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FIELD DESORPTION AND FAST ATOM BOMBARDMENT MASS SPECTROMETRY OF BLEOMYCINS AND THEIR DERIVATIVES

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SUMMARY: Earlier work using field desorption mass spectrometry for the characterisation of guanidino containing bleomycins is extended to include bleomycin A_2 and related substances. The new technique of fast atom bombardment mass spectrometry is assessed and compared with field desorption for the analysis of these complex antibiotics. Bleomycin A_2 is shown to be more reactive to acetic anhydride than bleomycin B_2. A procedure for in situ derivatisation for fast atom bombardment studies is described and unexpected catalysis in the glycerol matrix is reported.

INTRODUCTION

The bleomycins are a family of glycopeptide-derived antibiotics some members of which are used clinically for the treatment of squamous cell carcinomas and malignant lymphomas (1,2). The structures of two major naturally occurring members of the family, A_2 and B_2, are given in Fig. 1. A number of other bleomycins have been isolated which differ only in the C-terminal moiety. The structurally related phleomycins differ only in the absence of a double bond in one of the thiazole rings (3), but certain bleomycin group antibiotics isolated more recently (e.g. tallysomycin (4), cleomycin (5)) have been found to differ in structure more fundamentally. The bleomycins are very difficult substances to characterise due to their complexity, high molecular weight and thermal instability and the structures of additional isolated members of the family have yet to be determined (see e.g. 6,7).

As part of a long term programme aimed at the characterisation of both naturally occurring novel bleomycins and the products of chemical synthesis of the bleomycins

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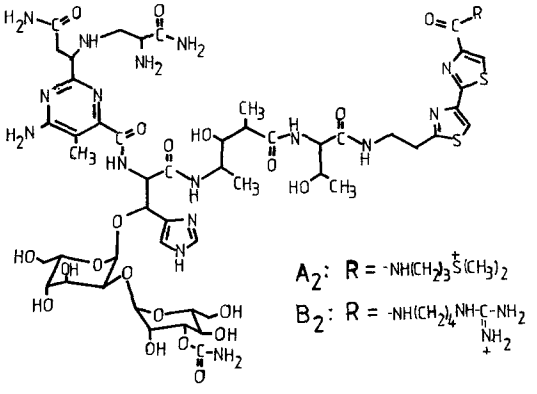

Fig. 1 Structures of bleomycins A_2 and B_2.

we have been developing mass spectrometric methods suitable for the analysis of these complex molecules (8). Previously we have demonstrated that field desorption mass spectrometry (FDMS) gives excellent spectra of guanidino containing bleomycins and their acetylated derivatives and have shown that a mono-acetyl product, potentially useful in the structure investigation of novel bleomycins, can be obtained in high yield and readily analysed by FDMS (9). We have recently extended this work to include all types of known bleomycins and in this paper we present the results of these investigations. In addition we are complementing our FDMS studies with fast atom bombardment (FAB) mass spectrometry, a new technique which is showing considerable promise for the analysis of complex, polar molecules (10). We have examined a representative selection of bleomycins using FAB MS and the results of this work are presented below, together with an assessment of the relative merits of FAB and FD for the structure determination of bleomycins and their derivatives.

MATERIALS AND METHODS

Blenoxane (the clinically used mixture of bleomycins) was obtained from Bristol Laboratories through the courtesy of Drs Stanley Crooke and William Bradner; it was fractionated as described (11) to provide bleomycins A_2, B_2 and demethyl A_2. Cu-Blenoxane was obtained from the National Cancer Institute with the assistance of Dr Harry Wood.

Field desorption mass spectrometry was carried out using a KRATOS MS 50 mass spectrometer fitted with a high field magnet (8). Spectra were obtained as described

previously (9). Samples were acetylated for FD experiments using the reported conditions (9). Fast atom bombardment mass spectrometry was carried out using a VG Analytical ZAB 1F mass spectrometer fitted with an extended range magnet giving a mass range of 1800 m.u. at 8 kV. An M-Scan FAB system was used which consisted of a Townsend discharge ion gun mounted on a Teflon probe inside the source housing via the laser port. The standard ZAB FD source and probe were used with minor modifications to the source optics. Samples were loaded in glycerol on a nickel/chromium ribbon target spot welded at a suitable angle onto the legs of an FD emitter. Argon was used as the bombarding gas and the gun was operated at 8-10kV.

Acetylation experiments in the glycerol matrix were performed as follows : a solution of acetic anhydride in methanol (1:3 v/v) was prepared using 1:1 $(CH_3CO)_2O$: $(C^2H_3CO)_2O$. A drop of glycerol was placed on the target and 1-2 µl of a solution of bleomycin in methanol or methanol/water (1:1 v/v; 1-5 µg/µl) was added. Approx. 0.5-1.0 µl of acetylating mixture was then added , the probe inserted into the mass spectrometer and FAB spectra were immediately recorded.

RESULTS AND DISCUSSION

Bleomycin A_2, whose terminal amide moiety contains a sulphonium cation, is the most difficult member of the bleomycin family to characterise unambiguously by mass spectrometry. Its behaviour under FD and FAB conditions is broadly similar, although the latter spectra are rather easier to interpret. Representative FD spectra of bleomycin A_2 are shown in Fig. 2a. The molecular ion (m/z 1414) is very weak and usually is only obtained in the first scan. Subsequently thermal demethylation and cationisation takes place on the FD wire giving rise to the sodium cationised molecular ion of the demethylated species (m/z 1422) and an abundant fragment ion at m/z 1380. The other signals present in Fig. 2a have not been assigned and, since they were not always present in the spectra of different batches of bleomycin A_2, they may arise from related substances. The demethylation/cationisation processes taking place on the surface of the FD wire generate different spectra from those obtained from genuine bleomycin demethyl A_2 whose FD spectrum is given in Fig 2b. Although there is some cationisation of demethyl A_2 (m/z 1422, $(M+Na)^+$), the pseudomolecular ion (m/z 1400, $(M+H)^+$) is the major species. Note the presence of signals 43 m.u. apart, e.g. m/z 1400 and m/z 1357, a pattern previously reported to be characteristic of the guanidino containing bleomycins (9), and which therefore must now be attributed to

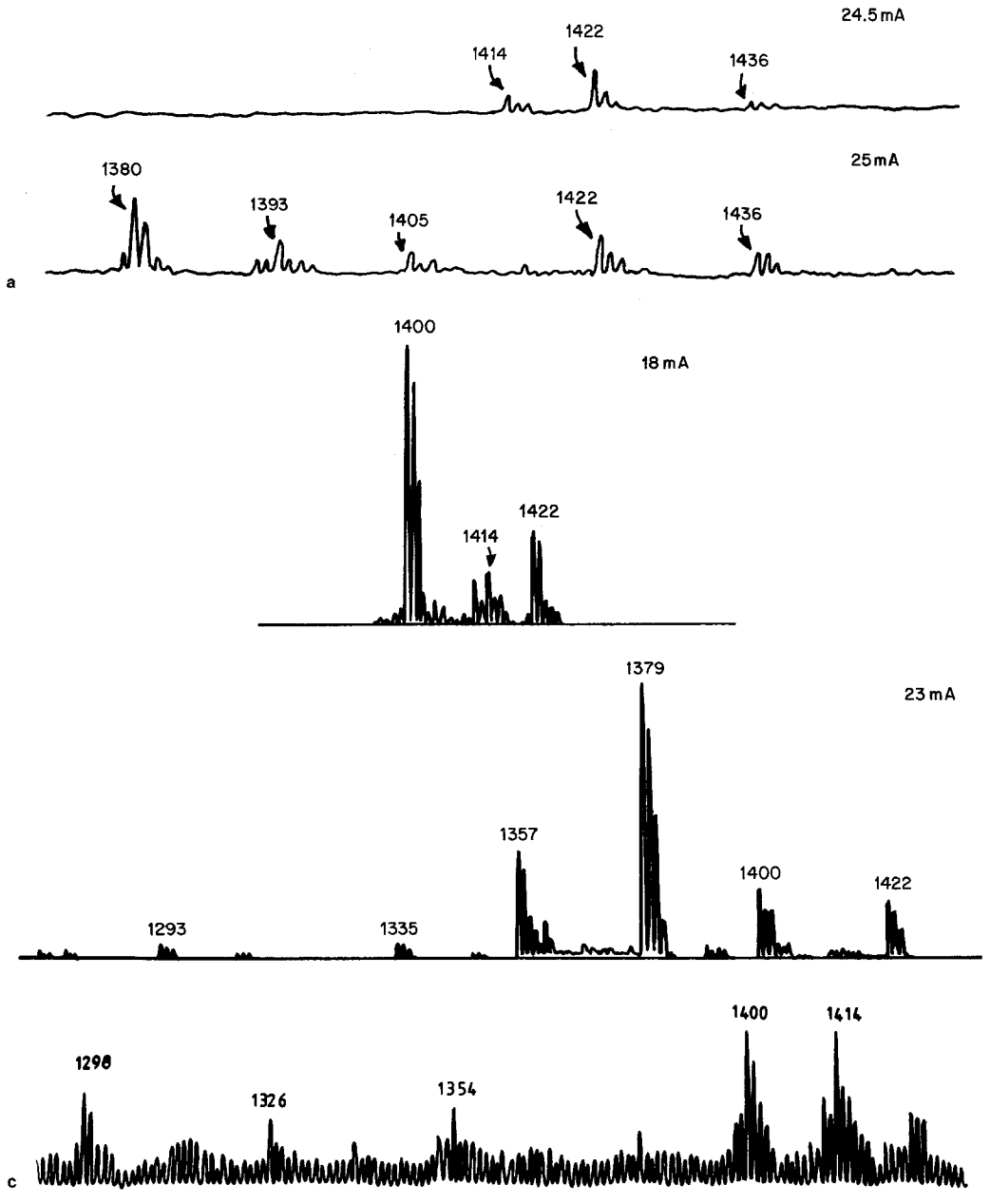

Fig. 2 (a) Field desorption mass spectrum of bleomycin A_2 obtained using the rapid heating procedure previously described(9); (b) field desorption mass spectrum of bleomycin demethyl A_2; (c) fast atom bombardment mass spectrum of bleomycin A_2.

fragmentation of the glycopeptide "core" rather than the terminal amide. The functional group giving rise to this fragmentation has not yet been identified.

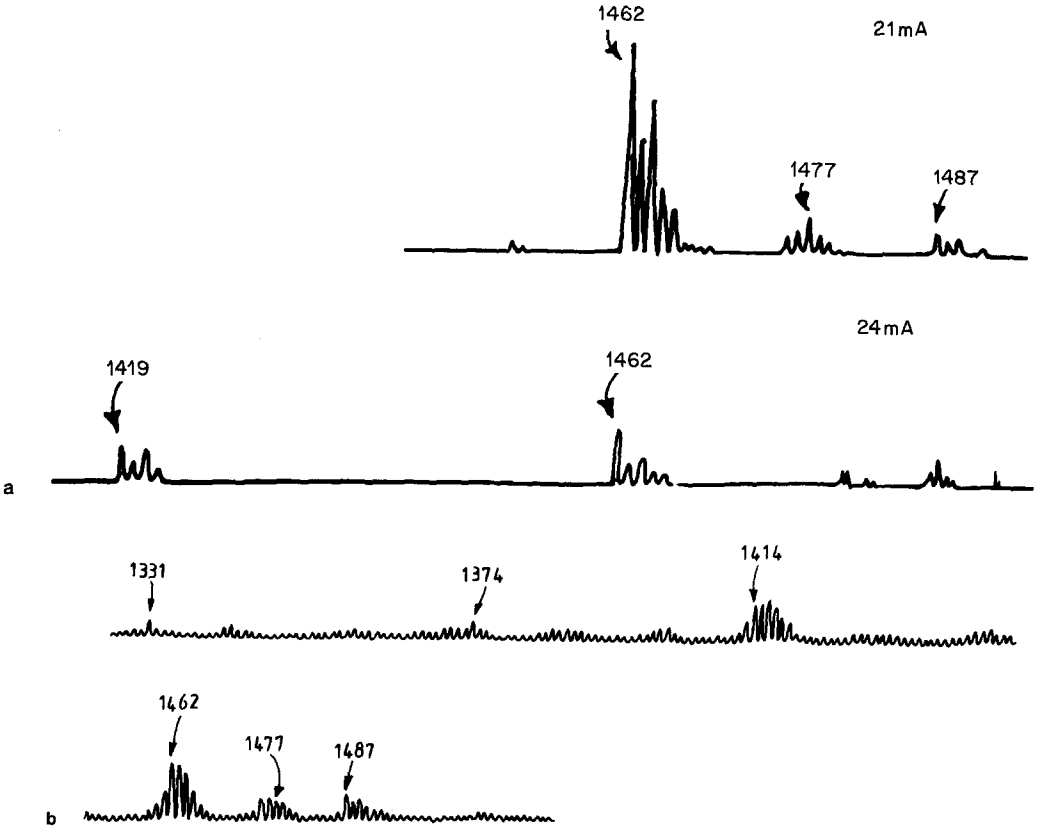

Fig. 3 (a) Field desorption mass spectrum of Cu-Blenoxane; (b) fast atom bombardment mass spectrum of Cu-Blenoxane.

Bleomycin A_2 also demethylates under FAB conditions (Fig 2c) but demethylation is less complete and a relatively abundant molecular ion is observed at m/z 1414. In contrast to the FD results the pseudomolecular ion (m/z 1400, $(M+H)^+$) is the major species obtained from the demethylated product. Bleomycin demethyl A_2 itself yields excellent FAB spectra, showing intense signals at m/z 1400 and 1298 (data not shown).

Metal complexes of the bleomycins are amenable to both FAB and FD MS and representative spectra of the copper complex of Blenoxane are given in Fig. 3. Blenoxane consists of a mixture of bleomycins A_2 and B_2 together with a number of minor components. The copper complexes ionise to yield molecular ions under FD conditions and a mixture of molecular and pseudomolecular ($(M+H)^+$) ions under FAB

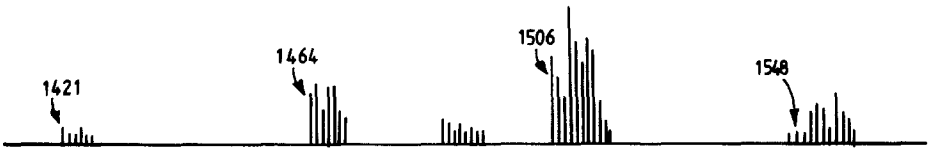

Fig. 4 Field desorption mass spectrum of the product of short acetylation of bleomycin A_2 ; demethylated cationised molecular ions are present at m/z 1464,1467 (1:1, mono-acetyl), 1506, 1509, 1512 (1:2:1, di-acetyl) and 1548, 1551, 1554, 1557 (1:3:3:1, tri-acetyl).

conditions. Consequently the copper isotope pattern is more clearly evident in the FD spectra. The major signal at m/z 1462 is assigned to the molecular ion of bleomycin A_2 after demethylation in the mass spectrometer, while m/z 1477 and 1487 are the true molecular ions of bleomycins A_2 and B_2 respectively.

In an earlier publication (9) we have described the advantages of incorporating acetyl:$^2H_3$-acetyl (1:1) groups into the bleomycin skeleton both in terms of providing an isotopic "handle" to aid interpretation of spectra and to improve the molecules' desorption properties. Acetylation experiments were vital aids in the interpretation of the complex FD spectra obtained from bleomycin A_2 and they also demonstrated that it is considerably more reactive than other bleomycins. The product of 1 min acetylation at room temperature gives the FD spectrum shown in Fig. 4. In contrast to bleomycin B_2, which specifically incorporates one acetyl group under these conditions, bleomycin A_2 yields mono-, di- (the major product) and tri-acetyl derivatives. Demethylation on the wire is complete and the major cationised molecular ions are observed at m/z 1464, 1467 (1:1, mono-acetyl) and m/z 1506, 1509, 1512 (1:2:1, di-acetyl). The reason(s) for the greater reactivity of bleomycin A_2 are unknown but may conceivably be due to intramolecular catalysis by the dimethylsulphonium cation or to differences in preferred conformation between bleomycin A_2 and the other species studied. In this context it may be mentioned that bleomycin demethyl A_2 which lacks the sulphonium cation, behaves analogously to bleomycin B_2, yielding a mono-acetyl derivative in high yield.

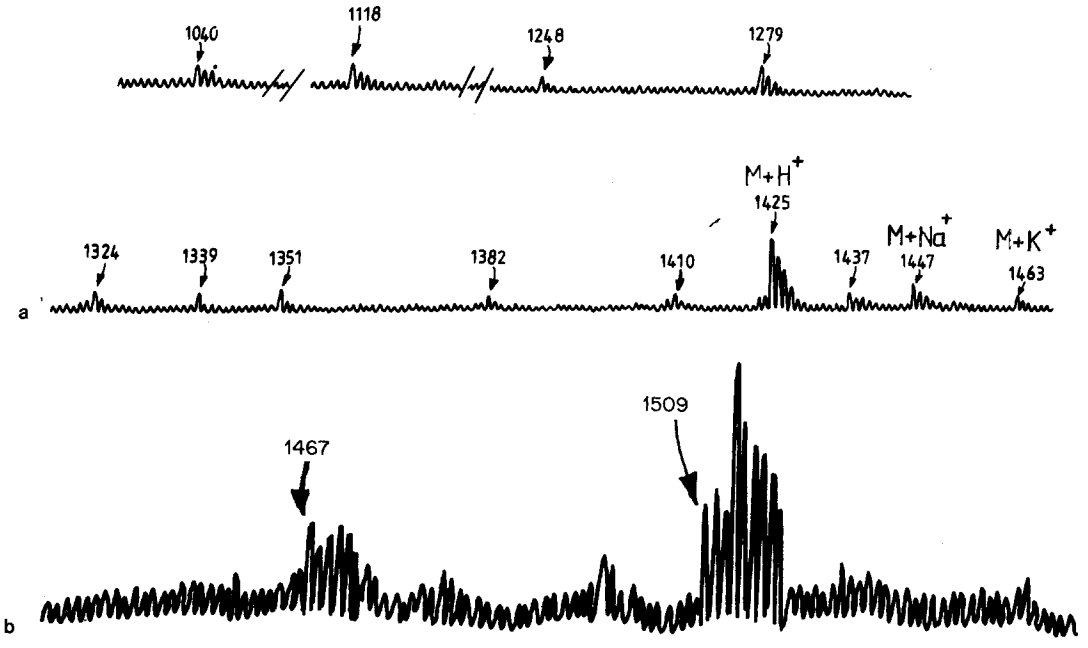

Fig. 5 Fast atom bombardment mass spectra of bleomycin B_2 (a), and the product of acetylation in the glycerol matrix (b); pseudomolecular ions for mono- and di-acetyl derivatives occur at m/z 1467, 1470 (1:1) and 1509, 1512, 1515 (1:2:1); the molecular ion region of the native material (a) shows sodium and potassium cationisation in addition to protonation.

Similar results are obtained if FABMS is used to examine bleomycins which have been treated with the acetylation mixture followed by removal of the reagents prior to loading in the glycerol matrix. However, different behaviour is observed if acetylation is performed in the glycerol matrix in the FAB source. In other work on peptides (12) we have shown that simple derivatisations such as acetylation can be very conveniently carried out in the glycerol matrix. The method has potential for the structure investigation of unknown compounds since FAB spectra of the native material can be first recorded followed by derivatisation and re-recording of the FAB spectrum. Several types of functional groups could be located in this manner. This type of protocol has been applied to the bleomycins and a typical result is shown in Fig. 5. The underivatised antibiotic yields excellent FAB spectra with abundant pseudomolecular ions (see Legend to Fig. 5). Acetylation in the glycerol matrix produces the di-acetyl

derivative as the major product (1:2:1). Clearly, under FAB conditions, an additional functional group is activated for reaction with acetic anhydride. Bleomycin demethyl A_2 reacts analogously to yield a di-acetyl derivative, while the FAB spectra of acetyl derivatives of bleomycin A_2 prepared in the glycerol matrix are similar to those obtained by pre-treatment with the reagents (the di-acetyl product predominates). The simplicity and speed of acetylation on the FAB target make this a valuable approach to the characterisation of novel bleomycins.

From our FAB and FD studies of the bleomycins and their acetyl derivatives the following conclusions can be drawn:

(i) FAB and FD MS gives molecular weight information on all the bleomycins so far examined both as the native material and as metal complexes. Technically FAB is a much easier technique to use than FDMS. The sensitivity of the two methods is comparable but more intense spectra can be obtained in FABMS by increasing the quantity loaded on the target. This is not possible in FDMS.

(ii) Acetyl derivatives can be conveniently prepared in situ for both FD and FAB and the techniques are complementary since different products are obtained. However, FAB offers greater scope for in situ derivatisation.

(iii) FABMS of bleomycin A_2 must be coupled with analytical procedures such as high voltage paper electrophoresis or HPLC in order to distinguish between the presence of its demethyl analog and demethylation in the mass spectrometer.

(iv) FAB spectra are more complex than FD spectra because of greater fragmentation and this may be a disadvantage if unknown substances are being examined. We therefore recommend that FAB and FD be used to complement one another in such circumstances. FAB spectra should be obtained first because data can be acquired so readily with this technique. Once the possible molecular weight distribution has been established FDMS can be used to distinguish molecular ions from fragment ions.

In conclusion, we have demonstrated that FD and FAB MS can be used to characterise a wide variety of bleomycins. We are now applying these methods to the structure elucidation of other members of the family.

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