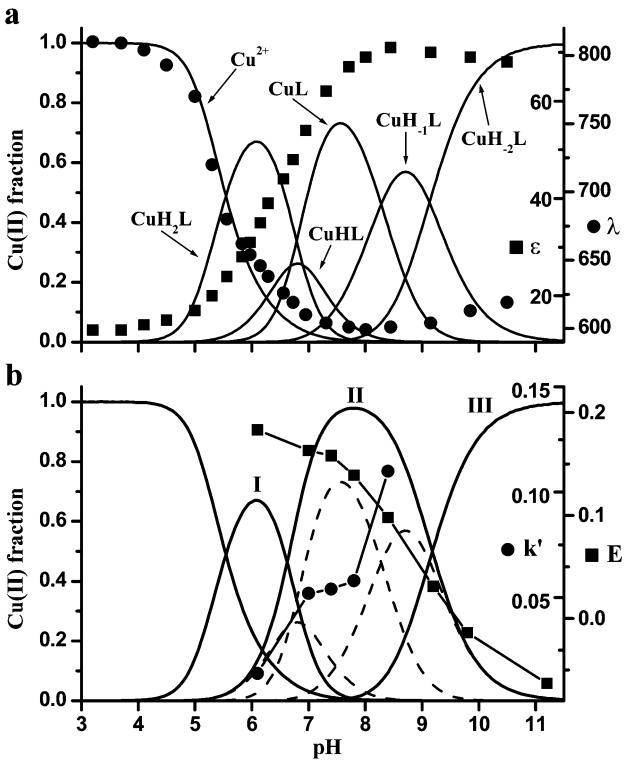

Fig. 2 The species distribution diagram for Cu(II) complexes of kanamycin A with d–d band parameters overlaid (a), λ units are nm, ε units are M^{-1} cm^{-1}. Reduction potential values and pseudo-first order rate values for dioxygen generation overlaid (b). I, II and III represent the consecutive spectroscopic complex forms.

C in geneticin. By analogy with other unmodified aminoglycosides, the chelate in the CuH_2L complex is thought to involve the deprotonated C3 amine and C4 hydroxyl donors of the kanosamine moiety (ring C). All other amino groups are protonated in this species. The stability of this initial complex can be compared among the aminoglycosides using values of $^*K$, the equilibrium constant of the proton displacement reaction:

$$\mathrm{Cu(II)} + \mathrm{H}_n\mathrm{L} = \mathrm{CuH}_{(n-2)}\mathrm{L} + 2\ \mathrm{H}^+$$

The log $^*K$ for the ring C complex of kanamycin A, −8.39, is very close to that of tobramycin (−8.32) and slightly higher than that of kanamycin B (−8.79), which has the identical ring C, but a different overall charge. This is probably due to an electrostatic interaction between the neighboring substituents of ring A of the antibiotic molecule: the A2 amine and the A3 hydroxyl groups in the case of kanamycin B. Tobramycin does not possess the A3 hydroxyl group and kanamycin A does not have the A2 amine function. The lack of the above mentioned interaction is responsible for similar log $^*K$ values (kanamycin A and tobramycin). Geneticin, although at the same charge, has the lowest value of log $^*K$ (−9.00) which results from the dissimilar donor arrangements in the A and C rings. The differences in these values however are not very high, supporting the essentially identical coordination fashion.

The identity of the CuH_2L complex can be deciphered from its spectroscopic parameters (Table 2). The CD spectrum of this complex is very similar to the analogous one of kanamycin B,[16] showing a weak d–d band at 705 nm and a mixed NH_2 and $O^- \rightarrow$ Cu(II) CT band at 286 nm. $A_\parallel$ and $g_\parallel$ values of 163 G and 2.29, respectively, obtained from EPR spectra for solution at pH ~ 6 are identical to those for a corresponding complex of tobramycin[17] (EPR parameters for such a species of kanamycin B could not be established due to complex aggregation and precipitation). The higher $A_\parallel$ value for the analogous Cu(II) complex of geneticin was due to the methylation of an anchoring amine donor.[19] The EPR parameters of the CuH_2L complex fit in between the characteristic values for complexes with 1N and 2N coordination, similarly to the systems reported previously.[17,25] Therefore, the coordination in CuH_2L is of the $\{NH_2, O^-\}$ type, with O^- provided by the sugar hydroxyl group, vicinal to the C-ring amine group, similar to other unsubstituted aminoglycosidic antibiotics.[16,17,19]

The above complex precedes the three following species belonging to the same coordination mode (type II): CuHL, CuL and $CuH_{-1}L$. The pK value for the formation of the CuL species is lower than that for CuHL by 0.2 log units. This inversion of order signifies the cooperativity of the two processes,[47] so that CuHL is only a minor species in solution. The average pK of formation of type II complex is thus 6.79, identical to that found previously for the corresponding process in the tobramycin complex[17] and only slightly higher than the one for kanamycin B[16] (see Table 3). This confirms similar Cu(II) chelation in CuL complexes of kanamycin A and the above mentioned antibiotics. The formation of CuL is accompanied by a significant increase of the d–d band energy (UV-Vis and CD), by a split of the CT band into two components, $O^- \rightarrow$ Cu(II) at 343 nm and $NH_2 \rightarrow$ Cu(II) at 269 nm, and by a drastic change of EPR parameters (Table 2). The latter ($A_\parallel$ of 190 G and $g_\parallel$ of 2.23, Table 2) are similar to those seen previously for $CuH_{-1}L_2$ complexes of aminosugars: 183 G, 2.24;[22] 182 G, 2.24,[24] and for the CuHL complex of amikacin: 187 G, 2.24.[18] All these changes indicate the introduction of the second nitrogen atom in the copper(II) coordination sphere and the $\{2 \times NH_2, O^-\}$ bonding. Due to sterical reasons this second nitrogen can only be provided by the A6 amine group of 6-D-glucosamine (ring A), all the more that the presence of the neighboring A4 hydroxyl group provides an opportunity for the formation of a 6-membered chelate ring. Unchanged spectroscopic parameters prove that the formation of the next complex form, $CuH_{-1}L$, brings no changes to the coordination mode, it being only the result of deprotonation of the remaining non-coordinating amine function of the central 2-deoxystreptamine ring.[16,17,19]

Above pH 9, the last potentiometric and spectroscopic form, $CuH_{-2}L$, predominates in solution. Its $A_\parallel$ and $g_\parallel$ values (188 G, 2.24) do not differ significantly from the ones of the previous complexes. The presence of an altered coordination mode is, however, confirmed by changes in the parameters of electronic absorption and circular dichroism spectra (Table 2). The pK value for this process (9.13) indicates the deprotonation of the bonded A4 hydroxyl group.

Table 3 Comparison of formation constants (pK values) of Cu(II) complexes of kanamycin A, geneticin,[19] kanamycin B[16] and tobramycin[17]

Reaction	Kanamycin A pK	Geneticin pK	ΔpK	Kanamycin B pK	Tobramycin pK
$CuH_3L \rightarrow CuH_2L$				5.79	6.35
$CuH_2L \rightarrow CuHL$	6.9	6.30	+0.60	6.61	6.79
$CuHL \rightarrow CuL$	6.68	6.87	−0.19	7.66	7.44
$CuL \rightarrow CuH_{-1}L$	8.28	7.65	+0.63	8.11	8.59
$CuH_{-1}L \rightarrow CuH_{-2}L$	9.13	8.59	+0.54	9.26	9.94

The comparison of formation constant values of Cu(II) complexes with kanamycin A and the previously studied geneticin[19] indicates that all significant kanamycin A complexes are weaker than the corresponding species of geneticin (values of ΔpK, Table 3). This is difficult to explain but may result from differences in the structures of both antibiotics, which lead to different conformational properties of their copper complexes.

Cyclic voltammetry of kanamycin A and its Cu(II) complexes

The cyclic voltammetry measurements were performed separately for cathodic and anodic ranges of potentials as a function of the scan rate and pH. The electrochemical data were collected at different pH values between pH 6.1 and 11.2, covering the range of variability of complex species. Kanamycin A alone was redox-inactive, in accordance with other aminosugar-containing antibiotics studied previously,[35–37] while its copper(II) complex exhibited irreversible reduction and oxidation peaks in a wide pH range. The reduction peak potential exhibited a significant pH dependence, while reduction currents increased linearly with the square root of the scan rate. The voltages of the major reduction peaks are overlaid on the speciation diagram in Fig. 2b. Clearly, there is little difference in reduction properties between forms I and II, while Cu(II) in complex form III is reduced with much more difficulty (*i.e.* at much lower negative potential). The reduction proceeds to Cu(0), and is accompanied by complex decomposition, as indicated by the irreversibility of the process, and the appearance of a broad reoxidation feature at *ca.* +0.3 V. Fig. 3 shows the peaks of the complex reduction and oxidation at pH 8.0, the latter attributable to the oxidation of the Cu(II) complex to a Cu(III) species. At this pH the complex form II predominates in solution. The well defined oxidation peak was visible only for low scan rates (25 mV s^{-1}). For higher values, it contributed to the total current increase at this edge of the potential range. This process, irreversible as well, and thus likely to involve ligand decomposition, could be seen at pH 7 and above, at the approximately constant potential of +1.48 V.

The CV studies demonstrated the accessibility of reduction and oxidation processes specifically to complex form II.

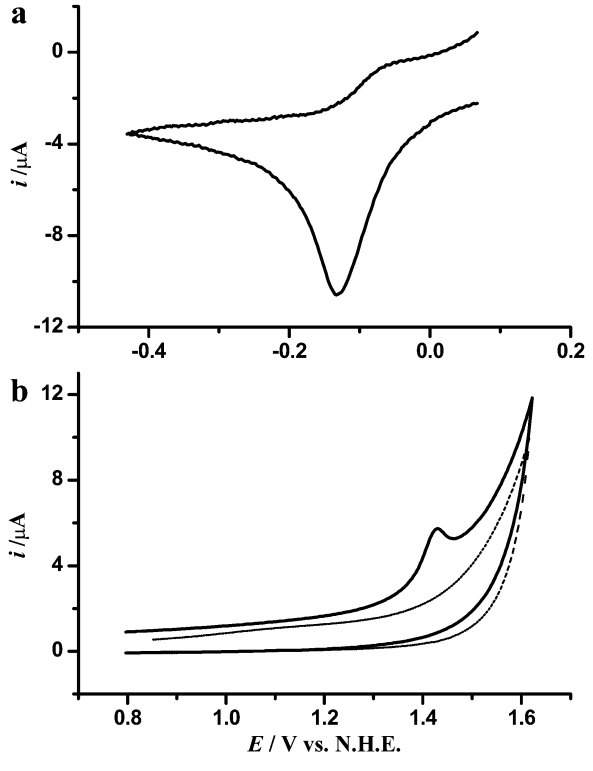

Fig. 3 Cathodic (a) and anodic (b) scans of Cu(II)–kanamycin A complexes at pH 8.0: (a) scan rate 250 mV s^{-1}, (b) scan rate 25 mV s^{-1}, background scan indicated.

Kinetics of formation of ROS generated by Cu(II)–kanamycin A complexes

As reported previously, Cu(II) complexes of aminosugar antibiotics disproportionate hydrogen peroxide to dioxygen and water through radical intermediates.[35–37] To identify possible radicals generated from H_2O_2 by Cu(II) complexes of kanamycin A we performed the reactions with two relatively specific reporter molecules: NBT, which reacts specifically with superoxide radicals, and NDMA, which is commonly regarded as specific towards hydroxyl radicals, but is also bleached by other strong oxidants and singlet oxygen.[48,49] The former reaction failed to demonstrate superoxide formation, but the time-dependent decomposition of NDMA demonstrated the formation of strongly oxidizing ROS following H_2O_2 addition. The kinetics of this reaction at various pH values was therefore studied, as shown in Fig. 4. It presents typical curves of NDMA decomposition by various combinations of components, demonstrating a specific role of the complex. The analysis of the curve shape demonstrated that initially the indicator molecule consumption followed the pseudo-first order kinetics, switching to a higher order and slowing down upon H_2O_2 consumption. The pH dependence of the 1st order rate constants for Cu(II)–kanamycin A complexes is presented in Fig. 5, along with that of the control of Cu(II) in the phosphate buffer. Our calculations of species distribution in the latter system, using literature data on binding constants, indicate that below pH 8 the control samples contain the $CuHPO_4$ complex,[50,51] and above that pH the soluble or slowly precipitating hydroxides predominate, represented by $Cu(OH)^+$ and $Cu_2(OH)_2^{2+}$.[52] This diagram indicates that complex form II (which exists to the maximum extent around physiological pH, see Fig. 2b) is the most efficient ROS generator, with the activity dropping to one-quarter of its peak value for the solution at pH around 10.5, where the complex form III predominates. The pH profile of the control samples is quite different, with a maximum at pH 9—where soluble Cu(II) hydroxides predominate over insoluble ones.

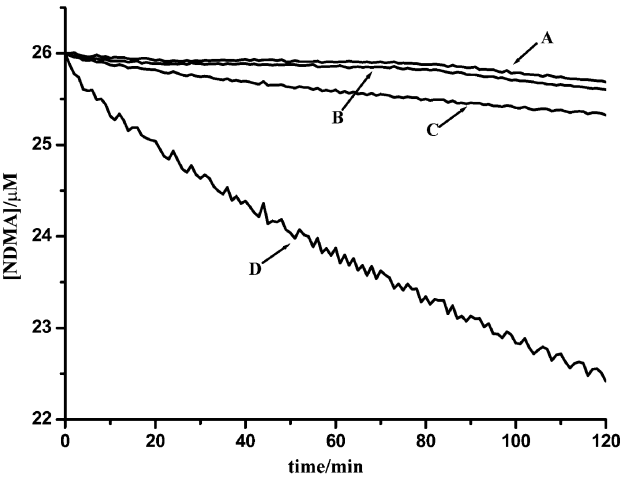

Fig. 4 The experimental curves of kinetic measurements of NDMA bleaching by the Cu(II)–kanamycin A–H_2O_2 system at 37 °C. The reaction mixtures contained sodium phosphate buffer, 50 mM, + NDMA, 25 μM, and: (A) H_2O_2, 0.5 mM; (B) kanamycin A, 50 μM, + H_2O_2, 0.5 mM; (C) Cu(II), 50 μM, + H_2O_2, 0.5 mM; (D) Cu(II), 50 μM, + kanamycin A, 50 μM, + H_2O_2, 0.5 mM.

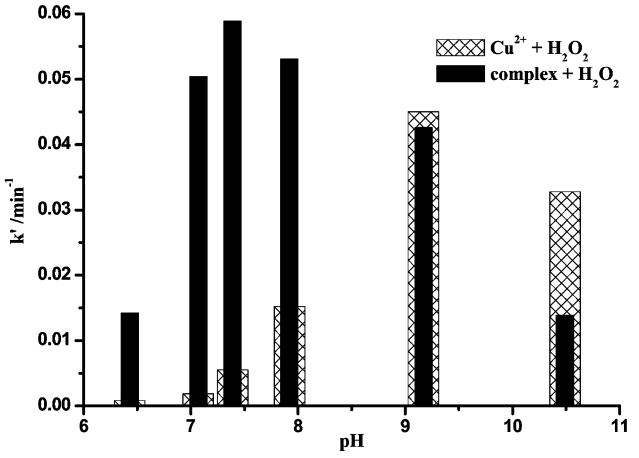

Fig. 5 The comparison of pH influence on the rates of hydroxyl radicals formation by Cu(II)–kanamycin A complex and Cu(II) ions, both in the presence of H_2O_2 at 37 °C. The reaction mixtures contained sodium phosphate buffer, 50 mM; kanamycin A and Cu(II) ions, 50 μM; H_2O_2, 0.5 mM; and NDMA, 25 μM.

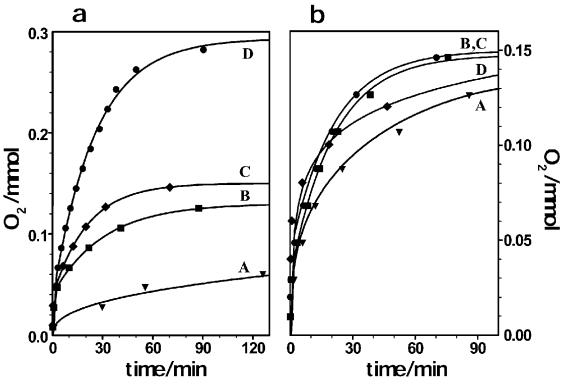

Fig. 6 Concentration dependences of dioxygen formation from H_2O_2, catalyzed by Cu(II)–kanamycin A at 37 °C, pH 7.4. (a) Dependences on H_2O_2 concentration: 25 mM (A), 37.5 mM (B), 50 mM (C), 100 mM (D); Cu(II)–kanamycin A constant at 0.5 mM. (b) Dependences on Cu(II)–kanamycin A concentration: 0.25 mM (A), 0.5 mM (B), 1 mM (C), 2.5 mM (D); H_2O_2 constant at 50 mM.

The value for the rate constant of NDMA bleaching at pH 7.4 and 37 °C was found to be $5.9 \pm 0.1 \times 10^{-2}$ min^{-1}, somewhat higher than that found previously[37] for amikacin in similar conditions (1.4×10^{-2} min^{-1}).

To carry out the unambiguous identification of the radical species, an analogous reaction was done in the presence of DMPO instead of NDMA. These experiments yielded spectra characteristic for the DMPO-•OH adduct. The control sample without kanamycin A exhibited a low intensity DMPO-•OH spectrum, while the complex with H_2O_2 provided a three-fold enhancement of the EPR signal for the adduct. Addition of excess ethanol (1.5 M) to the latter system resulted in complete quenching of DMPO-•OH radical formation. Ethanol is a specific scavenger for hydroxyl radicals,[53] and the complete quenching allows us to exclude the presence of even minute amounts of DMPO-•OOH, a product of superoxide reaction with DMPO, which could serve as a precursor of the DMPO-•OH adduct. The above experiments provide good evidence for the formation of moderate amounts of •OH radicals from H_2O_2 by the copper(II)–kanamycin A system.

Dioxygen generation and mechanistic considerations

The measurements were conducted using a gasometric apparatus, and the volumes of oxygen evolved were measured. Fig. 6 presents the course of oxygen evolution reactions for various concentrations of components, grouped according to the variation in concentrations of Cu(II)–kanamycin A (part a) and H_2O_2 (part b). For longer times, the reactions slowed down, apparently due to a decrease of H_2O_2 concentration and complex decomposition (evidenced by a decrease of pH value, despite the presence of phosphate buffer). Fig. 7 presents the dependence of initial rates (V_0) of the O_2 evolution reaction on H_2O_2 (part a) and Cu(II)–kanamycin A (part b) concentrations, while the correlation of kinetic parameters with speciation is presented in Fig. 2b. Table 4 presents the selectivity of dismutation reaction of H_2O_2 to O_2 and turnover numbers (TON) for these reactions, proving the catalytic role of the Cu(II)–kanamycin A complex. The activities of individual components were negligible below pH 9. The pace of both complex-assisted and spontaneous H_2O_2 disproportionation increased markedly at high pH, due to its lower redox potential under such conditions.[54] The dioxygen evolution was nearly explosive above pH 9, and the reaction rates could be estimated to increase by more than one order of magnitude (data not shown). Partial precipitation of $Cu(OH)_2$ was also apparent. This, as well as the pH drop, is attributable to oxidative decomposition of kanamycin A, which leads to a loss of Cu(II) binding, analogously to the effects seen previously in MS studies of complexes of lincomycin and amikacin.[36,55]

The reaction orders relative to Cu(II)–kanamycin A and H_2O_2 concentrations were calculated as 1.1 ± 0.1 for both. These results indicate that the initial rate of disproportionation of H_2O_2 (V_0) is linearly proportional to the concentration of the catalyst, which is the Cu(II)–kanamycin A complex, and also to H_2O_2 concentration. Thus, the 1:1 interaction between the complex and H_2O_2 is the rate-determining step of the catalysis. From the temperature dependences the activation energy $E_a = 120 \pm 4$ kJ mol^{-1} was determined using an Arrhenius plot. A comparison of the pseudo-1st order constants for NMDA bleaching and O_2 production (Table 5) indicated that these values were nearly identical for complex form II. This suggests that the formation of a NDMA-reactive species is an essential element of H_2O_2 disproportionation, rather than a mere side reaction. The known lack of specificity of reactions of •OH excludes it, however, as an element of a catalytic cycle.[53,56] The spin-trapping experiment demonstrated the •OH radical, but at a rather low level, only three times that detected for Cu(II) in phosphate buffer. The reaction-mediating ROS must thus be complex-bonded, likely at the metal ion. Such species were proposed previously, *e.g.* for Cu(I).[57] On the basis of less direct evidence, we have previously proposed

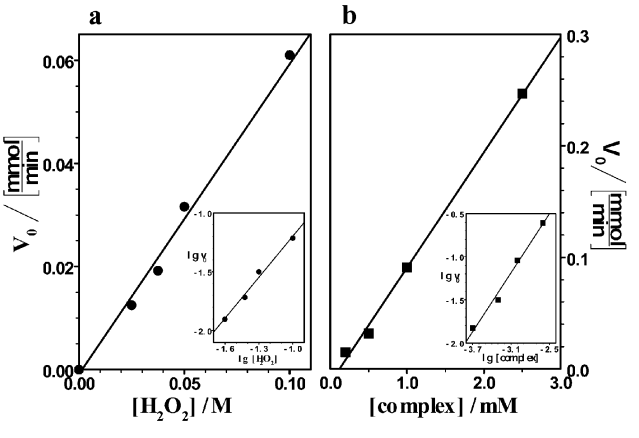

Fig. 7 Effect of H_2O_2 (a) and Cu(II)–kanamycin A (b) concentration on the initial rate of dioxygen formation at 37 °C, pH 7.4. Inserts present logarithmic plots, which indicate reaction orders with respect to individual components.

Table 4 Influence of concentrations of H_2O_2 and Cu(II)-kanamycin A (CuKan) complex on H_2O_2 dismutation

$[H_2O_2]$/mM	[Complex]/mM	S^a (%)	$V_0{}^b$/mmol min^{-1}	TON[c]
25	0.5	97.1	0.012	24.0
37.5	0.5	89.3	0.019	51.2
50	0.5	85.7	0.032	60.0
100	0.5	78.4	0.061	116.8
50	0.2	72.8	0.015	130.0
50	1	88.6	0.091	29.4
50	2.5	84.2	0.247	11.0

[a] *S* selectivity of dismutation reaction to O_2 is defined as the molar ratio (in %) of H_2O_2 decomposed to O_2 with respect to the total reacted amount of H_2O_2, knowing that two molecules of H_2O_2 are required in the dismutation reaction to generate one molecule of O_2. [b] V_0 initial rate of O_2 evolution reaction. [c] TON turnover number: ratio of the number of moles of H_2O_2 decomposed to O_2 over the number of moles of Cu(II)-kanamycin A.

a suitable set of reactions for the interaction between amikacin (L = Ami) and H_2O_2.[37]

$$\text{Cu(II)–L} + H_2O_2 = \text{Cu(I)–L–}^{\bullet}\text{OOH} + H^+$$

$$\text{Cu(II)–L} + H_2O_2 = \text{Cu(III)–L–}^{\bullet}\text{OH} + OH^-$$

$$\text{Cu(I)–L–}^{\bullet}\text{OOH} + \text{Cu(III)–L–}^{\bullet}\text{OH} = 2\ \text{Cu(II)–L} + O_2 + H_2O$$

$$2\ H_2O_2 = O_2 + 2\ H_2O \text{(sum reaction)}$$

These reactions correspond well with the reactivity and redox properties of complex form II of kanamycin A. The proposal of formation of an unprecedented Cu(I)-superoxide species from Cu(II) is perhaps a weak point in this scheme. Such a reaction, however, yields Cu(I) which is a necessary element in all reaction mechanisms proposed so far. We are not able to explain the catalytic cycle in a different way. The alternative mechanisms,[58–61] developed for iron, start from a low-valence species (Fe(II), corresponding to Cu(I)), include two-electron processes, and are poorly reversible. Reactive dimers could also be assumed, by analogy to dicopper centers in enzymes, in which coupled one-electron processes provide a two-electron reaction required to disproportionate H_2O_2. However, the formation of dimers with aminoglycosides is highly improbable at low concentrations for electrostatic reasons, as determined for amikacin.[37] Interestingly, the leaking of a radical species, as indicated by a deviation of *S* from 100% (Table 4), and confirmed by DMPO spin trapping studies, is proportional to TON with an excellent *R* factor of 0.99 (calculated for the first four experiments in Table 4, which were done at the same complex concentration). It therefore seems to be an intrinsic feature of the reaction system, rather than a result of catalyst damage, which seems to be more pronounced at higher concentrations of reagents (experiment 7 of Table 4).

To further test our hypothesis we studied the dependence of dioxygen evolution on NDMA at pH 7.4. The results presented in Fig. 8 demonstrated the concentration-dependent inhibition of O_2 formation by NDMA, and thus provided further evidence that the generation of NDMA-reactive ROS and dioxygen evolution are coupled processes, and so the above mechanism may be valid for kanamycin A. The shape of the resultant curve could not be reproduced by any simple mathematical function. This is not very surprising, because the mechanism of interaction between NDMA and copper-bound ROS may be quite complex. A steep descent of reaction rate for 0.5 mM NDMA, followed by a much weaker effect at higher concentrations, may suggest that there are alternative mechanisms of radical recombination, and only one of them is affected by NDMA. This idea remains, however, to be explored.

Table 5 Comparison of the pseudo-1st order rates of kinetics for ROS formation and O_2 evolution

pH	k'/min^{-1} for NDMA bleaching	k'/min^{-1} for O_2 evolution
6.45	1.4×10^{-2}	1.4×10^{-2}
7.05	5.0×10^{-2}	5.2×10^{-2}
7.4	5.9×10^{-2}	5.4×10^{-2}
7.8	5.3×10^{-2}	5.8×10^{-2}
8.4	—	1.1×10^{-1}
9.15	4.3×10^{-2}	—
10.5	1.4×10^{-2}	—

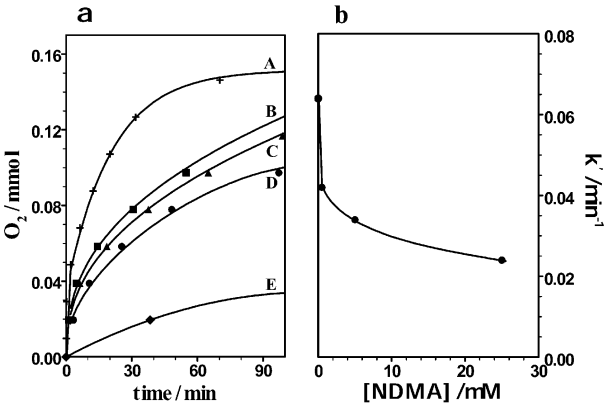

Fig. 8 Effect of NDMA concentration on dioxygen formation from H_2O_2, catalysed by Cu(II)–kanamycin A at 37 °C, pH 7.4 (a) and pseudo-first order constant dependences for these reactions (b). The reaction mixtures contained sodium phosphate buffer, 200 mM; kanamycin A, 1 mM; Cu(II) ions, 1 mM; H_2O_2, 50 mM and NDMA: 0 mM (A), 0.5 mM (B), 5 mM (C), 25 mM (D) and in the absence of Cu(II)–kanamycin A, NDMA, 0.5 mM (E).

Conclusions

The results presented above indicate that kanamycin A effectively binds Cu(II) ions at physiological pH. The binding modes are analogous to those seen previously for a range of aminoglycosidic antibiotics. The Cu(II) complex of kanamycin A is a catalyst of H_2O_2 disproportionation, which proceeds *via* copper-bound ROS. The mechanism of this process appears to be rather complicated, and has deleterious side-effects by leaking a hydroxyl radical species. The reactivity of kanamycin A is quite similar to that of amikacin, studied by us previously, and may be generally common to Cu(II) complexes of aminoglycosides.

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References

1 B. D. Davis, P. C. Tai and B. J. Wallace, *Complex Interactions of antibiotics with the ribosome*, in *Ribosome*, eds. M. Nomura, A. Tissieres and P. Lengyel, Cold Spring Harbor Laboratory, Cold Springs Harbor, New York, 1974, p. 771.

2 E. F. Gale, E. Cundliffe, P. E. Reynolds, M. H. Richmond and M. J. Waring, *The molecular basis of antibiotic action*, 2nd edn., Wiley Interscience, London, 1981, p. 278.
3 N. Tanaka, K. Matsunaga, H. Yamaki and T. Nishimura, *Biochem. Biophys. Res. Commun.*, 1984, **122**, 460.
4 B. D. Davis, *Microbiol. Rev.*, 1987, **51**, 341.
5 G. Laurent, M. B. Carlier, B. Rollman, F. Van Hoof and P. Tulkens, *Biochem. Pharmacol.*, 1982, **31**, 3861.
6 D. Carrier, N. Chartrand and W. Matar, *Biochem. Pharmacol.*, 1997, **53**, 401.
7 S. R. Kirk and Y. Tor, *Bioorg. Med. Chem.*, 1999, **7**, 1979.
8 F. Walter, Q. Vicens and E. Westhof, *Curr. Opin. Chem. Biol.*, 1999, **3**, 694.
9 S.-H. Sha and J. Schacht, *Hear. Res.*, 1999, **128**, 112.
10 S.-H. Sha and J. Schacht, *Free Rad. Biol. Med.*, 1999, **26**, 341.
11 A. Forge and J. Schacht, *Audiol. Neuro-Otol.*, 2000, **5**, 3.
12 H. Umezawa, M. Ueda, K. Maeda, K. Yagishita, S. Kondo, Y. Okami, R. Utahara, Y. Osato, K. Nitta and Y. Takeuchi, *J. Antibiotics*, 1957, **10A**, 181.
13 M. Nakajima, A. Hasegawa, N. Kurihara, H. Shibata, T. Ueno and D. Nishimura, *Tetrahedron Lett.*, 1968, **5**, 623.
14 *Aminoglycoside antibiotics: A Guide to Therapy*, ed. W. G. Barnes, CRC Press, Inc., Boca Raton, Florida, 1984.
15 G. Koyama, Y. Iitaka, K. Maeda and H. Umezawa, *Tetrahedron Lett.*, 1968, **15**, 1875.
16 M. Jeżowska-Bojczuk, W. Bal and H. Kozłowski, *Inorg. Chim. Acta*, 1998, **275–276**, 541.
17 M. Jeżowska-Bojczuk, A. Karaczyn and H. Kozłowski, *Carbohydr. Res.*, 1998, **313**, 265.
18 M. Jeżowska-Bojczuk and W. Bal, *J. Chem. Soc., Dalton Trans.*, 1998, 153.
19 M. Jeżowska-Bojczuk, A. Karaczyn and W. Bal, *J. Inorg. Biochem.*, 1998, **71**, 129.
20 B. Gyurcsik and L. Nagy, *Coord. Chem. Rev.*, 2000, **203**, 81.
21 H. Kozłowski, P. Decock, I. Olivier, G. Micera, A. Pusino and L. D. Pettit, *Carbohydr. Res.*, 1990, **197**, 109.
22 A. Pusino, D. Droma, P. Decock, B. Dubois and H. Kozłowski, *Inorg. Chim. Acta*, 1987, **138**, 5.
23 M. Jeżowska-Bojczuk, H. Kozłowski, L. D. Pettit, G. Micera and P. Decock, *J. Inorg. Biochem.*, 1995, **57**, 1.
24 M. Jeżowska-Bojczuk, E. Chruścińska, T. Trnka and G. Micera, *J. Inorg. Biochem.*, 1996, **63**, 231.
25 G. Micera and H. Kozłowski, *Metal ion–aminosugar complexes*, in *Handbook of Metal–Ligand Interactions in Biological Fluids*, ed. G. Berthon, Marcel Dekker, New York, 1995, vol. 1, p. 707.
26 M. Jeżowska-Bojczuk, S. Lamotte and T. Trnka, *J. Inorg. Biochem.*, 1996, **61**, 213.
27 M. Jeżowska-Bojczuk, H. Kozłowski, T. Trnka and M. Cerny, *Carbohydr. Res.*, 1994, **253**, 19.
28 S. Yamabe, *Jpn. J. Pharmacol.*, 1967, **17**, 120.
29 S. Hanessian and G. Patil, *Tetrahedron Lett.*, 1978, **12**, 1031.
30 S. Hanessian and G. Patil, *Tetrahedron Lett.*, 1978, **12**, 1035.
31 I. Grapsas, I. Massova and S. Mobashery, *Tetrahedron*, 1998, **54**, 7705.
32 T. L. Nagabhushan, A. B. Cooper, W. N. Turner, H. Tsai, S. McCombie, A. K. Mallams, D. Rane, J. J. Wright, P. Reichert, D. L. Boxler and J. Weinstein, *J. Am. Chem. Soc.*, 1978, **100**, 5253.
33 A. Sreedhara, A. Patwardhan and J. A. Cowan, *Chem. Commun.*, 1999, 1147.
34 M. Jeżowska-Bojczuk, W. Bal and K. S. Kasprzak, *J. Inorg. Biochem.*, 1996, **64**, 231.
35 M. Jeżowska-Bojczuk and W. Leśniak, *J. Inorg. Biochem.*, 2001, **85**, 99.
36 M. Jeżowska-Bojczuk, W. Leśniak, W. Szczepanik, K. Gatner, A. Jezierski, M. Smoluch and W. Bal, *J. Inorg. Biochem.*, 2001, **84**, 189.
37 M. Jeżowska-Bojczuk, W. Leśniak, W. Bal, H. Kozłowski, K. Gatner, A. Jezierski, J. Sobczak, S. Mangani and W. Meyer-Klaucke, *Chem. Res. Toxicol.*, 2001, **14**, 1353.
38 A. Sreedhara and J. A. Cowan, *Chem. Commun.*, 1998, 1737.
39 A. Sreedhara, J. D. Freed and J. A. Cowan, *J. Am. Chem. Soc.*, 2000, **122**, 814.
40 A. Sreedhara and J. A. Cowan, *J. Biol. Inorg. Chem.*, 2001, **6**, 337.
41 H. Irving, M. G. Miles and L. D. Pettit, *Anal. Chim. Acta*, 1967, **38**, 475.
42 P. Gans, A. Sabatini and A. Vacca, *J. Chem. Soc., Dalton Trans.*, 1985, 1195.
43 Z. Hartman and P. E. Hartman, *Chem.–Biol. Interact.*, 1992, **84**, 153.
44 *CRC Handbook of Methods for Oxygen Radical Research*, ed. E. A. Greenwald, CRC Press, Inc., Boca Raton, Florida, 1985.
45 R. Belal, M. Momenteau and B. Meunier, *J. Chem. Soc., Chem. Commun.*, 1989, 412.
46 B. Noszal, *Acid-base properties of bioligands*, in *Biocoordination C: Coordination Equilibria in Biological Active Systems*, ed. K. Burger, Ellis Horwood, Chichester, 1990.
47 H. Sigel and R. B. Martin, *Chem. Rev.*, 1982, **82**, 385.
48 W. Bors, C. Michel and M. Saran, *Eur. J. Biochem.*, 1979, **95**, 621.
49 K. Muller-Breitkreutz, H. Mohr, K. Brivitz and H. Sies, *J. Photochem. Photobiol. B*, 1995, **30**, 63.
50 H. Sigel, K. Becker and D. McCormick, *Biochim. Biophys. Acta*, 1967, **148**, 655.
51 S. Ramamoorthy and P. Manning, *J. Inorg. Nucl. Chem.*, 1973, **35**, 1279.
52 C. Childs, *Inorg. Chem.*, 1970, **9**, 2465.
53 B. Halliwell and J. M. C. Gutteridge, *Free Radicals in Biology and Medicine.*, 3rd edn., Oxford University Press, Oxford, 1999.
54 A. J. Masson, *Sch. Sci. Rev.*, 1987, **69**, 84.
55 M. Jeżowska-Bojczuk, W. Szczepanik, W. Bal, W. Leśniak, J. Ciesiołka and J. Wrzesiński, *Eur. J. Biochem*, submitted.
56 A. O. Hill, *Philos. Trans. R. Soc. London, Sect. B*, 1985, **311**, 605.
57 M. Masarwa, H. Cohen, D. Meyerstein, D. L. Hickman, A. Bakac and J. H. Espenson, *J. Am. Chem. Soc.*, 1988, **110**, 4293.
58 M. L. Kremer, *Phys. Chem. Chem. Phys.*, 1999, **1**, 3595.
59 J. D. Rush and D. E. Cabelli, *Radiat. Phys. Chem.*, 1997, **49**, 661.
60 S. Goldstein, D. Meyerstein and G. Czapski, *Free Rad. Biol. Med.*, 1993, **15**, 435.
61 E. Luzzatto, H. Cohen, C. Stockheim, K. Wieghardt and D. Meyerstein, *Free Rad. Res.*, 1995, **23**, 453.