Preparative, physico-chemical and cytotoxicity studies of prodrugs activated in hypoxia to give metal-binding analogues of bleomycin

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The synthesis of 2,6-disubstituted pyridines **10**, **23**, **27a**–**28b** is reported. These compounds are expected to complex iron(II) and yield hydroxyl radicals by interaction of the aqueous complex with oxygen. In addition a second series of 2,6-disubstituted pyridines **24a**, **24b** and **29** having additional features (nitro or *N*-oxide groups), which are expected to prevent complexation of iron, is described. These deactivated compounds are expected to be reduced in hypoxic tumour cells to yield products **25a**, **25b** and **10**, respectively, which are able to complex metals and yield hydroxyl radicals. EPR and fluorescence spectroscopy provide evidence for the production of hydroxyl radicals from all the compounds except the prodrugs **10**, **25a** and **25b** and the compounds not having an imidazole nucleus **27a**–**28b**. The prodrugs were not cytotoxic in air alone to Chinese hamster V79 cells *in vitro*. However, when the prodrug was added to the cells and then exposed to hypoxia followed by air, the nitro compounds **24a** and **24b** showed slightly increased cytotoxicity. However, the *N*-oxide **29** showed marked cytotoxicity similar to that of the corresponding *N*-deoxygenated compound **10**.

Bleomycin-A_2 (Fig. 1) is a glycopeptide antibiotic first isolated as a copper complex from *Streptomyces verticillus*.[1,2] Blenoxane, clinically useful in the treatment of Hodgkin's lymphoma and carcinomas of the skin, head, neck and testis,[3,4] is a mixture of related compounds but is mainly bleomycin-A_2. The drug has no significant bone marrow toxicity but has dose limiting pulmonary toxicity and is used in combination chemotherapy.

The bleomycin (BLM) molecular structure can be considered to be made up of four parts: (i) the amine–pyrimidine–imidazole section which at the marked (*) nitrogen atoms complexes with iron (Fig. 1), (ii) the bithiazole and terminal cation which bind with DNA, (iii) the threonine–aminovalerate dipeptide linker, and (iv) the disaccharide unit. The complexed iron(II) centre binds oxygen at the vacant sixth co-ordination site to form an oxygenated-iron–BLM complex (Fig. 2). The means by which BLM exerts its cytotoxic effect has been studied extensively and is known to be an oxidative strand scission of DNA mediated by metal chelates of bleomycin.[5–8] The greatest activation and the only *in vivo* activity is said to be when iron is the complexed metal.[9] It has been shown that both hydroxyl radical and superoxide radical ions are generated by the Fe(II)–BLM system[10,11] and that cleavage of double-stranded DNA occurs by abstraction of the C-4′ hydrogen atom of the deoxyribose moiety of the pyrimidine nucleoside.[6,12] However, there is some evidence that attack by hydroxyl and superoxide radicals is not the only cause of DNA degradation in the presence of Fe(II)–BLM, water and oxygen.[13] The detailed structure of the active species which involves bleomycin, iron and a dioxygen is still uncertain.[14]

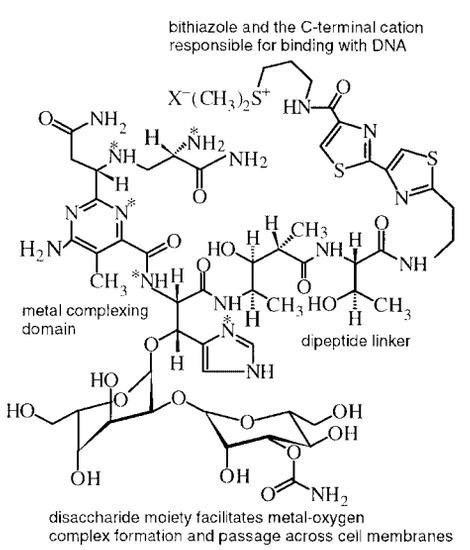


Fig. 1 Bleomycin A_2.

A number of analogues of the metal binding portion of BLM have been prepared and the evidence from structure–activity relationship (SAR) studies reviewed.[15] Many of the essential structural requirements for oxygen activation by BLM analogues have been determined. Several 2,6-disubstituted pyrimidine analogues **1**–**4** of bleomycin have been studied.[16] However, the pyrimidine nucleus is not essential and more attention has been paid to 2,6-disubstituted pyridines which also show oxygen activation effects in the presence of Fe(II).[17–21] In this series of compounds it has been found that an electron withdrawing 4-substituent (*e.g.* in **5**) decreases the oxygen activating ability whereas electron donating 4-substituents (*e.g.* OMe in **6** and NMe_2 in **7**) increases the effect.

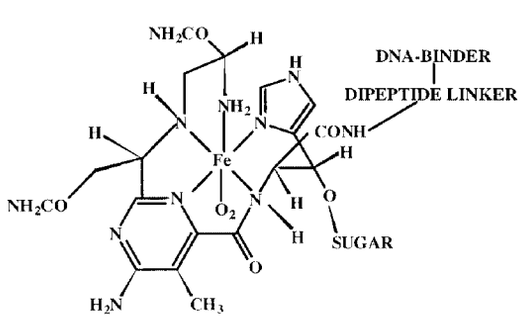
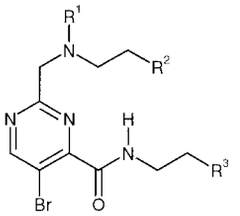
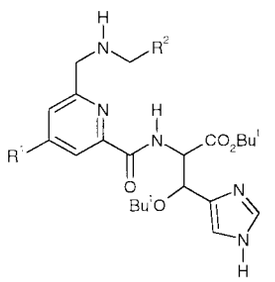
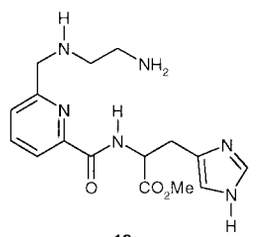


Fig. 2 Bleomycin–iron–oxygen complex.



1 R^1 = H, R^2 = NH_2, R^3 = imidazol-4(5)-yl
2 R^1 = Me, R^2 = NHMe, R^3 = imidazol-4(5)-yl
3 R^1 = H, R^2 = R^3 = NH_2
4 R^1 = Me, R^2 = NMe_2, R^3 = imidazol-4(5)-yl





10

5 R′ = Cl, R^2 = $CH(NH_2)CONH_2$
6 R′ = OMe, R^2 = $CH(NH_2)CONH_2$
7 R′ = NMe_2, R^2 = $CH(NH_2)CONH_2$
8 R′ = OMe, R^2 = CH_2-imidazol-4(5)-yl
9 R′ = OMe, R^2 = imidazol-4(5)-yl

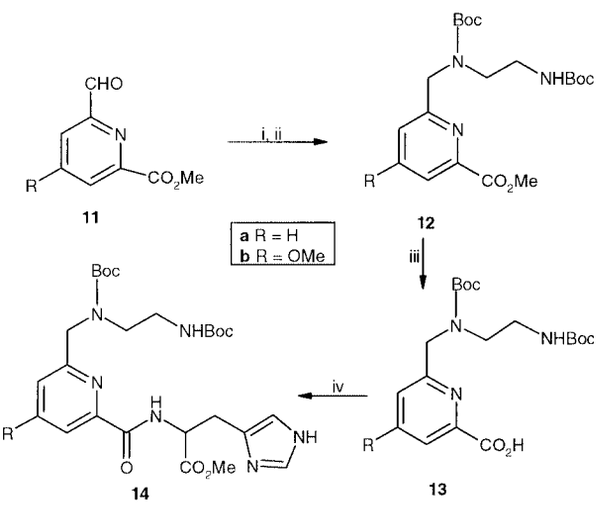
In pyridine[19,20] or pyrimidine[16] analogues the 2-substituents (hereafter referred to as chain A) having a terminal primary (**1**, **6** and **7**) or secondary amine (**2**)[16] or an imidazole nucleus (**8** and **9**)[21] have been found to have oxygen activating ability. However, the iron complexes of **8** and **9** were different in properties from the bleomycin–iron complexes. Evidence from the pyrimidine series of BLM analogues indicates that a terminal dimethylamino group is not satisfactory.[16] The carboxamide group which occurs in chain A of bleomycin is not essential for antitumour activity.[22] One of the simplest BLM analogues is **10**, "methyl 2-(2-aminoethyl)aminomethylpyridine-6-carboxyl-histidinate" abbreviated to AMPHIS.[23]

The amide chain in BLM (hereafter referred to as chain B) contains a histidine residue which serves as part of the metal complexation unit. The BLM analogues usually contain either a histidine or histamine residue. To the best of our knowledge there is little evidence whether the imidazole nucleus can be replaced by either an aliphatic amine or a hetereoaromatic residue. However, what little evidence there is concerning the aliphatic amine indicates that the presence of two methylene groups between the amide and amine nitrogen atoms in **3** does not provide a structure which is capable of activating oxygen.[16]

Solid tumours contain regions of oxic, hypoxic and necrotic cells. The lethal X-ray dose required for hypoxic cells is about three times that necessary to kill normal cells. The hypoxic cells are thought to survive radiotherapy and later to become reoxygenated upon catabolism of the killed tumour cells.[24] These reoxygenated surviving tumour cells may become the foci of metastases at the site of the primary tumour or elsewhere in the body. However, hypoxic but not oxic cells are able to cause reduction of certain functional groups, in particular, the partial or complete reduction of certain aromatic nitro groups and some *N*-oxides. Since normal cells are oxic, this effect can be used to produce cytotoxic drugs through bioreductive activation[25] by hypoxic cells at the site of the tumour. This paper gives an account of our application of this idea to bleomycin analogues in order to develop drugs whose cytotoxic effects are targeted to the tumour site. A preliminary communication has been published.[26]

Our objectives have been to synthesise two types of analogues of the metal complexing region of bleomycin. The first type of compounds have in chain B a terminal aliphatic amine or a heteroaromatic nucleus other than imidazole. The second type have a substituent designed to prevent metal complexation until reduction of substituent has occurred. We also proposed to assess the hydroxyl radical production by the two types of analogues, and to assay the cytotoxicity of these compounds to mammalian cells under (a) entirely oxic conditions and (b) in oxygen after prior exposure of the mixture of cells and compound to hypoxia.

The synthesis of **13a** was accomplished by the reported procedure of Huang *et al.*,[27] from the aldehyde **11a** *via* the protected amine **12a**. The corresponding 4-methoxy analogue **13b** was synthesised by a similar method from the known[19] aldehyde **11b**. The method described[27] for the preparation of **12a** was used to synthesise **12b** from **11b**. Reaction of **11b** with *tert*-butyl *N*-(2-aminoethyl)carbamate in methanol in presence of 10% palladium on charcoal in an atmosphere of hydrogen followed by the protection of the secondary amine group with di-*tert*-butyl dicarbonate provided the ester **12b** in an overall yield of 72% from the aldehyde **11b**. Hydrolysis of **12b** afforded the acid **13b** in a high yield of 95%. Separate condensation of **13a** and **13b** with histidine methyl ester hydrochloride in the presence of diphenylphosphoryl azide (DPPA)[28] afforded the amides **14a** and **14b** in 70% and 83% yield respectively (Scheme 1). Although good yields were obtained, the isolation of the



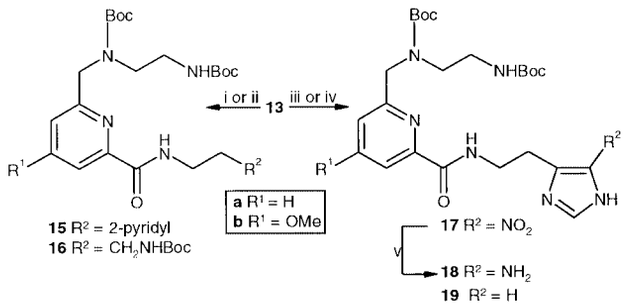
Scheme 1 *Reagents and conditions*: (i) $H_2N(CH_2)_2NHBoc$, H_2, Pd/C, MeOH, rt; (ii) $(Boc)_2O$, DCM, rt, 1 h; (iii) aq. NaOH; (iv) histidine methyl ester hydrochloride, DPPA, Et_3N, DMF, 0 °C, 4 h then rt, 4 days.

products proved to be difficult because the byproducts had R_f values similar to those of the peptides and hence column chromatography had to be repeated several times until the pure compounds were obtained. Subsequent condensations of the acids **13a** and **13b** with amines were carried out using 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI) and 1-hydroxybenzotriazole (HOBT) as activating agents.

Compounds with a pyridine nucleus **15a** and **15b** and an aliphatic amine **16a** and **16b** on chain B were synthesised by the coupling reaction of **13a** and **13b** with 2-(2-aminoethyl)pyridine and *tert*-butyl *N*-(3-aminopropyl)carbamate respectively. Monoprotected propylamine was used in order to provide the same number of carbon atoms between the nitrogen atoms involved in the complexation with iron as that which occurs in bleomycin.

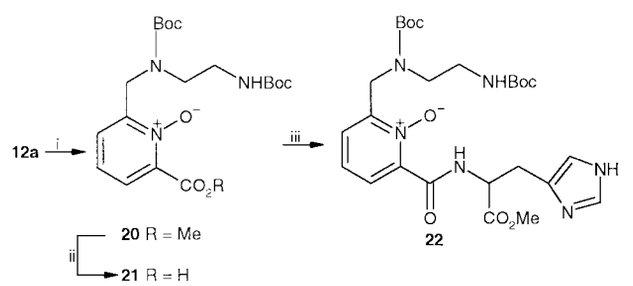
In order to prepare an analogue which was expected to be bioreductively activated at the tumour site a 4(5)-nitroimidazol-5(4)-yl unit was incorporated into the molecule by the reaction of 4(5)-nitrohistamine[29] with **13a** and **13b** to form the amides **17a** and **17b**, respectively. Hydrogenation of **17a** and **17b** separately in the presence of palladium on charcoal gave the

corresponding amines **18a** and **18b**. As reference compounds, the two amides **19a** and **19b** were prepared from histamine and **13a** and **13b** (Scheme 2).



Scheme 2 *Reagents and conditions*: (i) 2-(2-aminoethyl)pyridine, EDCI, HOBT, DMF, 24 h; (ii) *tert*-butyl *N*-(3-aminopropyl)carbamate, EDCI, HOBT, DMF, 24 h; (iii) 4(5)-nitrohistamine hydrochloride, EDCI, HOBT, DMF, 24 h; (iv) histamine, EDCI, HOBT, DMF, 24 h; (v) H_2, Pd/C, methanol, rt.

Another approach to prevent metal complexation is to introduce an *N*-oxide group on the pyridine ring. *N*-Oxidation on the pyridine nitrogen atom in the system used in this work was difficult due to the steric crowding by the bulky 2- and 6-substituents. Attempts to oxidise the pyridine nitrogen atom in **13a** by dimethyldioxirane were unsuccessful.[30] However, oxidation of **12a** was achieved by treatment with *m*-chloroperbenzoic acid (MCPBA) to give **20** (Scheme 3). This reagent



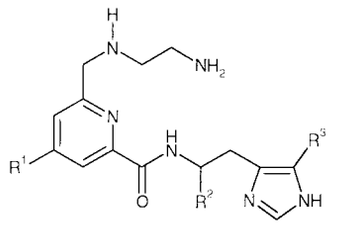
Scheme 3 *Reagents and conditions*: (i) MCPBA, DCM, rt, 7 days; (ii) aq. NaOH, MeOH, rt, 1 day; (iii) histidine methyl ester hydrochloride, EDCI, HOBT, DMF, Et_3N, rt.

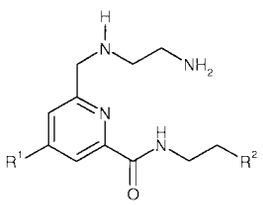
was used because it does not cause side reactions associated with the use of hydrogen peroxide and acetic acid. Basic hydrolysis of **20** afforded **21** in excellent yield. Peptide coupling of **21** with histidine methyl ester hydrochloride in presence of EDCI and HOBT gave **22** in 50% yield.

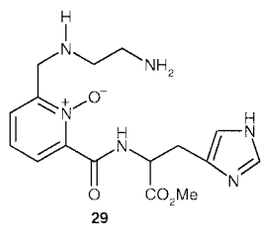
The protecting *tert*-butoxycarbonyl groups were removed from **14a**, **14b**, **15a**, **15b**, **16a**, **16b**, **17a**, **17b**, **18a**, **18b**, **19a**, **19b** and **22** by treatment with trifluoroacetic acid at 0 °C. The corresponding free amines **10**, **23**, **27a**, **27b**, **28a**, **28b**, **24a**, **24b**, **25a**, **25b**, **26a**, **26b** and **29**, respectively, were isolated in high yields by passing an aqueous solution of the peptide trifluoroacetate through an ion exchange resin.

Studies of the ability of AMPHIS (**10**) to produce radicals in aqueous ethanolic solution and in the presence of iron(II) and oxygen were made by use of 5,5-dimethyl-1-pyrroline 1-oxide (DMPO) and α-phenyl-*N*-*tert*-butylnitrone (PBN)† as spin traps. The spin adducts observed had line patterns[31,32] and hyperfine splitting in agreement with the values reported for the α-hydroxyethyl radical ($H_3C(HO)CH^{\bullet}$) adduct of DMPO and PBN.[23,33] The same EPR spectrum was obtained from DMPO, Fe(II) and hydrogen peroxide. The intensity of the spectrum was increased in the presence of ethylenediaminetetraacetic acid

† IUPAC names: 2,2-dimethyl-3,4-dihydro-2*H*-pyrrole 1-oxide and *N*-benzylidene-*tert*-butylamine respectively.







(EDTA) but not when AMPHIS (**10**) was used as the chelator. Previously,[23,27] the activity of bleomycin analogues was measured by the relative height of the EPR signals produced by spin-trapped hydroxyl radicals and compared with results obtained under similar conditions in the presence of bleomycin. Measurement of the activity by this method may be inaccurate and is dependent on the time allowed between the preparation of the test solution and the measurement of the EPR spectrum. If the solution of an analogue and iron is exposed to oxygen for too long a period before the EPR signal is recorded, the iron is oxidised and precipitated as iron(III) hydroxide. However, if the EPR spectrum is recorded too quickly, the analogue–iron–oxygen complex may not have had time to form. In the present work, the activity of the metal complexing analogues was measured as the quantity of hydroxyl radicals produced over a period of time. The assay of hydroxyl radicals was obtained through their reaction with the benzoate to produce hydroxybenzoates, which was quantitatively estimated by fluorescence spectroscopy of the 2-hydroxybenzoate (salicylate), the only isomer showing fluorescence.[34,35] This hydroxylation assay was calibrated using ^{60}Co irradiation of an aqueous benzoate solution saturated with nitrous oxide (N_2O), when the formation of salicylate is proportional to the radiation dose.[34]

Hydroxylation of benzoate occurred in the presence of iron(II) and hydrogen peroxide but increased on the addition of EDTA and to a larger extent on the addition of **23**. Hydroxylation of benzoate in a solution of iron(III) and hydrogen peroxide was again increased in the presence of EDTA or **23** but this time the former was the more effective. The analogues **25a** and **25b** and bleomycin gave curves similar to that obtained with Fe(II) and hydrogen peroxide alone. The presence of bleomycin or **28a** did not markedly affect the hydroxylation of benzoate by Fe(III) and hydrogen peroxide. Little or no hydroxylation occurred in solutions of either Fe(II) or Fe(III) and hydrogen peroxide in the presence of the other analogues **10**, **24a**, **24b**, **25b**, **26a**, **27a**, **27b**, **28b** and **29**. No hydroxylation was produced by the Fe(III)–chelator systems in the absence of hydrogen peroxide.

The concentration of hydroxyl radicals produced with time from an aqueous solution of a bleomycin analogue, Fe(II) and nitrogen or oxygen or air was measured by fluorescence spectroscopy through the hydroxylation of benzoate. As expected,

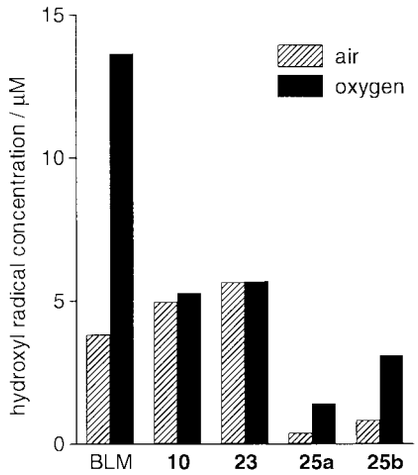


Fig. 3 Production of hydroxyl radicals assayed by benzoate hydroxylation (salicylate formation). BLM = bleomycin; other compounds, see text. Concentrations were measured 5 min after addition of Fe(II).

no hydroxyl radicals were detected in the presence of nitrogen. However, in the presence of oxygen, the BLM analogue–iron–oxygen complex may form and yield hydroxyl radicals. The analogues **10** and **23** gave yields of hydroxyl radicals greater than those obtained with bleomycin in air (Fig. 3) but BLM gave more hydroxyl radicals in oxygen than **10** and **23** though these were the most efficient of the analogues. Typical concentration–time profiles for hydroxyl radical production for some compounds have been reported.[26] In general, the bleomycin analogues carrying a 4-methoxy substituent on the pyridine ring were more active than the corresponding compounds without the substituent. As expected, the nitro compounds **24a** and **24b** gave little or no radicals but the corresponding amines **25a** and **25b** were slightly more effective. The nitro compounds were not expected to complex with the iron but it was hoped that the corresponding amines would do so and, after activation of oxygen, produce hydroxyl radicals. The results obtained were qualitatively as expected but the differential ability of the nitro and amino compounds to activate oxygen was not as great as had been anticipated. However, the *N*-oxide **29** produced few hydroxyl radicals whereas its deoxygenated product **10** was an active agent for the production of hydroxyl radicals. Increasing the iron concentration caused the concentration of hydroxyl radicals to increase up to an apparent maximum as measured by fluorescence. Further increases in iron concentration caused a decrease in the observed fluorescence which was thought to be due to hydroxylation of the fluorescent salicylate to give non-fluorescent products.

The binding of the bleomycin analogues with DNA was studied by measurement of the loss of the fluorescence of the ethidium bromide–DNA complex as a function of increasing BLM analogue concentration.[36] At pH 7.5, the BLM analogues containing an amino group had binding constants in the range 1.1×10^4 to 4.0×10^4 M^{-1} (Table 1) compared with bleomycin-A_2 which had a binding constant of 1.2×10^5 M^{-1}. The higher value for bleomycin is expected since this molecule contains the bithiazole unit whose function is to bind to DNA. Thus far, the bleomycin analogues prepared in this work do not have a DNA-binding unit.

The cytotoxicity of some of the bleomycin analogues was assessed after exposure of mammalian cells (V79 hamster fibroplast-like) to a solution of the BLM analogue in an atmosphere of either air for four hours or nitrogen (2 h) followed by air (2 h). A range of drug concentrations was used. The nitro prodrugs **24a** and **24b** and the *N*-oxide prodrug **29** were not expected to show cytotoxicty in air resulting from bleomycin-like action involving attack on DNA because metal complexation would not occur. In fact, the prodrugs showed little

Table 1 Binding constants of bleomycin analogues to DNA

BLM analogue	DNA binding constant at pH 7.5/10^4 M^{-1}	DNA binding constant at pH 6.0/10^4 M^{-1}
BLM-A_2 **1**	12.0[a]	14.0
AMPHIS **10**	1.2	2.3
23	1.2	2.0
24a	4.0	7.5
24b	1.9	7.5
26a	1.7	2.5
26b	1.5	3.5
27a	1.1	1.4
27b	1.1	1.5
28a	2.8	6.0
28b	2.0	4.2
29	1.4	1.8

[a] At pH 8.4 the binding constant is 1.2×10^5 M^{-1}.

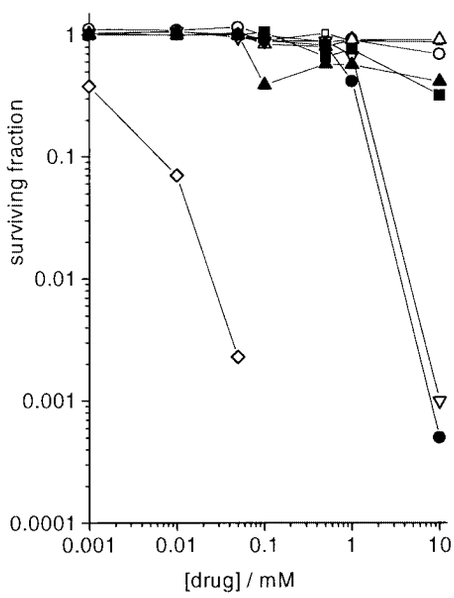


Fig. 4 Cytotoxicity to Chinese hamster V79 cells. Open symbols refer to exposure in air (4 h) and solid symbols refer to incubation in hypoxia (2 h) followed by exposure in air (2 h); bleomycin (◇), **10** (▽), **24a** (△), **24b** (□) and **29** (○).

toxicity up to 10 mM levels whereas **10**, the deoxygenated product from **29**, showed marked toxicity at about 1 mM concentration (Fig. 4). It was expected that the prodrugs **24a**, **24b** and **29** on exposure to cells under hypoxic conditions would be bioreductively converted to active forms which would complex with Fe(II) and, on subsequent exposure to oxygen, would form the cytotoxic "active complex". Disappointingly, after exposure of cells to the nitro prodrugs **24a** and **24b** under nitrogen and subsequent exposure to air, there was only a non-significant increase in the cytotoxicity compared with that already observed when cells plus **24a** and **24b** were exposed to air alone. However, importantly, the killing of cells exposed to the *N*-oxide **29** under a nitrogen and subsequently under an air atmosphere was marked and almost identical to the cell killing found for the deoxygenated compound **10** in air. It seems likely that under the conditions of the cytotoxicity test the *N*-oxide group was reduced by the hypoxic cells. Other monofunctional aromatic *N*-oxides are selectively toxic to hypoxic cells.[37]

Experimental

Melting points were determined on an electrothermal digital apparatus and are uncorrected. ^{1}H NMR spectra were obtained on a JEOL FX200 (200 MHz) or a Bruker AM360 (360 MHz) spectrometer using TMS as internal standard. *J* values are

given in hertz (Hz) for solutions in DMSO-d_6. Accurate mass measurements were carried out at the EPSRC National Mass Spectrometry Service Centre, University of Wales, Swansea.

Thin layer chromatography was performed on silica gel plates (0.25 mm with fluorescent indicator UV_{254}) obtained from Camlab. Column chromatography was carried out using silica gel MPD 60 Å (40–60 μm) and developed under slight positive pressure. Petroleum ether refers to light petroleum (bp 40–60 °C) and ether refers to diethyl ether. All solvents were redistilled before use.

Histidine methyl ester hydrochloride, histamine dihydrochloride, 2-(2-aminoethyl)pyridine, *tert*-butyl *N*-(3-aminopropyl)carbamate, α-phenyl-*N*-*tert*-butylnitrone and 5,5-dimethyl-1-pyrroline *N*-oxide were obtained from Aldrich Chemical Company. Eagle's minimum essential medium, Earle's salt and Hank's saline were obtained from Sigma.

Fluorescence spectra were measured using a Perkin Elmer LS50B luminescence spectrometer. Electron paramagnetic resonance (EPR) spectra were obtained on a Bruker EMX6/1 X-band spectrometer equipped with a TE_{102} resonator. The microwave power was 20 mW and the magnetic field was modulated at 100 KHz and 0.6 G amplitude. A sweep rate of 1.3 G s^{-1}, a time constant of 40.96 ms and the receiver gain at 1×10^5 were used and 5 scans were averaged.

Methyl 6-(*N*-{2-[(*tert*-butoxycarbonyl)amino]ethyl}-*N*-(*tert*-butoxycarbonyl)aminomethyl)-4-methoxypyridine-2-carboxylate 12b

A solution of methyl 6-formyl-4-methoxypyridine-2-carboxylate[19] **11b** (1.66 g, 0.0085 M) and *tert*-butyl *N*-(2-aminoethyl)-carbamate[27] (1.36 g, 0.0085 M) in methanol (75 cm^3) was stirred at room temperature for 30 min and then hydrogenated under atmospheric pressure in the presence of 10% palladium on carbon (2 g) until the uptake of hydrogen had ceased. The catalyst was filtered off and the filtrate concentrated under reduced pressure. To the solution of the residue in dichloromethane (100 cm^3) containing 4-(dimethylamino)pyridine (50 mg) was added dropwise di-*tert*-butyl dicarbonate (3.75 g, 0.017 M) in dichloromethane (15 cm^3). The resulting solution was stirred at room temperature for 1 h and evaporated under diminished pressure. The residue was purified by column chromatography (ethyl acetate–petroleum ether, 1:1) to give the *title compound* **12b** (2.68 g, 72%) as a yellow oil (Found: M^+ (EI), 439.231. $C_{21}H_{33}N_3O_7$ requires M^+, 439.231); δ_H (200 MHz) 1.36 (18H, s, 2 × $C(CH_3)_3$), 3.11 (2 H, m, CH_2), 3.19 (2 H, m, CH_2), 3.86 (3 H, s, OCH_3), 3.88 (3 H, s, CH_3), 4.43 (2 H, s, CH_2), 6.72 (1 H, br s, NH exchanged with D_2O), 6.93 (1 H, d, *J* 2, 5-H), 7.44 (1 H, d, *J* 2, 3-H); *m*/*z* (EI) 440 ($M^+ + 1$, 3%), 439 (M^+, 12.8), 366 (16), 322 (18), 181 (12), 85 (65), 83 (100).

Methyl 6-(*N*-{2-[(*tert*-butoxycarbonyl)amino]ethyl}-*N*-(*tert*-butoxycarbonyl)aminomethyl)pyridine-2-carboxylate 1-oxide 20

The ester **12a**[27] (0.3 g, 0.73 mM) was stirred in dichloromethane at room temperature with a large excess of *m*-chloroperbenzoic acid (7 mM) for 7 days. Dichloromethane was removed *in vacuo* and the residue was purified by column chromatography (ethyl acetate–petroleum ether, 1:9) to afford the *title compound* **20** as a colourless viscous oil (0.17 g, 55%) (Found: $M^+ - OH$ (EI), 408.2135. $C_{20}H_{30}N_3O_6$ requires $M^+ - OH$, 408.2133); ν_{max}/cm^{-1} 3376 (NH), 1692 (CO), 1246 (N^+–O^-); δ_H (360 MHz) 1.36 (18 H, s, 2 × $C(CH_3)_3$), 3.09 (2 H, m, CH_2), 3.32 (2 H, m, CH_2), 3.88 (3 H, s, CH_3), 4.49 (2 H, s, CH_2), 6.89 (1 H, br s, NH exchanged with D_2O), 7.55 (1 H, t, *J* 7, 4-H), 7.71 (1 H, d, *J* 8, 5-H), 7.90 (1 H, dd, *J* 2, 7, 3-H); *m*/*z* (EI) 409 ($M^+ - O$, 26%), 408 ($M^+ - OH$, 100).

General procedure for the hydrolysis of esters 12b and 20

A solution containing the appropriate ester (0.006 M) in 0.4 M sodium hydroxide (30 cm^3) and methanol (10 cm^3) was stirred at room temperature for 3 (for **13b**) or 24 h (for **20**). The methanol was evaporated *in vacuo* and the solution diluted with water (10 cm^3) and extracted with ether. The organic layer was back extracted with saturated aqueous sodium bicarbonate solution. The combined aqueous layers were acidified with aqueous citric acid and extracted with dichloromethane. The organic layer was dried (Na_2SO_4), concentrated *in vacuo* and triturated with petroleum ether to give the crude acid which was crystallised.

2-(*N*-{2-[(*tert*-Butoxycarbonyl)amino]ethyl}-*N*-(*tert*-butoxycarbonyl)aminomethyl)-4-methoxypyridine-6-carboxylic acid 13b. Yield: 95%, mp 93–94 °C (chloroform–petroleum ether) (Found: C, 55.95; H, 7.38; N, 9.56. $C_{20}H_{31}N_3O_7$ requires C, 56.56; H, 7.34; N, 9.86; Found: M^+ (EI), 425.216. $C_{20}H_{31}N_3O_7$ requires M^+, 425.216); ν_{max}/cm^{-1} 3276 (OH), 1692 (CO); δ_H (200 MHz) 1.35 (18 H, s, 2 × $C(CH_3)_3$), 3.09 (2 H, m, CH_2), 3.24 (2 H, m, CH_2), 3.87 (3 H, s, OCH_3), 4.43 (2 H, s, CH_2), 6.73 (1 H, br s, NH exchanged with D_2O), 6.89 (1 H, d, *J* 2, 5-H), 7.43 (1 H, d, *J* 3, 3-H); *m*/*z* (EI) 425 (M^+, 3.5%), 325 ($M^+ - CO_2C(CH_3)_3$, 32), 308 (100).

2-(*N*-{2-[(*tert*-Butoxycarbonyl)amino]ethyl}-*N*-(*tert*-butoxycarbonyl)aminomethyl)pyridine-6-carboxylic acid 1-oxide 20. Yield: 80%, mp 165–166 °C (dichloromethane–petroleum ether) (Found: MH^+ (FAB, 3-nitrobenzyl alcohol (NOBA)), 412.2107. $C_{19}H_{30}N_3O_7$ requires MH^+, 412.2082); ν_{max}/cm^{-1} 3380 (OH), 1690 (CO), 1252 (N^+–O^-); δ_H (200 MHz) 1.37 (18 H, m, 2 × $C(CH_3)_3$), 3.15 (2 H, m, CH_2), 3.36 (2 H, m, CH_2), 4.64 (2 H, s, CH_2), 6.70 (1 H, br s, NH exchanged with D_2O), 7.62 (1 H, d, *J* 7, 5-H), 7.88 (1 H, t, *J* 8, 4-H), 8.25 (1 H, dd, *J* 2, 7, 3-H); *m*/*z* (EI) 394 ($M^+ - OH$, 4%), 350 (11), 294 (10), 194 (21), 44 (100).

General procedure to give 14a and 14b from 13a and 13b using diphenylphosphoryl azide

Triethylamine (0.5 cm^3), diphenylphosphoryl azide[28] (0.5 cm^3) and further triethylamine (0.5 cm^3) were successively added to a suspension of the appropriate acid **13a**[27] or **13b** (1.2 mM) and histidine methyl ester hydrochloride (1.3 mM) in dry DMF (20 cm^3) at 0 °C under nitrogen with vigorous stirring. The reaction mixture was stirred at 0 °C for 4 h and then at room temperature for 4 days. The solvent was evaporated *in vacuo* and the residue purified by column chromatography (dichloromethane–methanol, 19:1) to give a colourless oil which on trituration with ether gave the amide as a hygroscopic solid.

2-(*N*-(*tert*-Butoxycarbonyl)-*N*-{2-[(*tert*-butoxycarbonyl)-amino]ethyl}aminomethyl)-*N*-[2-(imidazol-4-yl)-1-(methoxycarbonyl)ethyl]pyridine-6-carboxamide 14a. Yield: 70% (Found: M^+ (EI), 546.280. $C_{26}H_{38}N_6O_7$ requires M^+, 546.278); δ_H (200 MHz) 1.36 (18 H, s, 2 × $C(CH_3)_3$), 3.11 (4 H, m, 2 × CH_2), 3.38 (2 H, m, CH_2), 3.62 (3 H, s, CH_3), 4.52 (2 H, s, CH_2), 4.76 (1 H, dt, *J* 8, 6, collapses to t with D_2O, *J* 6, CH), 6.68 (1 H, br s, NH exchanged with D_2O), 6.85 (1 H, s, 5-H of imidazole), 7.41 (1 H, dd, *J* 2, 8, 3-H), 7.54 (1 H, s, 2-H of imidazole), 7.88 (1 H, dd, *J* 2, 8, 5-H), 7.96 (1 H, t, *J* 8, 4-H), 8.99 (1 H, m, NH exchanged with D_2O), 11.76 (1 H, br s, NH exchanged with D_2O); *m*/*z* (EI) 547 ($M^+ + 1$, 33%), 546 (M^+, 91), 446 (68), 417 (100).

2-(*N*-(*tert*-Butoxycarbonyl)-*N*-{2-[(*tert*-butoxycarbonyl)-amino]ethyl}aminomethyl)-4-methoxy-*N*-[2-(imidazol-4-yl)-1-(methoxycarbonyl)ethyl]pyridine-6-carboxamide 14b. Yield: 83% (Found: M^+ (EI), 576.291. $C_{27}H_{40}N_6O_8$ requires M^+, 576.2905); δ_H (200 MHz) 1.36 (18 H, s, 2 × $C(CH_3)_3$), 3.1 (4 H, m, 2 × CH_2), 3.36 (2 H, m, CH_2), 3.62 (2 H, s, CH_3), 3.88 (3 H, s, OCH_3), 4.45 (2 H, s, CH_2), 4.74 (1 H, dt, *J* 8, 6, collapses to t with D_2O, *J* 6, CH), 6.67 (1 H, br s, NH exchanged with D_2O), 6.84 (1 H, s, 5-H of imidazole), 6.89 (1 H, d, *J* 2, 3-H), 7.41

Table 2 Yields and spectroscopic data for **15a**–**19b** and **22**

Compound (Formula)	Yield (%)	Amine	Acc. mass Found (Required)	δ_H (200 MHz)
15a ($C_{26}H_{37}N_5O_5$)	60[a]	2-(2-Amino-ethyl)pyridine	499.2795 (499.2792)	1.37 (18 H, s, 2 × $C(CH_3)_3$), 2.94–3.15 (4 H, m, 2 × CH_2), 3.31 (2 H, m, CH_2), 3.71 (2 H, m, CH_2), 4.49 (2 H, s, CH_2), 6.65 (1 H, br s, NH exchanged with D_2O), 7.18–7.29 (2 H, m, 3′- and 5′-H), 7.37 (1 H, dd, *J* 2, 7, 3-H), 7.69 (1 H, m, 4′-H), 7.87–7.98 (2 H, m, 4- and 5-H), 8.53 (1 H, dd, *J* 2, 7, 6′-H), 8.60 (1 H, br s, NH exchanged with D_2O)
15b ($C_{27}H_{39}N_5O_6$)	75[a]	2-(2-Amino-ethyl)pyridine	529.290 (529.289)	1.37 (18 H, s, 2 × $C(CH_3)_3$), 2.99–3.13 (4 H, m, 2 × CH_2), 3.30 (2 H, m, CH_2), 3.70 (2 H, m, CH_2), 3.88 (3 H, s, OCH_3), 4.44 (2 H, s, CH_2), 6.66 (1 H, br s, NH exchanged with D_2O), 6.87 (1 H, d, *J* 3, 3-H), 7.18–7.29 (2 H, m, 3′- and 5′-H), 7.44 (1 H, d, *J* 2, 5-H), 7.70 (1 H, m, 4′-H), 8.54 (1 H, d, *J* 6, 6′-H), 8.58 (1 H, br s, NH exchanged with D_2O)
16a ($C_{27}H_{45}N_5O_7$)	42[b]	*tert*-Butyl *N*-(3-aminopropyl)-carbamate	551.332 (551.332)	1.37 (27 H, s, 3 × $C(CH_3)_3$), 2.85–2.99 (4 H, m, 2 × CH_2), 3.10 (2 H, m, CH_2), 3.27–3.40 (4 H, m, 2 × CH_2), 4.51 (2 H, s, CH_2), 6.65 (1 H, br s, NH exchanged with D_2O), 7.37 (1 H, dd, *J* 2, 7, 3-H) 7.86–7.99 (2 H, m, 4- and 5-H), 8.45 (1 H, br s, NH exchanged with D_2O), 8.57 (1 H, br s, NH exchanged with D_2O)
16b ($C_{28}H_{47}N_5O_8$)	80[b]	*tert*-Butyl *N*-(3-aminopropyl)-carbamate	581.342 (581.342)	1.37 (27 H, s, 3 × $C(CH_3)_3$), 2.91 (4 H, m, 2 × CH_2), 3.12 (2 H, m, CH_2), 3.29–3.38 (4 H, m, 2 × CH_2), 3.87 (3 H, s, OCH_3), 4.44 (2 H, s, CH_2), 6.65 (1 H, br s, NH exchanged with D_2O), 6.85 (1 H, d, *J* 2, 3-H), 7.44 (1 H, d, *J* 2, 5-H), 8.41 (1 H, br s, NH exchanged with D_2O), 8.54 (1 H, br s, exchanged with D_2O)
17a ($C_{24}H_{36}N_7O_7$)	61[c]	4(5)-Nitro-histamine hydrochloride	534.2661[d] (534.2674)	1.37 (18 H, s, 2 × $C(CH_3)_3$), 3.08–3.40 (6 H, m, 3 × CH_2), 3.67 (2 H, m, CH_2), 4.49 (2 H, s, CH_2), 6.65 (1 H, br s, NH exchanged with D_2O), 7.39 (1 H, dd, *J* 2, 5, 3-H), 7.64 (1 H, s, 2-H of imidazole), 7.86 (1 H, dd, *J* 2, 7, 5-H), 7.95 (1 H, t, *J* 8, 4-H), 8.55 (1 H, br s, NH exchanged with D_2O), 12.96 (1 H, br s, NH exchanged with D_2O)
17b ($C_{25}H_{38}N_7O_8$)	60[c]	4(5)-Nitro-histamine hydrochloride	565.2897[e] (565.2860)	1.35 (18 H, s, 2 × $C(CH_3)_3$), 3.11 (2 H, m, CH_2), 3.21–3.33 (4 H, m, 2 × CH_2), 3.66 (2 H, m, CH_2), 3.87 (3 H, s, OCH_3), 4.43 (2 H, s, CH_2), 6.64 (1 H, br s, NH exchanged with D_2O), 6.84 (1 H, d, *J* 2, 3-H), 7.40 (1 H, d, *J* 2, 5-H), 7.65 (1 H, s, 2-H of imidazole), 8.53 (1 H, br s, NH exchanged with D_2O)
19a ($C_{24}H_{36}N_6O_5$)	60[f]	Histamine dihydro-chloride	488.2747 (488.2745)	1.36 (18 H, s, 2 × $C(CH_3)_3$), 2.76 (2 H, m, CH_2), 3.11 (2 H, m, CH_2), 3.33 (2 H, m, CH_2), 3.56 (2 H, m, CH_2), 4.49 (2 H, s, CH_2), 6.67 (1 H, br s, NH exchanged with D_2O), 6.81 (1 H, s, 5-H of imidazole), 7.37 (1 H, dd, *J* 2, 7, 3-H), 7.51 (1 H, s, 2-H of imidazole), 7.88–7.99 (2 H, m, 4- and 5-H), 8.57 (1 H, br s, NH exchanged with D_2O), 11.74 (1 H, br s, NH exchanged with D_2O)
19b ($C_{25}H_{38}N_6O_6$)	69[f]	Histamine dihydro-chloride	518.285 (518.286)	1.36 (18 H, s, 2 × $C(CH_3)_3$), 2.76 (2 H, m, CH_2), 3.10 (2 H, m, CH_2), 3.32 (2 H, m, CH_2), 3.55 (2 H, m, CH_2), 3.88 (3 H, s, OCH_3), 4.43 (2 H, s, CH_2), 6.66 (1 H, br s, NH exchanged with D_2O), 6.81 (1 H, s, 5-H of imidazole), 6.86 (1 H, d, *J* 3, 3-H), 7.44 (1 H, d, *J* 2, 5-H), 7.51 (1 H, s, 2-H of imidazole), 8.53 (1 H, br s, NH exchanged with D_2O), 11.71 (1 H, br s, NH exchanged with D_2O)
22 ($C_{26}H_{38}N_6O_8$)	50[g]	Histidine methyl ester hydrochloride	563.2839[d] (563.2827)	1.36 (18 H, s, 2 × $C(CH_3)_3$), 3.11–3.16 (4 H, m, 2 × CH_2), 3.31–3.40 (2 H, m, CH_2), 3.65 (3 H, s, CH_3), 4.52 (2 H, s, CH_2), 6.68 (1 H, br s, NH exchanged with D_2O), 6.86 (1 H, s, 5-H of imidazole), 7.37 (1 H, d, *J* 8, 3-H), 7.49 (1 H, s, 2-H of imidazole), 7.58 (1 H, t, *J* 8, 4-H), 8.15 (1 H, dd, *J* 2, 8, 5-H), 9.00 (1 H, br s, NH exchanged with D_2O), 11.76 (1 H, br s, NH exchanged with D_2O)

[a] Eluant: petroleum ether–ethyl acetate, 1:9. [b] Eluant: petroleum ether–ethyl acetate, 1:1. [c] Crystallised from methanol and ether. [d] $(M + H)^+$ ion. [e] $(M + 2H)^+$ ion. [f] Eluant: ethyl acetate–methanol, 19:1. [g] Dichloromethane–methanol, 19:1.

(1 H, d, *J* 2, 5-H), 7.54 (1 H, s, 2-H of imidazole), 8.92 (1 H, m, NH exchanged with D_2O), 11.77 (1 H, br s, NH exchanged with D_2O); *m*/*z* (EI) 576 (M^+, 17.6%), 530 (8), 360 (12), 318 (15), 149 (17), 41 (100).

General procedure for the preparation of 15a–19b and 22 from 13a, 13b and 20 using 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride and 1-hydroxybenzotriazole

A mixture of the appropriate acid (0.00127 M), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride[27] (0.0016 M) and 1-hydroxybenzotriazole (0.0016 M) in anhydrous DMF (12 cm^3) was stirred under nitrogen at room temperature for 30 min. To this solution was added dropwise triethylamine (2 cm^3) and subsequently the appropriate amine or amine hydrochloride (0.00133 M). The solution was stirred overnight, solvent removed *in vacuo* and the residue dissolved in ethyl acetate. The solution was washed with saturated aqueous sodium bicarbonate, dried (Na_2SO_4) and the solvent evaporated. The residue was purified by column chromatography or crystallisation. The physical data are reported in Table 2.

General procedure for the reduction of nitro compounds 17a and 17b

A solution of the appropriate nitro compound (0.1 mmol) in methanol (10 cm^3) was hydrogenated at room temperature in the presence of 10% palladium on carbon (0.1 g) for 48 h. The catalyst was filtered off and the solvent evaporated *in vacuo* to yield a yellow viscous oil.

2-(*N*-(*tert*-Butoxycarbonyl)-*N*-{2-[(*tert*-butoxycarbonyl)-amino]ethyl}aminomethyl)-*N*-[2-(5-aminoimidazol-4-yl)ethyl]-pyridine-6-carboxamide 18a. Yield: 95% (Found: MH^+ (FAB, NOBA), 504.2884. $C_{24}H_{39}N_7O_5$ requires MH^+, 504.2932); ν_{max}/cm^{-1} 3384 (NH), 1688 (CO); δ_H (200 MHz) 1.36 (18 H, s, 2 × $C(CH_3)_3$), 2.95 (2 H, m, CH_2), 3.10 (4 H, m, 2 × CH_2), 3.32 (2 H, m, CH_2), 3.80 (2 H, br s, NH_2 exchanged with D_2O), 4.50 (2 H, s, CH_2), 6.64 (1 H, br s, NH exchanged with D_2O), 7.39 (1 H, dd, *J* 2, 7, 3-H), 7.57 (1 H, s, 2-H of imidazole), 7.95 (2 H, m, 4- and 5-H), 8.48 (1 H, br s, NH exchanged with D_2O); *m*/*z* (FAB) 504 (MH^+, 34%), 395 (63), 295 (28), 195 (100).

2-(*N*-(*tert*-Butoxycarbonyl)-*N*-{2-[(*tert*-butoxycarbonyl)-amino]ethyl}aminomethyl)-4-methoxy-*N*-[2-(5-aminoimidazol-4-yl)ethyl]pyridine-6-carboxamide 18b. Yield: 90% (Found: MH^+ (FAB, NOBA), 534.3042. $C_{25}H_{40}N_7O_6$ requires MH^+, 534.3038); ν_{max}/cm^{-1} 3375 (NH), 1684 (CO); δ_H (360 MHz) 1.35 (18 H, s, 2 × $C(CH_3)_3$), 3.09 (2 H, m, CH_2), 3.35 (8 H, m, NH_2 and 3 × CH_2), 3.88 (3 H, s, OCH_3), 4.43 (2 H, s, CH_2), 6.83 (2 H, m, 3- and 5-H), 7.42 (1 H, s, 2-H of

Table 3 Yields and spectroscopic data for **10**, **23**, **24a**–**28b** and **29**

Compound (Formula)	Yield (%)	Acc. mass [a,b] Found (Required)	δ_H
10 ($C_{16}H_{22}N_6O_6$)	96	347.1829 (347.1830)	[c] 2.84–2.93 (4 H, m, 2 × CH_2), 3.12 (2 H, m, CH_2), 3.63 (3 H, s, CH_3), 3.94 (2 H, s, CH_2), 4.74 (1 H, m, CH), 6.16 (2 H, br s, NH_2 exchanged with D_2O), 6.88 (1 H, s, 5-H of imidazole), 7.58 (1 H, s, 2-H of imidazole), 7.63 (1 H, d, *J* 7, 3-H), 7.85–7.98 (2 H, m, 4- and 5-H), 9.21 (1 H, br s, NH exchanged with D_2O)
23 ($C_{17}H_{24}N_6O_4$)	90	377.1947 (377.1935)	[d] 2.96 (2 H, m, CH_2), 3.03 (2 H, m, CH_2), 3.17 (2 H, m, CH_2), 3.64 (3 H, s, CH_3), 3.90 (3 H, s, OCH_3), 4.12 (2 H, s, CH_2), 4.77 (1 H, m, CH), 7.00 (1 H, s, 5-H of imidazole), 7.24 (1 H, d, *J* 2, 3-H), 7.43 (1 H, d, *J* 2, 5-H), 7.87 (1 H, s, 2-H of imidazole), 9.32 (1 H, br s, NH exchanged with D_2O)
24a ($C_{14}H_{19}N_7O_3$)	97	334.1628 (334.1626)	[c] 3.01 (2 H, m, CH_2), 3.09 (2 H, m, CH_2), 3.28 (2 H, m, CH_2), 3.67 (2 H, m, CH_2), 4.21 (2 H, s, CH_2), 7.62 (1 H, d, *J* 7, 3-H), 7.70 (1 H, s, 2-H of imidazole), 7.93 (1 H, d, *J* 7, 5-H), 8.01 (1 H, t, *J* 7, 4-H), 9.15 (1 H, br s, NH exchanged with D_2O)
24b ($C_{15}H_{21}N_7O_4$)	91	364.177 (364.173)	[d] 2.71 (2 H, m, CH_2), 2.89 (2 H, m, CH_2), 3.26 (2 H, m, CH_2), 3.64 (2 H, m, CH_2), 3.82 (2 H, s, CH_2), 3.88 (3 H, s, OCH_3), 7.17 (1 H, s, 3-H), 7.37 (1 H, d, *J* 2, 5-H), 7.68 (1 H, s, 2-H of imidazole), 8.94 (1 H, br s, NH exchanged with D_2O)
25a ($C_{14}H_{21}N_7O$)	95	304.1890 (304.1884)	[d] 2.76 (2 H, m, CH_2), 2.90 (2 H, m, CH_2), 3.14 (2 H, m, CH_2), 3.25 (2 H, m, CH_2), 3.89 (2 H, s, CH_2), 4.52 (2 H, br s, NH_2 exchanged with D_2O), 7.58 (1 H, d, *J* 7, 3-H), 7.66 (1 H, br s, NH exchanged with D_2O), 7.88–7.95 (2 H, m, 4- and 5-H), 8.29 (1 H, s, 2-H of imidazole), 8.33 (1 H, br s, NH exchanged with D_2O)
25b ($C_{15}H_{23}N_7O_2$)	95	334.1992 (334.1990)	[c] 2.75 (2 H, m, CH_2), 2.88 (2 H, m, CH_2), 3.38 (2 H, m, CH_2), 3.55 (2 H, m, CH_2), 3.81 (2 H, m, CH_2), 3.88 (3 H, s, OCH_3), 7.16 (1 H, s, 3-H), 7.38 (1 H, s, 5-H), 8.33 (1 H, s, 2-H of imidazole), 9.00 (1 H, br s, NH exchanged with D_2O)
26a ($C_{14}H_{20}N_6O$)	96	311.1595 [e] (311.1595)	[c] 2.85–2.93 (2 H, m, CH_2), 2.97–3.07 (4 H, m, 2 × CH_2), 3.60 (2 H, m, CH_2), 4.15 (2 H, s, CH_2), 5.75 (2 H, br s, NH_2 exchanged with D_2O), 7.12 (1 H, s, 5-H of imidazole), 7.62 (1 H, dd, *J* 2, 7, 3-H), 7.91–8.02 (2 H, m, 4- and 5-H), 8.20 (1 H, s, 2-H of imidazole), 9.03 (1 H, br s, NH exchanged with D_2O)
26b ($C_{15}H_{22}N_6O_2$)	90	319.1891 (319.1881)	[d] 2.78 (4 H, m, 2 × CH_2), 2.91 (2 H, m, CH_2), 3.54 (2 H, m, CH_2), 3.84 (2 H, m, CH_2), 3.89 (3 H, s, OCH_3), 6.85 (1 H, s, 5-H of imidazole), 7.17 (1 H, d, *J* 2, 3-H), 7.41 (1 H, d, *J* 2, 5-H), 7.56 (1 H, br s, NH exchanged with D_2O)
27a ($C_{16}H_{21}N_5O$)	97	300.1824 (300.1823)	[d] 2.80 (2 H, m, CH_2), 2.94 (2 H, m, CH_2), 3.04 (2 H, m, CH_2), 3.68 (2 H, m, CH_2), 3.94 (2 H, s, CH_2), 7.22–7.31 (2 H, s, 3′- and 5′-H), 7.61 (1 H, d, *J* 7, 3-H), 7.72 (1 H, m, 4′-H), 7.89–8.00 (2 H, m, 4- and 5-H), 8.52 (1 H, d, *J* 5, 6′-H), 9.09 (1 H, br s, NH exchanged with D_2O)
27b ($C_{17}H_{23}N_5O_2$)	90	330.1930 [f] (330.1928)	[d] 2.76 (2 H, m, CH_2), 2.90 (2 H, m, CH_2), 3.03 (2 H, m, CH_2), 3.67 (2 H, m, CH_2), 3.84 (2 H, s, CH_2), 3.89 (3 H, s, OCH_3), 7.19 (1 H, s, 3-H), 7.27 (2 H, m, 3′- and 5′-H), 7.41 (1 H, d, *J* 1, 5-H), 7.72 (1 H, m, 4′-H), 8.51 (1 H, d, *J* 4, 6′-H), 9.04 (1 H, br s, NH exchanged with D_2O)
28a ($C_{12}H_{21}N_5O$)	97	252.1824 (252.1823)	[c] 2.90–2.98 (8 H, m, 4 × CH_2), 3.50 (2 H, m, CH_2), 4.00 (2 H, s, CH_2), 6.18 (2 H, br s, NH_2 exchanged with D_2O), 7.69 (1 H, d, *J* 6, 3-H), 7.99–8.06 (2 H, m, 4- and 5-H), 9.09 (1 H, br s, NH exchanged with D_2O)
28b ($C_{13}H_{23}N_5O_2$)	92	282.1930 (282.1928)	[d] 2.66 (2 H, m, CH_2), 2.77 (2 H, m, CH_2), 2.84 (2 H, m, CH_2), 3.37 (4 H, m, 2 × CH_2), 3.81 (2 H, s, CH_2), 3.89 (3 H, s, OCH_3), 7.17 (1 H, s, 3-H), 7.40 (1 H, s, 5-H), 9.04 (1 H, br s, NH exchanged with D_2O)
29 ($C_{16}H_{22}N_6O_4$)	97	363.18 (363.19)	[d] 2.81 (2 H, m, CH_2), 2.93 (2 H, m, CH_2), 3.11 (2 H, m, CH_2), 3.66 (3 H, s, CH_3), 3.98 (2 H, s, CH_2), 4.86 (1 H, m, CH), 6.98 (1 H, s, 5-H of imidazole), 7.62 (1 H, t, *J* 8, 4-H), 7.83 (1 H, s, 2-H of imidazole), 7.86 (1 H, d, *J* 7, 3-H), 8.16 (1 H, dd, *J* 4, 8, 5-H), 11.64 (1 H, br s, NH exchanged with D_2O)

[a] Ionisation by FAB unless stated otherwise. [b] *m*/*z* for $M^+ + H$ unless stated otherwise. [c] 200 MHz. [d] 360 MHz. [e] $(M + Na)^+$ ion. [f] Ionisation by EI.

imidazole), 8.70 (1 H, br s, NH exchanged D_2O); *m*/*z* (FAB) 534 (MH^+, 100%).

General procedure for the removal of the protecting *tert*-butoxycarbonyl groups to provide 10, 23–28b and 29

To the appropriate protected compound (0.1 g), trifluoroacetic acid (5 cm^3) was added and the mixture stirred for 1 h at 0 °C. Trifluoroacetic acid was then removed *in vacuo* and the residue dissolved in water. The aqueous solution was charged on a column of Amberlite IRA 93 (as free base, 7 cm height of the ion exchange resin in the column). The column was eluted with water. The first five fractions were combined and freeze-dried to give the *target* compounds as hygroscopic colourless foams. The physical data are reported in Table 3.

Spin-trapping experiments

Two spin traps, α-phenyl-*N*-*tert*-butylnitrone (PBN) and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) were used to detect the production of hydroxyl radicals. AMPHIS **10**–Fe(II) complex was prepared by the addition of stoichiometric amounts of ammonium iron(II) sulfate hexahydrate to AMPHIS. The complex is air sensitive and is readily oxidised to Fe(III) complexes with the production of radicals.

The reaction mixture for spin-trapping experiments consisted of 1 : 1 AMPHIS–Fe(II) complex (10 mM in aqueous solution) and PBN or DMPO (80 mM ethanolic solution) in buffered solution at pH 6.9. The spectrum was obtained for solutions separately under atmospheres of air, nitrogen and oxygen. Control experiments were made to ensure that the EPR spectra neither resulted from spin-trap alone, nor from the separate addition of Fe(II) or AMPHIS **10**.

The effect of AMPHIS **10** compared to EDTA on the Fe(II)–H_2O_2 system was also studied. Solutions of DMPO (10 mM) in phosphate buffer (10 mM) at pH 6.9 containing 1 M of ethanol were treated with ammonium ferrous sulfate (250 μM), with or without 1 mM H_2O_2, and with or without chelator (275 μM). EPR spectra were recorded after the solution had been allowed to stand for 5 min.

Benzoate hydroxylation assay

The formation of hydroxyl radicals was also detected by treatment of aqueous sodium benzoate (5 mM) with the system being tested and detection of the 2-hydroxybenzoate produced with time by fluorescence spectroscopy at λ_{exc} 295 nm and λ_{em} 406 nm. In order to calibrate the assay hydroxyl radicals were produced by ^{60}Co irradiation of N_2O-saturated aqueous benzoate (5 mM in 10 mM phosphate buffer, pH 5.0) solution.

Steady-state γ-irradiations were performed using a ^{60}Co source with a nominal activity of 2000 Ci and a dose rate of 0.0941 Gy s^{-1}, determined by Fricke dosimetry.

Production of hydroxyl radicals with time

A mixture of sodium benzoate (5 mM) and BLM-analogue (110 μM) in phosphate buffer (10 μM, pH 5) was treated with ammonium ferrous sulfate (100 μM in buffer) and the change in fluorescence intensity measured with time.

The effect of oxygen or nitrogen on the reaction system was investigated by passing the respective gas through the reaction solution for 10 min. The ammonium ferrous sulfate solution was then added to give a final concentration of 100 μM and the change in fluorescence intensity measured with time.

The change in fluorescence intensity was also measured using ammonium ferric sulfate in place of ammonium ferrous sulfate to give a final concentration of 100 μM in the reaction solution.

Effect of metal chelators on the Fenton reaction

A mixture of sodium benzoate (5 mM) and hydrogen peroxide (1 mM) in phosphate buffer (10 mM, pH 5) was treated with ammonium ferrous sulfate (100 μM in buffer) or ammonium ferric sulfate (100 μM) in buffer and the change in fluorescence intensity measured with time.

A similar aqueous solution of benzoate, hydrogen peroxide and buffer but also containing a metal chelator (110 μM) or ammonium ferric sulfate (100 μM in buffer) and the change in fluorescence was measured with time.

Effect of changes in iron concentration

A mixture of sodium benzoate (5 mM), BLM-A_2 or BLM analogue (1 mM) and ammonium ferrous sulfate (0.05–0.9 mM) in phosphate buffer (10 mM, pH 5) was allowed to stand in air for 30 min. The fluorescence intensity at 406 nm was then measured.

Effect of drug concentration

A mixture of sodium benzoate (5 mM), ammonium ferrous sulfate (0.2 mM) and BLM-A_2 or BLM analogue (0.1–1 mM) in phosphate buffer (10 mM, pH 5) was allowed to stand in air for 30 min. The fluorescence intensity at 406 nm was measured.

DNA Binding assay

Binding to DNA was measured by recording the loss of the fluorescence of ethidium bromide (EB) fluorescence (λ_{exc} 523 nm, λ_{em} 600 nm) with increasing concentration of BLM analogue or BLM-A_2 using a Perkin Elmer LS50B luminescence spectrometer.

The reaction mixture consisted of 8 μM ethidium bromide in an aqueous solution of 2-[tris(hydroxymethyl)methylamino]-ethanesulfonic acid (10 mM) and 0.5 mM EDTA buffer at pH 7.5 or pH 6.0 (adjusted with HCl) containing 9.92 μM DNA.

The effect of the drug in quenching the EB–DNA fluorescence was then measured by the addition of consecutive amounts of the drug, leaving 5 min intervals between the addition of the drug and measurement of the fluorescence. Increasing amounts of the drug were added until the fluorescence was quenched by over 50%. All fluorescence readings were corrected by the blank value (*i.e.* the fluorescence recorded for EB in buffer alone).

Cell survival assay

Chinese hamster cells were chosen for the study and the line used has a doubling time of 12 h in Eagle's minimum essential medium supplemented with Earle's salts and 10% foetal calf serum. The pH of the medium during subculture and colony growth was controlled by adding 2.2 g L^{-1} of sodium bicarbonate and maintaining a 5% CO_2 atmosphere in the 37 °C incubator.

Exposure in air

Cells were plated to yield 2000 and 200 cells per dish and were incubated for 1 h prior to exposure. The medium was then removed and replaced by 2 cm^3 of the drug solution in the medium and the control treated with medium alone. The cells were exposed for 4 h, except for those treated with **1**, **10**, **24a**, **24b** and **29** which were exposed for only 1 h, while still attached and in the incubator.

At the end of treatment the drug containing medium was removed and each culture dish washed once with 2 cm^3 Hank's saline. The cells were then incubated for 7 days in 4 cm^3 culture medium. After 7 days the cell colonies were stained with crystal violet and the number of surviving colonies counted with a colony counter.

Exposure in hypoxia followed by air

The cell suspension in Eagle's spinner medium (SMEM) was diluted to a concentration of 2×10^5 cells per cm^3. The suspension was then split into 4 cm^3 portions and spun down at 2000 rpm for 5 min. The pellet was resuspended in 4 cm^3 of various concentrations of drug (**24a**, **24b** and **29**) solutions in SMEM and a control suspended in 4 cm^3 of SMEM. The cell samples were then incubated at 37 °C with a 95% N_2–5% CO_2 gas mixture continuously passed over the dishes for 2 h. After 2 h, the cell samples were incubated under a 95% air–5% CO_2 gas mixture for a further 2 h. A 0.5 cm^3 sample was then removed from each and spun down at 2000 rpm. The pellet was washed with 2 cm^3 Hank's saline and resuspended in 5 cm^3 of SMEM to give a cell concentration of 2×10^4 cells per cm^3. The cell suspension was diluted by adding 1 cm^3 of the suspension to 9 cm^3 SMEM and 1 cm^3 plated in 3 cm^3 culture medium to yield 2000 cells per dish and 100 μL in 4 cm^3 culture medium to yield 200 cells per dish. The cells were then incubated for 7 days and survival assayed as above.

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