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SYNTHESIS AND PROPERTIES OF PRODRUGS ACTIVATED IN HYPOXIA TO GIVE BLEOMYCIN ANALOGUES

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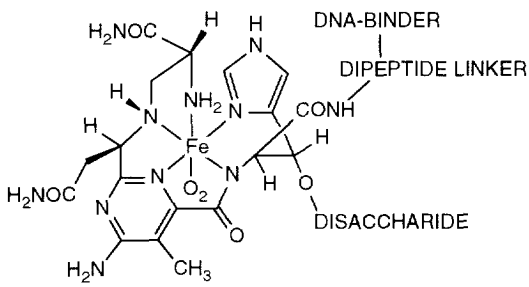
Abstract: Prodrugs bioreductively activated to bleomycin analogues are reported. The production of hydroxyl radicals in the presence of Fe(II) and dioxygen by both the prodrugs and the activated products are determined and their *in vitro* cytotoxicity measured.

Bleomycins are a family of glycopeptide antitumour antibiotics discovered by Umezawa's group in 1966.[1] Bleomycin A_2 is the main constituent of the mixture of bleomycins used clinically in combination chemotherapy for the treatment of Hodgkin's lymphoma, carcinomas of the head and neck and tumours of the testis.[2] Important factors in the clinical use of bleomycin are that the drug does not cause damage to bone marrow but, unfortunately, the drug does have pulmonary toxicity, which is a dose limiting factor and as a consequence bleomycins are used in low doses in synergistic combinations.[3]

Bleomycin A_2 (BLM) can be considered to be composed of four parts: (i) a metal ion complexing unit (Figure 1), (ii) a peptide linker, (iii) a substituted bithiazole residue which binds with DNA and (iv) a disaccharide unit which facilitates complex formation and passage across cell membranes. It is the variation of the substitutents on the bithiazole which provides the family of bleomycins. The therapeutic action of bleomycin occurs when an iron(II) complex is formed which can activate molecular oxygen and lead to oxygen radical species which cause degradation of DNA (Figure 1).[4, 5] A full and satisfactory explanation for the unique oxygen chemistry associated with Fe(II)BLM complex remains to be found.[6]

Figure 1. Metal-oxygen complex of Bleomycin



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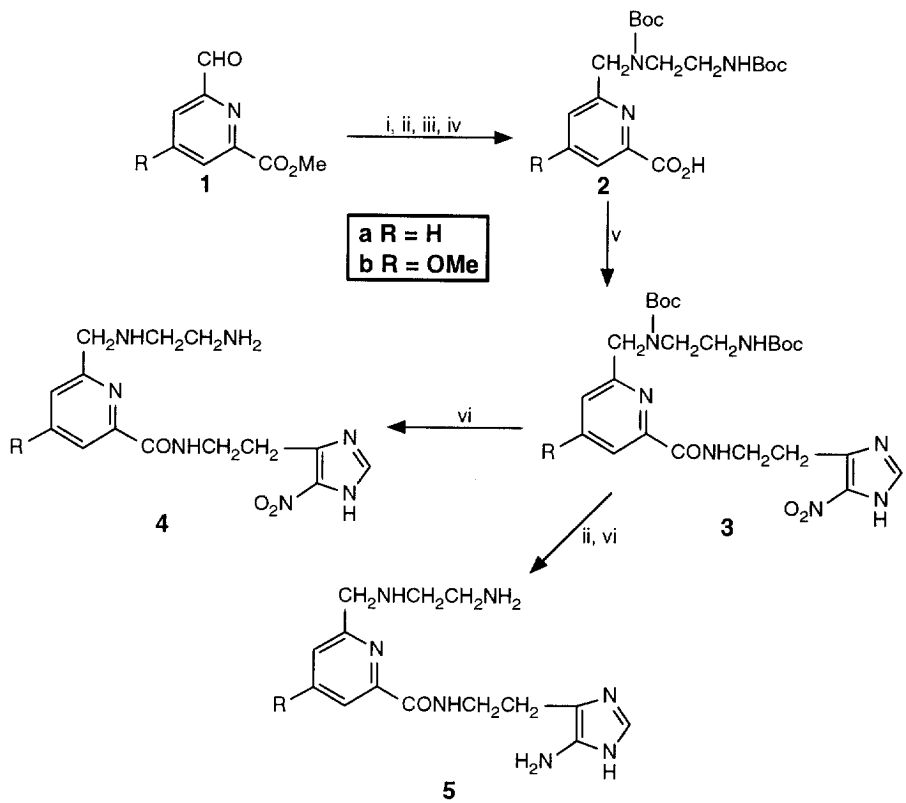
Much synthetic chemistry has been done in order that the properties of bleomycin analogues might be investigated with a view to establishing mechanistic details of the oxygen activation process and the minimum requirement for iron complexation leading to oxygen activation. An important aspect of preparative findings relevant to our work is that a pyridine nucleus can replace the pyrimidine ring in bleomycin. The activity of the iron complex with a pyridine analogue is greater when there is an electron donating 4-substituent on the pyridine ring.[7]

Our objective has been to design and synthesise prodrugs which do not complex with iron and hence do not produce activated oxygen species until after the prodrug is reduced to a bleomycin-like product. This process is expected to occur in the hypoxic cells of solid tumours which are known to be effective at reducing bioreducible agents.[8] Thus, by incorporation of suitable reducible groups at crucial sites in the bleomycin analogues it was hoped that the active species would be produced only in the hypoxic region of the tumour. This region is known to be resistant to killing by radiation, and surviving dormant oxygen-deficient cells are thought to become oxygenated and to provide foci for secondary tumours.[9] At this crucial stage, it was expected that the now reduced prodrug would bind iron, activate oxygen and degrade the DNA of these malignant cells. We report here our findings on the synthesis of both the prodrugs and the bioreductively activated drugs, oxygen activation by both the prodrug and drug as measured by hydroxyl radical production, and some preliminary biological data.

In order to deactivate the complexation unit of the molecule we have introduced either a nitro group on the imidazole nucleus or an *N*-oxide group on the pyridine ring. Examples of the reduction of both nitro and *N*-oxide substituents on heteroaromatic nuclei in hypoxic cells are known.[8] The nitro group was introduced in the imidazole nucleus in the expectation that its effect would be to decrease the electron density on the ring nitrogen atom and so lessen the ease of complexation to the metal ion. Reduction of the nitro group to either the hydroxylamine or amine was expected to restore the metal ion-binding ability of the ligand.[8] It is known that a terminal dimethylamino group on the other chain of the ligand prevents complexation of a metal ion.[10] Production of the *N*-oxide at the pyridine nitrogen atom was chosen but, unfortunately, only one *N*-oxide **7** was obtained (Scheme 2). The *N*-oxide corresponding to **7** in the series with a 4-methoxy substituent was not obtained because oxidation of the ester of **2b** did not afford the *N*-oxide.

The known methyl 2-formylpyridine-6-carboxylate **1a**[11] (Scheme 1) and the 4-methoxy derivative **1b**[7] were prepared from commercially available pyridine 2,6-dicarboxylic acid and chelidamic acid. The aldehydes **1a** and **1b** were condensed with a monoprotected 1,2-diaminoethane and the resultant imines reduced in a one-pot process to give the secondary amine. Protection of these amines and hydrolysis of the esters afforded the protected diamino acids **2a** and **2b**, respectively. Peptide coupling of the acids using 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI) and 1-hydroxybenzotriazole (HOBT) with 4(5)-nitrohistamine[12] gave the protected peptides **3a** and **3b**, and deprotection then gave the nitro prodrugs **4a**, and **4b**. Catalytic hydrogenation of the nitro group in **3a** and **3b** afforded the corresponding amines **5a** and **5b** after deprotection.[13]

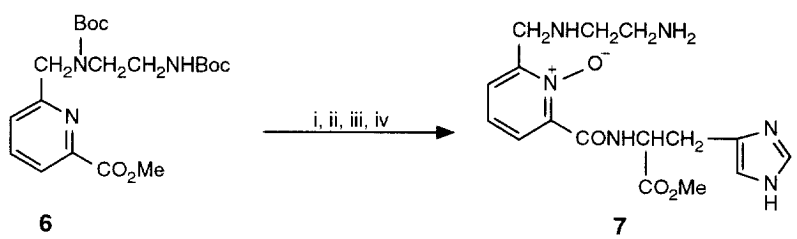
Scheme 1



Reagents: (i) $H_2N(CH_2)_2NHBoc$, (ii) H_2, Pd/C, (iii) $(Boc)_2O$, (iv) aq. NaOH, (v) EDCI, HOBT, Et_3N, 4(5)-nitrohistamine, (vi) TFA

The methyl ester (**6**), obtained as an intermediate in the interconversion of **1a** to **2a**, was oxidised with *m*-CPBA to give the pyridine *N*-oxide and then saponified to give the acid. Histidine methyl ester was coupled with the acid and the product deprotected to afford the *N*-oxide prodrug (**7**) (Scheme 2).

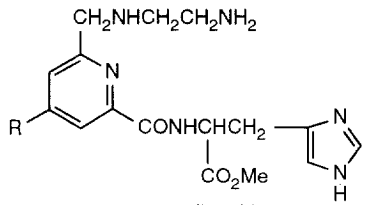
Scheme 2



Reagents: (i) *m*-CPBA, (ii) aq. NaOH, (iii) EDCI, HOBT, Et_3N, histidine methyl ester, (iv) TFA

The pyridine derivative **8a**[13] was prepared by a known method[10] and the corresponding novel 4-methoxy

derivative **8b** was obtained in a similar way.



8a R = H
8b R = OMe

The ability of the 'deactivated' prodrugs **4a**, **4b** and **7** and of their reduced 'activated' counterparts **5a, 5b** and **8a**, respectively, to produce hydroxyl radicals when in the presence of iron and dioxygen was measured using ESR spectroscopy. Both the techniques of spin-trapping, with phenyl-*N*-*t*-butylnitrone (PBN)[14, 15] and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO),[15] and the hydroxylation of benzoate to form fluorescent salicylate[16, 17] were used to qualitatively and quantitatively assay the yield of hydroxyl radicals. As expected, production of hydroxyl radicals from the deactivated prodrugs in the presence of Fe(II) and dioxygen was very small whereas the corresponding reduced and activated compounds, which are expected to be formed in tumours under hypoxic conditions, gave significant quantities of hydroxyl radicals, though more slowly and less efficiently than bleomycin (Figure 2). When dioxygen was passed through the solution of the amines and Fe(II) ions the concentration of hydroxyl radicals was doubled as compared to air alone. None of the compounds gave hydroxyl radicals in a deaerated solution under a nitrogen atmosphere which is consistent with the idea that hydroxyl radicals are produced by activation of dioxygen by the metal complex. The amine **5b** gave approximately three times the quantity of hydroxyl radicals produced by **5a**, which is probably a result of electron donation by the methoxy group giving a higher electron density on the pyridine ring thus increasing complexation and the oxygen activation. Similarly, compound **8b** gave a higher yield of hydroxyl radicals more rapidly than the corresponding compound **8a** without the methoxy group.

In the presence of Fe(III) alone no hydroxyl radicals were produced by these bleomycin analogues because the Fe(III) is not reduced in this system.

As expected the prodrugs were not cytotoxic in air alone to chinese hamster V79 cells *in vitro*. When the prodrugs **4a** and **4b** were mixed with hypoxic cells *in vitro* followed by incubation of the cells in an air-carbon dioxide mixture (95:5%) a small, barely significant difference in cytotoxicity was observed (data not shown). However, the *N*-oxide **7**, which showed essentially no cell kill in air alone, gave, after prior exposure to hypoxia, a similar cell kill to the corresponding deoxygenated compound **8a** in air (Figure 3).

The differential effects observed for both hydroxyl radical production and cytotoxicity of the prodrugs and their reduction products suggests a new basis for improving the therapeutic ratio of bleomycin analogues.

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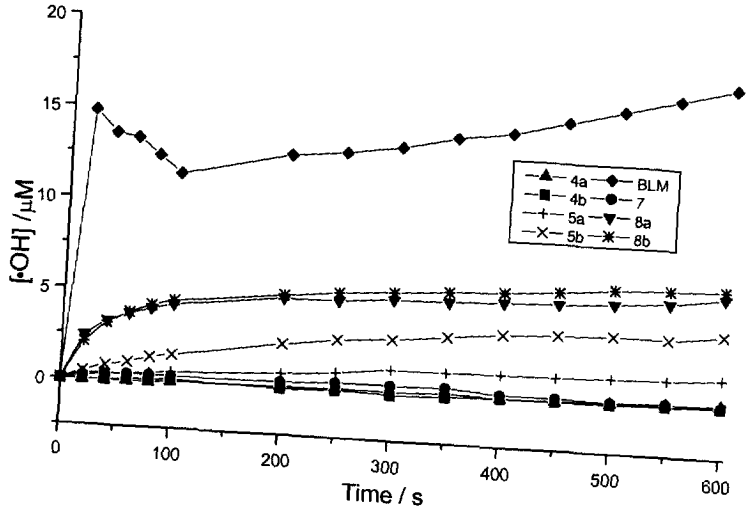
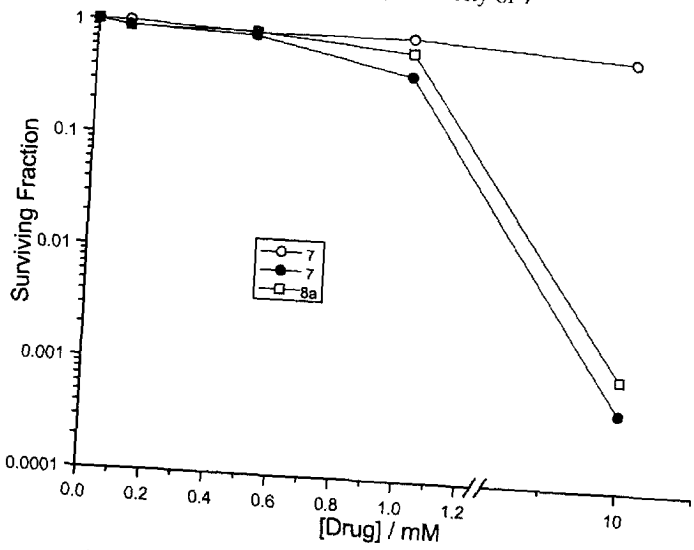
Figure 2. Production of hydroxyl radicals with time



The concentration of reagents were : 5 mM sodium benzoate, 100 µM Fe(II) and 110 µM BLM analogue. Oxygen was passed through the mixture for 10 min. prior to addition of Fe(II).

Figure 3. Cytotoxicity of **7**



open symbols: exposure to air for 4 hours
closed symbols: exposure to hypoxia (2 hours) followed by air (2 hours)

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