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The role of the gulose–mannose part of bleomycin in activation of iron–molecular oxygen complexes

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A comparison of the complexing properties of metal ions and O_2 activation by bleomycin-A_2 (BLM-A_2) and deglyco-BLM-A_2 is presented. Deglyco-BLM-A_2 is obtained from the parent derivative by HF cleavage of the sugar moiety followed by h.p.l.c. purification. Complexing of Cu(II) and Fe(III) is studied by using c.d. and e.s.r. spectroscopy. Spin-trapping experiments in the presence of phenyl *N*-t-butylnitrone indicated lower production of free radicals by deglyco-BLM-A_2. Finally, a proposal is made to explain this discrepancy, focusing on the probable role of the gulose–mannose moiety acting as a protecting pocket, comparable with the pocket and picket-fence porphyrins described for haemoproteins.

INTRODUCTION

Bleomycin (BLM) is the name of a group of glycopeptide antibiotics with potent anti-neoplastic properties (Crooke, 1978; Carter, 1985). Investigations into the biochemical mechanism of BLM action have revealed a variety of explanations which may account for the observed therapeutic as well as toxic effects of the drug. BLM has been shown to bind to DNA (Chien *et al.*, 1977; Povirk *et al*, 1979; Chen *et al.*, 1980) by a process that is different from intercalation (Hénichart *et al.*, 1985*b*) and to cause DNA strand scission (Sausville *et al.*, 1978) by a mechanism involving the formation of oxygenated free radicals (Sausville *et al.*, 1976; Lown & Sim, 1977) and/or the cleavage of deoxyribose by an activated intermediary (Burger *et al.*, 1979*a*, 1981).

The bithiazole end-part of BLM (Fig. 1) is undoubtedly implied in the binding of BLM to DNA (Hénichart *et al.*, 1985*b*). The pseudo-peptide part of the molecule has been shown to complex copper and iron ions, thus explaining, to some extent, the production of radicals. By the design of synthetic models, copper ligands have been clearly delineated (Otsuka *et al.*, 1981; Hénichart *et al.*, 1985*a*). The production of free radicals in the presence of Fe(II) and O_2 have been well defined (Otsuka *et al.*, 1981; Hénichart *et al.*, 1985*a*). Nevertheless, the exact role of the gulose–mannose sugar portion (Fig. 1) in BLM action remains uncertain. In particular, the effect of the disaccharide moiety on iron co-ordination and O_2 activation is questioned.

In the aim to determine a putative role for this part of the structure, in a molecular mechanism of DNA breakage, we have compared the properties of Cu(II)–BLM, Fe(II)–BLM and Fe(III)–BLM complexes with the corresponding metal–deglyco-BLM complexes, and, in particular, the effect of the disaccharide moiety on O_2 activation of ferrous complexes is discussed. The Fe(II) complex undoubtedly plays a role in the biological activity of BLM, whereas the involvement of the Cu(II)–BLM complex in the DNA degrading process is questionable (Ehrenfeld *et al.*, 1985, 1987; Suzuki *et al.*, 1985). BLM-A_2, the more potent component of clinical BLM, has been chosen for this study, and a novel and very easy method of preparation of deglyco-BLM-A_2 has been used.

MATERIALS AND METHODS

Materials

BLM-A_2 was obtained from Roger–Bellon Laboratories (Neuilly-sur-Seine, France). Available samples contain a small percentage of *S*-desmethyl-BLM-A_2, and, for physical and biological studies, crude BLM-A_2 was purified by h.p.l.c. (Kratos h.p.l.c. system equipped with a Spectroflow 400 solvent-delivery system, a Spectroflow 783 detector and a Spectroflow 480 injector). Deglyco-BLM-A_2 was obtained by HF solvolysis using a classical HF line designed by Sakakibara (1971) for the cleavage of peptides from the resin support in solid-phase synthesis. Crude dried BLM-A_2 (40 mg) and anisole (1.5 ml) were mixed in a Kel-F reactor at 0 °C, and dry HF (Matheson, Ovel, Belgium) (15 ml) was added. After stirring for 1 h, HF was removed in vacuo, and anhydrous ether was added to the oily residue in order to extract anisole. Deglyco-BLM-A_2 was obtained from ether as a white solid which was purified by h.p.l.c. For this purification, a Cu(II) complex was prepared, and a linear gradient of 30–50 % methanol in water [both containing 5.0 mM-pentanesulphonic acid and 0.5 % (v/v) acetic acid, pH of water adjusted at 4.3] was used. A flow rate of 0.8 ml/min was maintained for 25 min through a 150 mm × 4.6 mm Ultrasphere ODS (Altex) column (5 μm particle size). Detection was at 292 nm. In this way, unchanged BLM-A_2 (less than 20 %), desmethyl-BLM-A_2 and deglycodesmethyl-BLM-A_2

Abbreviations used: BLM, bleomycin; PBN, phenyl *N*-t-butylnitrone; f.a.b., fast atom bombardment.

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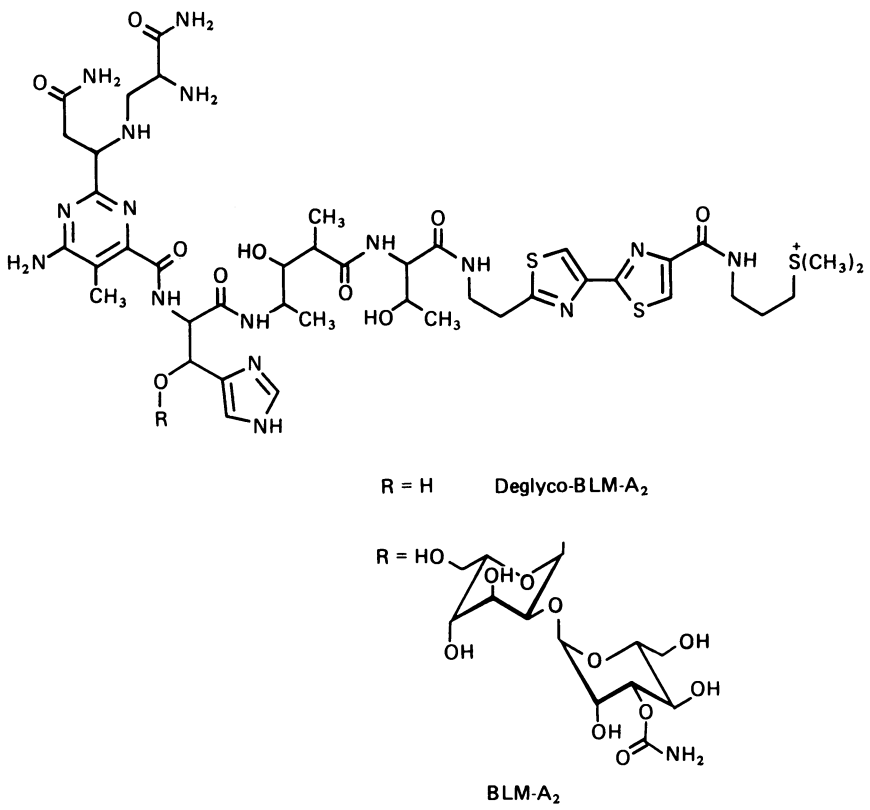

Fig. 1. Structures of BLM-A_2 and deglyco–BLM-A_2

Fig. 2. Elution profiles of (*a*) a BLM-A_2–Cu(II) complex and (*b*) a deglyco-BLM-A_2–Cu(II) complex by h.p.l.c.

A C-18 column was used with a linear gradient of methanol (30–50 %) in water containing 0.005 M-pentanesulphonic acid, pH 4.3. Flow rate was 0.8 ml/min. Detection was at 292 nm. Peak 1, BLM-A_2–Cu(II); peak 2, deglyco-BLM-A_2–Cu(II).

which appeared on the chromatogram (Fig. 2) were discarded. Free deglyco-BLM-A_2 was analysed by ^1H n.m.r. and ^{13}C n.m.r. in ^2H_2O with a Brucker AM 400 WB instrument.

An f.a.b. mass spectrum (Fig. 3) was obtained with a Kratos MS-50 RF mass spectrometer arranged in an electric field–magnetic field–electric field geometry. The sample was bombarded with a beam of Xe with a kinetic energy of 7 keV. The mass spectrometer was operated at 8 kV accelerating voltage with a mass resolution of 3000.

Sugar analysis was performed on BLM-A_2 and deglyco-BLM-A_2. Samples were methanolysed with 1.5 M-methanolic HCl for 24 h at 85 °C and trimethylsilylated with Sylon HTP in the presence of *myo*-inositol as an internal standard. The trimethylsilylated methyl glycosides were analysed by g.l.c. using a Hewlett–Packard model 5840 gas chromatograph equipped with a dual-flame ionization detector and a glass column (180 cm × 0.3 cm) containing 3 % OV-17 on Chromosorb WHP (80–100 mesh). The oven temperature was programmed from 120 °C to 200 °C at a rate of 8 °C/min.

E.s.r. measurements

E.s.r. measurements were recorded on a Brucker ESP 300 X-band spectrometer with a TE 102 cavity. A 100 kHz high-frequency modulation was used with a 50 mW microwave power and g values were determined with reference to $\alpha\alpha'$-diphenyl-β-picrylhydrazyl (g = 2.0036). The sample solutions were dispensed into a flat quartz tube.

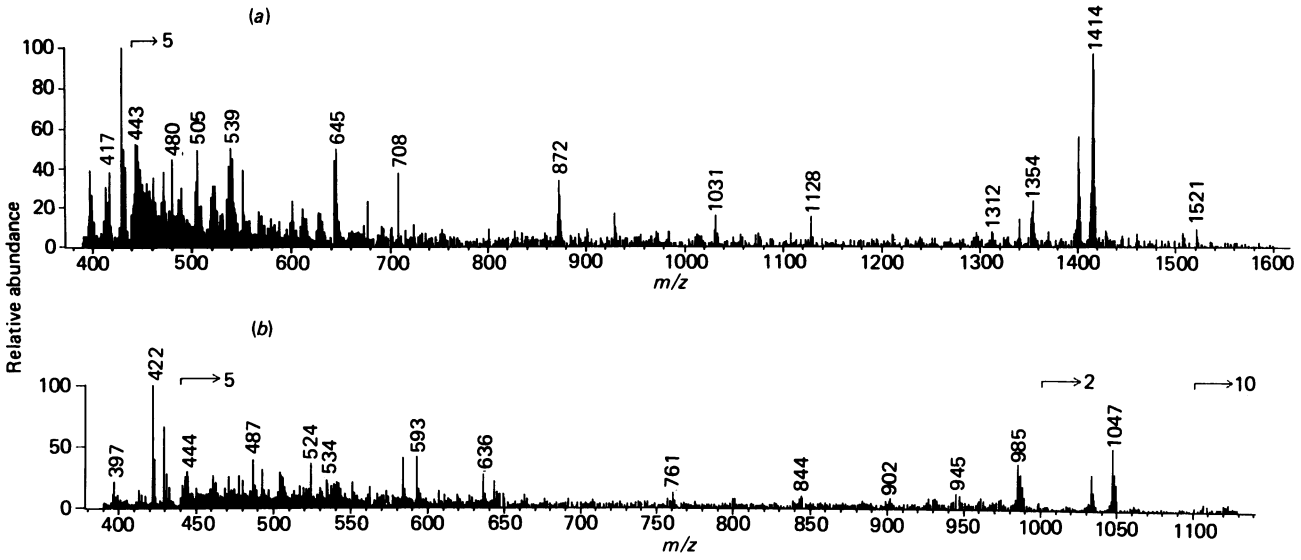

Fig. 3. F.a.b. mass spectra of (*a*) BLM-A_2 (molecular peak at 1414) and (*b*) deglyco-BLM-A_2 (molecular peak at 1047)

Copper complexes

BLM-A_2–Cu(II) and deglyco-BLM-A_2–Cu(II) complexes were prepared by addition of cupric perchlorate (1 mM) to a phosphate buffer, pH 6.9, containing the compounds in a 1:1 ratio. E.s.r. analyses were conducted at 77 K and 30% (v/v) glycerol was added to aid in the formation of a good quality glass.

Iron complexes

BLM-A_2–Fe(II) and deglyco-BLM-A_2–Fe(II) complexes were prepared by the addition of stoichiometric amounts of $Fe(NH_4)_2(SO_4)_2,6H_2O$ (freshly prepared) to the drugs at 4 °C. Then 30% (v/v) glycerol was added and samples were transferred to 3 mm quartz tubes and frozen in liquid N_2 within 20 s. The spectrum of an activated complex was immediately recorded.

The spectra of BLM-A_2–Fe(III) and deglyco-BLM-A_2–Fe(III) complexes, formed either by air oxidation or by adding stoichiometric amounts of Fe(III) to BLM-A_2 and deglyco-BLM-A_2, were recorded either in 0.05 M-Hepes buffer, pH 7.4, or 0.05 M-acetate buffer, pH 4.0.

Spin-trapping technique

The production of oxygenated free radicals during the formation of Fe(III) complexes was followed by the spin-trapping technique. An ethanolic solution (80 mM) of phenyl *N*-t-butylnitrone (PBN) spin trap (Janzen & Blackburn, 1968) was added to an aqueous solution (10 mM) of BLM-A_2 and deglyco-BLM-A_2, and pH was adjusted to 6.9. The 'OH adducts were detected by e.s.r. Control experiments were made to ensure that e.s.r. spectra did not result from nitrone spin trap alone, nor from the separate addition of Fe(II) or the drugs.

C.d. measurements

The c.d. spectra were recorded with a Jobin–Yvon dichrograph R.J. Mark III in quartz cells of appropriate path lengths in order to have an absorbance of less than 1.5. The ellipticity was expressed in $deg.cm^2.dmol^{-1}$. BLM-A_2 and deglyco-BLM-A_2 were dissolved in water at a concentration of 1 mM. Equimolar Cu(II) or Fe(III) complexes were obtained by adding a calculated quantity of $CuSO_4$ or $Fe(NH_4)_2(SO_4)_2,6H_2O$ dissolved in water. The pH was adjusted by adding either HCl or NaOH.

Base propenal quantification by thiobarbituric assay

To 0.2 ml of DNA_{CT} reaction mixture, containing an equimolecular amount of Fe(II)–BLM-A_2, was added 0.8 ml of 35 mM-2-thiobarbituric acid. The solution was heated at 92 °C for 20 min, allowed to cool, and its A_{532} measured.

RESULTS

Characterization of deglyco-BLM-A_2

The deglycosylation procedure by HF cleavage was found to be a very convenient method, providing deglyco-BLM-A_2 with a high yield (80%), as calculated on the h.p.l.c. chromatogram (Fig. 2). It is clear that HF cleavage of BLM-A_2 keeps the peptide part unchanged. This technique is therefore more adequate than the previously described method using alkaline conditions (Omoto *et al.*, 1972), which leads to the formation of dehydrohistidine residues.

Sugar analysis of BLM-A_2 before and after HF treatment clearly confirmed the high proportion of cleavage.

After purification by h.p.l.c., the purity of deglyco-BLM-A_2 was shown by f.a.b. m.s. Molecular peaks appeared at 1414 for BLM-A_2 and 1047 for deglyco-BLM-A_2 (Fig. 3). More proof of the complete deglycosylation was afforded by the comparison of the BLM-A_2 with the deglyco-BLM-A_2 n.m.r. spectra. ^{13}C and ^1H n.m.r. data were in accordance with previous reports (Chen *et al.*, 1977; Naganawa, 1979). A ^1H n.m.r. deglyco-BLM-A_2 spectrum analysis clearly indicated that δ 5.25 and δ 5.02 signals, attributed to anomeric protons of gulose and mannose respectively in the BLM-A_2 spectrum, were missing. The δ 98.1 and δ 98.7 ^{13}C signals, corresponding to the anomeric carbons of gulose and mannose, were also absent. Other differences attributed to the disaccharide cleavage, and not detailed

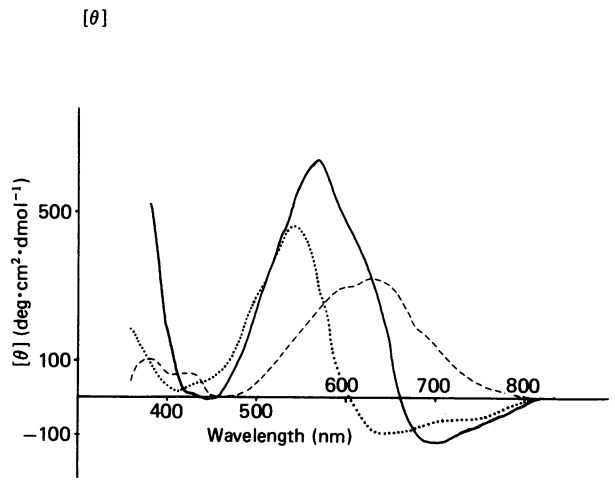

Fig. 4. C.d. spectra of the deglyco-BLM-A_2–Cu(II) complex in the visible region (800–350 nm) at pH 1.5 (-----), 3.1 (·····) and 5.8 (——)

Concentration of the drug was 1 mM.

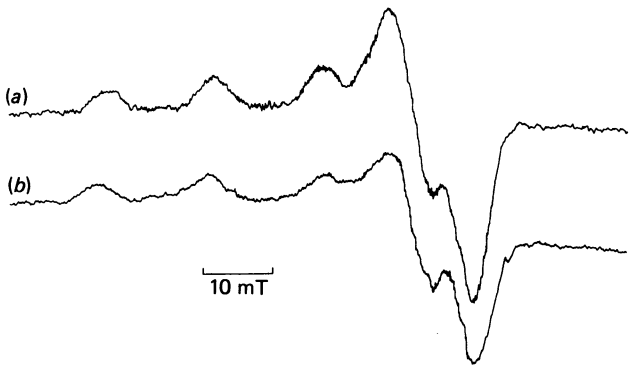

Fig. 5. E.s.r. spectra of (*a*) BLM-A_2–Cu(II) and (*b*) deglyco-BLM-A_2–Cu(II) complexes at pH 7.4

here, have been analysed. All these determinations were necessary to be able to assume the perfect purity of the prepared deglyco-BLM-A_2, and allowed the following studies.

Copper complexes

In c.d., such as that previously described for BLM-A_2 (Albertini, 1984), deglyco-BLM-A_2 in aqueous solution exhibits different spectra with varying pH.

In the visible region, the spectrum of deglyco-BLM-A_2–Cu(II) looks the same as the one obtained for BLM-A_2–Cu(II). On the contrary, they are very different in the u.v. region. However it was noted that all over the spectra the ellipticities exhibited by the deglyco-BLM-A_2–Cu(II) complex are smaller than the ones observed for the glycosylated parent derivative. As previously observed (Sugiura *et al.*, 1983), two *d–d* transitions can be visualized in the Cu(II) absorption region, the most intense one being located near 550 nm (d_{xz}, $d_{yz} \rightarrow d_{x^2-y^2}$) except at acidic pH.

In fact, at pH 1.5 (Fig. 4), only one broad band ($[\theta] = +280$ deg·cm²·dmol⁻¹) is observed in the visible region, centred at 630 nm {BLM-A_2–Cu(II) complex, pH 1.2, $\lambda_{max.}$ 680 nm, $[\theta] = +360$ deg·cm²·dmol⁻¹}. It can be attributed to a transition $d_{xy} \rightarrow d_{x^2-y^2}$, and

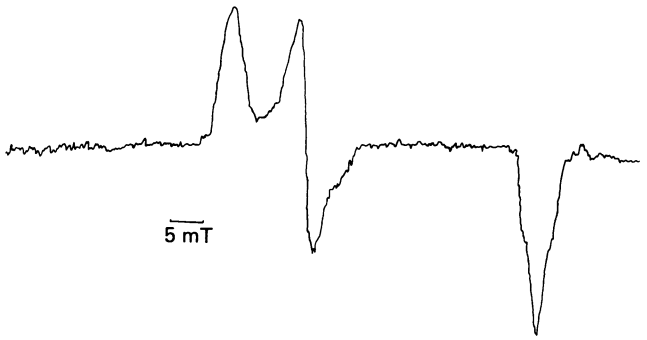

Fig. 6. E.s.r. spectrum of 'activated BLM' at pH 7.4

For further details see the text.

corresponds to a penta-atomic chelate ring (positive sign of the band; Garnier *et al.*, 1980) involving the same chromophores as BLM-A_2 (pyrimidinyl N and peptidic NH of the pyrimidinyl–histidyl bond).

At pH 3.1, some changes occur in the c.d. spectrum of the deglyco-BLM-A_2–Cu(II) complex. In particular, the visible region of the spectrum (Fig. 4) is characterized by a positive c.d. band, located at 540 nm ($[\theta] = +420$ deg·cm²·dmol⁻¹; d_{xz}, $d_{yz} \rightarrow d_{x^2-y^2}$), and a smaller negative one near 700 nm ($[\theta] = -100$ deg·cm²·dmol⁻¹; $d_{xy} \rightarrow d_{x^2-y^2}$). One more chromophore, i.e. the N^{π}-imidazole, is involved in the complex, which can then be considered to be similar to the one involving BLM-A_2 (pH 2.5, $\lambda_{max.}$ 650 nm, $[\theta] = -990$ deg·cm²·dmol⁻¹; $\lambda_{max.}$ 550 nm, $[\theta] = +990$ deg·cm²·dmol⁻¹), since it is well known (Schlafer & Gliemann, 1969) that the magnitude of the ligand field around the central Cu(II) is reflected in the d_{xz}, $d_{yz} \rightarrow d_{x^2-y^2}$ transition, and the planarity of the complex in the $d_{xy} \rightarrow d_{x^2-y^2}$ transition.

At higher pH (5.8), the positive band previously located at 540 nm undergoes a red-shift (λ 560 nm) and a rather weak enhancement in intensity ($[\theta] = +680$ deg·cm²·dmol⁻¹; d_{xz}, $d_{yz} \rightarrow d_{x^2-y^2}$), whereas no spectacular change occurs for the other visible c.d. band ($\lambda_{max.}$ 700 nm, $[\theta] = -120$ deg·cm²·dmol⁻¹). However the u.v. c.d. region reflects a dramatic change characterized by the occurrence of two main bands of very high intensity, one located at 285 nm ($[\theta] = +8200$ deg·cm².dmol⁻¹), and the other at 237 nm ($[\theta] = -13800$ deg·cm²·dmol⁻¹), reflecting the co-ordination of another ligand, namely, the α-NH_2 of β-aminoalanine. The occurrence of the intense negative band at 237 nm was not perceptible for the BLM-A_2–Cu(II) complex and thus reveals a different geometry for the deglyco-BLM-A_2–Cu(II) complex. The higher intensity of the bands reflects steric constraints that are more important at this pH (Bereman & Winkler, 1980).

E.s.r. spectroscopy also gives interesting information about the binding of the Cu ion by BLM-A_2 and deglyco-BLM-A_2 at neutral pH. The X-band spectra are presented in Fig. 5, and e.s.r. parameters have been measured. The $g_{\parallel}$ (2.204) and $A_{\parallel}$ (17.75 mT) of BLM-A_2–Cu(II) correspond to values previously reported (Dabrowiak *et al.*, 1978), and were similar to those of copper complexes containing four in-plane N atoms, on the basis of a comparison with $g_{\parallel}$ and $A_{\parallel}$ values of simpler synthetic models (Peisach & Blumberg, 1974). Moreover, the similarity of the e.s.r. spectrum of BLM-

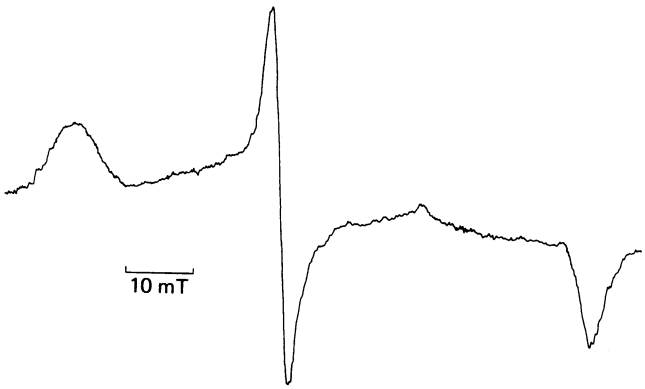

Fig. 7. E.s.r. spectrum of a low-spin BLM-A_2–Fe(III) complex at pH 7.4

For further details see the text.

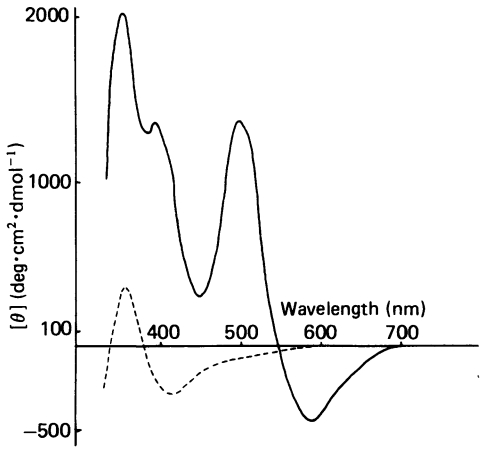

Fig. 8. C.d. spectra of the deglyco-BLM-A_2–Fe(III) complex in the visible region (700–350 nm) at pH 4.1 (-----) and 7.5 (——)

Concentration of the drug was 1 mM.

A_2–Cu(II) to the Cu(II) complex spectrum of a synthetic analogue of the chelating part of BLM-A_2 (AMPHIS) (Hénichart *et al.*, 1985*a*) is in favour of a square-pyramidal geometry for the BLM-A_2 complex, where the N^{π} and the deprotonated amide N of histidine, a heterocyclic N of the pyrimidine ring and the secondary amino group occupy the square basal positions. The involvement of the primary amino group of β-amino-alanine as an apical ligand has been postulated on the basis of previous comparative studies (Sugiura, 1979; Hénichart *et al.*, 1985*a*). Slight differences have been noted between deglyco-BLM-A_2 e.s.r. spectrum parameters ($g_{\parallel} = 2.210$ and $A_{\parallel} = 16.32$ mT), and can be explained in terms of deformation of the co-ordination geometry and not by loss of one ligand [the larger $g_{\parallel}$ and smaller $A_{\parallel}$ are associated with an off-planar distortion, probably into the pyramidal geometry (Miyoshi *et al.*, 1983)].

Iron complexes

At pH 7.4, the e.s.r. spectrum of the aerobic mixture Fe(III)–BLM-A_2, immediately frozen at 77 K, exhibited g values ($g_z = 2.26$; $g_y = 2.17$; $g_x = 1.94$) (Fig. 6) attributable to an 'activated BLM' (Sugiura, 1980; Burger *et al.*, 1981), corresponding to a low-spin Fe(III) complex. This complex was found to be very unstable. In a few minutes, another stable BLM-A_2–Fe(III) complex was formed. Its e.s.r. spectrum exhibited g values ($g_z = 2.43$; $g_y = 2.18$; $g_x = 1.89$) (Fig. 7) corresponding to a rhombic low-spin Fe(III) complex (Burger *et al.*, 1979*b*; Sugiura, 1980; Albertini & Garnier-Suillerot, 1984). This complex exhibited a good stability and it can be kept for several days without modification of its spectroscopic characteristics. In the case of deglyco-BLM-A_2, an e.s.r. spectrum corresponding to the so-called 'activated BLM' was detected at pH 7.0 with only a weak intensity (5% of the BLM-A_2 signal intensity). This signal was found to decay quickly, not displaying an Fe(III) e.s.r.-detectable species, as observed for BLM-A_2 (low-spin Fe(III) type).

Table 1. C.d. constants for the deglyco-BLM-A_2–Fe(III) complex at pH 4.1 and 7.5

Complex	λ (nm)	$[\theta]$ (deg·cm²·dmol⁻¹)
BLM-A_2–Fe(III), pH 4.0	410	+10560
	340	−5940
	275	−7590
	240	+13200
BLM-A_2–Fe(III), pH 7.4	620	−1980
	515	+5940
	390	+8250
	350	+9240
	325	+5280
	295	−10560
	275	−15180
	235	+14850
Deglyco-BLM-A_2–Fe(III), pH 4.1	420	−300
	360	+350
	315	−3100
	265	−3500
	240	−3100
Deglyco-BLM-A_2–Fe(III), pH 7.5	595	−400
	500	+1350
	400	+1375
	360	+2000
	310	−1800
	290	−2300
	255	−1600
	235	+2600

The BLM-A_2–Fe(III) complex was also studied at pH 4.0 in acetate buffer and gave rise to a high-spin ferric spectrum ($g = 4.3$). Such a high-spin ferric spectrum was not detected by e.s.r. at pH 4.0 with deglyco-BLM-A_2.

By c.d., no evidence for a high-spin form could be found at acidic pH for deglyco-BLM-A_2–Fe(III), whereas the low-spin form still existed at physiological pH (Fig. 8). Characteristic features of the spectra are given in Table 1 together with these relative to the BLM-A_2–Fe(III) complex. To be noted are the lower ellipticity values obtained for the deglycosylated molecule, together with a blue shift of the spectrum at pH 7.5. This characterizes a less polar environment for the complex, although the ligands implicated seem to be the same for both complexes. This observation suggests that the sugar moiety plays a role in the complexation process.

On the other hand, at pH 4.1, the spectrum obtained

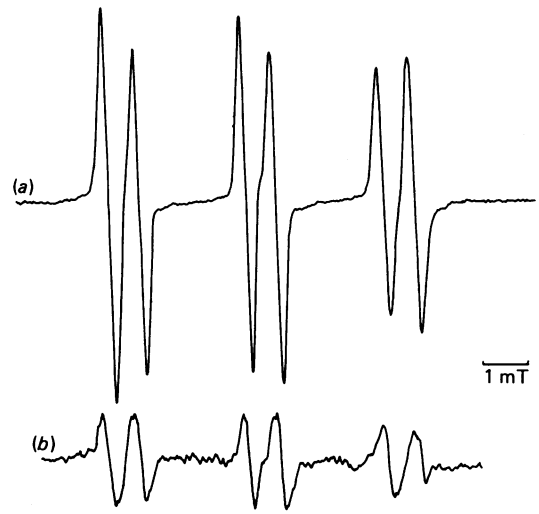

Fig. 9. E.s.r. spectra obtained by air oxidation of the (*a*) BLM-A_2–Fe(II) and (*b*) deglyco-BLM-A_2–Fe(II) complexes in the presence of PBN at pH 6.9

Concentrations of the drugs and of PBN were respectively 10 mM and 80 mM.

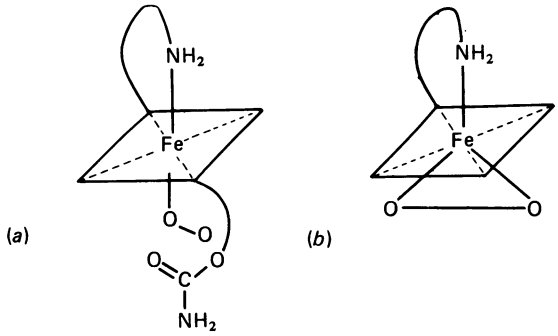

Fig. 10. Schematic diagrams of the superoxo structure of the BLM-A_2–Fe(III)–O_2 complex (*a*) and of the peroxo structure of the deglyco-BLM-A_2–Fe(III)–O_2 complex (*b*)

for deglyco-BLM-A_2–Fe(III) is characterized by a negative band located at 420 nm, exhibiting a very small ellipticity. This spectrum does not evolve on time. Once more, a smaller ellipticity together with a blue-shift is observed in the case of the deglycosylated molecule, leading to the same conclusion as above on the role played by the sugar moiety.

Free radical production

The e.s.r. signals generated by air oxidation of the BLM-A_2–Fe(II) system in the presence of PBN spin trap (triplet of doublets with $g = 2.006$ and $a_N = 1.53$ mT) have been attributed to the formation of $^{\cdot}OH$ radical adducts, as previously reported (Sugiura & Kikuchi, 1978; Oberley & Buettner, 1979; Sugiura, 1980). Fig. 9 shows the decrease in signal intensity when experiments were carried out in the presence of deglyco-BLM-A_2 in the same conditions. The radical-spin concentration in the presence of deglyco-BLM-A_2 was estimated to be approx. 35% compared with the spin density of radicals produced in the presence of BLM-A_2.

DISCUSSION

Copper complexes

From c.d. results obtained with BLM-A_2–Cu(II) and deglyco-BLM-A_2–Cu(II) complexes, it can be concluded that the same ligands are involved in the Cu(II) complexes of both drugs. However, the smaller ellipticities obtained for deglyco-BLM-A_2–Cu(II) reflect a lower rigidity in the structure of the complex.

This finding is confirmed by e.s.r. results. The slight differences observed between e.s.r. parameters of BLM-A_2 and deglyco-BLM-A_2, $g_{\parallel} = 2.204$, $g_{\perp} = 2.050$, $A_{\parallel} = 17.75$ mT and $g_{\parallel} = 2.211$, $g_{\perp} = 2.054$, $A_{\parallel} = 17.5$ mT respectively, are in favour of a variability of co-ordination geometry. For the square-pyramidal five-co-ordinate complex of BLM-A_2, the Cu(II) centre allows dictation of geometry by the ligands defined above, and also by forces induced by the disaccharide moiety. But, owing to the Jahn–Teller effect, in the absence of the sugar part, a slight modification of the Cu(II) complex structure occurs with displacement of the Cu(II) centre out of plane toward the fifth ligand. Further evidence of this study is that the carbamoyl group of the disaccharide part is not involved as a ligand as is claimed by other authors (Bereman & Winkler, 1980).

Iron complexes

Concerning the formation of iron–BLM-A_2 complexes in aerobic conditions, the present report confirms the previous results on the basis of c.d. and e.s.r. studies. It has already been reported that the BLM-A_2–Fe(III) final complex includes a ferric iron at low-spin state and pentaco-ordinates inducing a square-pyramidal geometry. In the case of deglyco-BLM-A_2–Fe(III), the transition low-spin→high-spin is no longer observed since no high-spin complex has been seen in evidence at pH 4.1, either by c.d. or by e.s.r. However, a c.d. spectrum typical of the low-spin form is obtained for deglyco-BLM-A_2–Fe(III) at pH 7.5. Though no complex could be seen by e.s.r. at pH 7.0, it is noticeable that this molecule gives a ternary complex with N_3^- ($g_x = 1.82$; $g_y = 2.22$; $g_z = 2.53$), similar to the BLM-A_2–Fe(III)–N_3^- complex ($g_x = 1.83$; $g_y = 2.23$; $g_z = 2.55$) at pH 7.0 (Sugiura, 1980). Therefore it can be inferred that deglycobleomycin is able to complex iron though no high-spin or low-spin complex could be seen in evidence in the conditions used here. The absence of a high-spin species in the case of deglyco-BLM-A_2 can be explained by the larger ionic radius of Fe, which can no longer fit inside the centre of the square when no stabilization is given by the sugar moiety.

Free radical production and DNA degradation

The concentration of free radicals produced in the presence of deglyco-BLM-A_2, Fe(II) and O_2, estimated by the spin-trapping technique, was found to be approx. 35% of that of the corresponding experiments with BLM-A_2, Fe(II) and O_2, the experiments being carried out with exactly the same initial concentration. From these observations, it can be stated that the gulose–mannose part undoubtedly plays a role both in the stabilization of the BLM-A_2–Fe(III)–O_2 complex and in the production of oxygenated free radicals.

Moreover, the consequence of the instability of the intermediary complex, and the decrease of radical production, was the observed decrease of DNA

degradation when the disaccharide moiety was missing. This result corresponds well with the observation that the deglyco-BLM–Fe(II) complex showed about half as much [^{3}H]thymine release from PM-2 DNA as the BLM–Fe(II) complex system (Oppenheimer *et al.*, 1982).

Studies including (i) deglyco-BLM-A_2–Fe(III)–O_2 complex evidence, (ii) production of reactive oxygen radicals and (iii) degradation of DNA have not been reported until recently. The previous results reported were on either spin-trapping experiments (Sugiura *et al.*, 1983) or DNA nicking (Oppenheimer *et al.*, 1982); they were not related to the formation of an activated complex. The present report shows that if deglyco-BLM-A_2 is able to form a deglyco-BLM-A_2–Fe(III)–O_2 complex, this species is still less stable than the BLM-A_2–Fe(III)–O_2-activated complex since only a weak-intensity e.s.r. signal was observed.

Thus the gulose–mannose portion of BLM-A_2 can be thought to contribute to the more effective O_2 activation which finds expression in formation of the BLM-A_2–Fe(III)–O_2 complex and in the production of free radicals, both phenomena being related. The nature of the species actually responsible for DNA cleavage remains questionable.

Moreover, it seems reasonable to suggest that the gulose–mannose moiety could act in the stabilization of BLM-A_2–Fe(III)–O_2 complex as a protecting pocket similar to the pocket and picket-fence porphyrins described for haemoproteins (Collmann *et al.*, 1983). This substantiates the decrease in intensity of the signal of the e.s.r. spectrum of the so-called 'activated' deglyco-BLM-A_2 complex. The lower intensity of this signal is correlated with lower production of active oxygen species, which reflects an affiliation between both phenomena.

In accordance with the weak stability of the deglyco-BLM complex, release of hydroperoxide can be envisaged, which may take the form of 'OH production able to induce DNA strand scission via a Fenton-type reaction.

The binding of O_2 to a transition metal is accompanied by the transfer of charge density to the O_2 molecule and, as a result, the O_2 ligand acquires basic and nucleophilic character.

The slightly basic character of O_2, assumed to have superoxo structure, or η_1 structure according to Vaska's (1976) classification, in BLM-A_2–Fe(III)–O_2 complex (Fig. 10*a*), explains its ability to form hydrogen bonds, as postulated for peroxo O_2 bound to iron in oxygen carriers (Shaanan, 1982). It is also reasonable to assume the establishment of hydrogen bonds between O_2 and the gulose–mannose part of BLM. The superoxo structure has been also reported to induce electron transfer reactions (Welborn *et al.*, 1981), i.e. in favour of the mechanism proposed above.

In the absence of a hydrogen-donor sugar environment, O_2 is supposed to have a peroxo structure, or η_2 according to Vaska's (1976) classification, and it can be suggested that this structure is favoured in the structure of the deglyco-BLM-A_2–Fe(III)–O_2 complex (Fig. 10*b*).

In the great majority of reactions, the co-ordinated O_2 with a peroxo structure behaves as a nucleophile. Nevertheless, the electronic structure does not show the same delocalization as found in superoxo complexes, and the mechanism of O_2 activation mentioned above is not favoured.

In conclusion, the mechanism of DNA cleavage by BLM-A_2 involves the activation of an O_2 ligand of a BLM-A_2–Fe(III) complex. For this activation, two characteristics are necessary. First, the presence of the sugar part plays a role of a stabilizing function and allows the homolytic scission of O_2, a key step in the abstraction of a hydrogen atom from deoxyribose. Secondly, this reaction must occur in the vicinity of the cleaving site, and the bithiazole part is able to bind the molecule selectively, assuring the specific cleavage at G-C and G-T sequences.

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