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CHARACTERISATION OF GUANIDINO-CONTAINING ANTIBIOTICS: FIELD DESORPTION MASS SPECTROMETRY OF BLEOMYCIN B_2 AND PHLEOMYCINS D_1 AND E.

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SUMMARY: Because of their complexity bleomycins and their relatives are difficult molecules to structurally characterise. In this paper the first successful field desorption mass spectrometric investigations of these molecules are reported. The recently corrected structure for the glycopeptide skeleton is confirmed and the preparation of a monoacetyl derivative suitable for both mass spectrometric and biological studies is described. The procedures outlined in this paper enable the unambiguous characterisation of closely related members of the bleomycin family and their derivatives, separately or in mixtures.

INTRODUCTION

The bleomycins are a family of glycopeptide antibiotics (see Fig. 1) some members of which are used clinically as anti-tumour agents (1,2). The closely related phleomycins (Fig. 1) have not been developed for clinical utilisation because of the renal toxicity associated with the earliest isolated derivatives (3). The bleomycins are extremely complex and the structure originally proposed (4) for the basic glycopeptide skeleton was established only after an extensive series of degradation experiments. The difficulties inherent in such an approach to structure elucidation, particularly in the absence of

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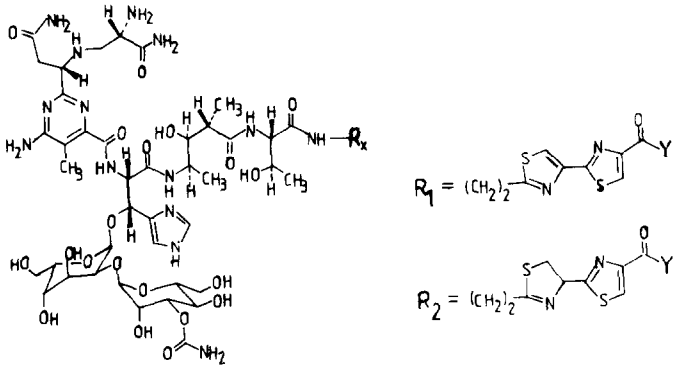

Fig. 1 Structures of the bleomycins ($R_x = R_1$) and phleomycins ($R_x = R_2$). Bleomycin B_2 and phleomycin D_1 have Y = $NH(CH_2)_4NH\text{-}C(=NH)\text{-}NH_2$; phleomycin E has Y = $NH(CH_2)_4NH\text{-}C(=NH)\text{-}NH(CH_2)_4NH\text{-}C(=NH)\text{-}NH_2$.

corroborative molecular weight and atomic composition data, were exemplified by the necessity to correct the original assignment of a β-lactam to an open chain amide (5). Even in the later work (5) the authors were not able to define the molecular formula or molecular weight, but arrived at the new structure by comparison of spectral (esp. NMR) data with those derived from P-3A, a biosynthetic intermediate whose structure was established by X-ray crystallography. In view of the importance of the bleomycins as therapeutic agents, the increasing interest in their mode of action (see, e.g. (6-8) and references cited therein) and the existence of other bleomycin-phleomycin group antibiotics (e.g., tallysomycin (9) and YA-56X (10)), it became apparent to us that it was essential to have an analytical method that could be used to characterise additional structurally related compounds and their derivatives.

Field desorption mass spectrometry (FDMS) is now a well established technique for defining the molecular weight of polar, thermally labile molecules (11). Indeed in our early studies (12) a field desorption molecular weight determination of the antibiotic Echinomycin was the crucial factor leading to the structure revision of this molecule; this was the earliest successful application of FDMS to the solution of a biochemical

problem. As part of a long term programme aimed at the chemical synthesis of bleomycin derivatives (13) we are investigating the applicability of FDMS for characterising these complex substances. A previous attempt (5) to obtain FD spectra of the bleomycins was unsuccessful, and this was probably due, in part, to the problems associated with obtaining FD spectra of molecules of molecular weight outside the mass range of conventional mass spectrometers operated at full sensitivity. ^{252}Cf-Plasma desorption mass spectra of bleomycin B_1 have recently been reported (14). The experiments described in the present paper were performed using a mass spectrometer especially developed for the study of high mass molecules (15). We now report our investigations on guanidino-containing members of the bleomycin and phleomycin families. These substances are the first guanidino-containing antibiotics to be successfully characterised by field desorption mass spectrometry.

MATERIALS AND METHODS

Blenoxane (a mixture of bleomycins consisting primarily of A_2 and B_2) and the phleomycins were obtained from Bristol Laboratories through the courtesy of Drs. Stanley Crooke and William Bradner. Bleomycin B_2 was obtained by fractionation of blenoxane as described (16).

Field desorption mass spectrometry was carried out using a KRATOS MS 50 mass spectrometer fitted with a 2.3 Wb m^{-2} magnet giving a mass range of 3000 a.m.u. at 8 kV (15). Spectra were obtained on chart paper and calibrated, using a crystal time marker, by comparison with the electron impact spectrum of Fomblin oil (17). High temperature activated carbon emitters with microneedles of length 15-25μ were used. Samples were dissolved in methanol/water (1:1 v/v) containing a trace of NaCl and were loaded by dipping the emitter into one drop of the solution (10μl, 2-5 μg/μl) placed on a strip of aluminium foil. The same sample could be used for numerous repeat loadings if necessary, and in some cases the short acetylation experiment described below was performed during the dipping procedure by adding a small amount of the reagent to the solution on the foil immediately prior to loading. FD spectra were obtained by rapidly increasing the wire current to a value just below that at which desorption takes place, followed by a carefully controlled increase of 1-2 mA in current as the sample desorbed. Bleomycin B_2 was acetylated as follows: - (1) Short acetylation: approx. 20 μg of bleomycin B_2 was dissolved in 10 μl of water to which was added 50 μl of a 1 : 3 (v/v) mixture of acetic anhydride/methanol. After 3 min. at R.T. the sample was dried down in vacuo. (2) Base catalysed acetylation: the sample was dissolved in a 2 : 1 (v/v) mixture of water/triethylamine (15 μl) and acetylated according to the procedure given in (1) except that reaction was allowed to proceed for 3 h before drying down.

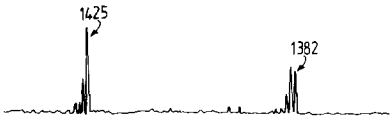

Fig. 2 Field desorption mass spectrum of bleomycin B_2. The spectrum was obtained at 22-24 mA using the rapid heating procedure given in Materials and Methods.

RESULTS AND DISCUSSION

A typical FD mass spectrum of bleomycin B_2 is given in Fig. 2. The molecule exhibits a pseudomolecular ion at m/e 1425 which is consistent with its recently corrected structure (5). In addition a major fragment ion is present at m/e 1382. An analogous pattern is observed for phleomycins D_1 and E (see later); hence pairs of signals separated by 43 m.u. appear to be diagnostic of the guanidino-containing members of the bleomycin family. Despite the polarity, high mass and thermal instability of bleomycin B_2, spectra similar to that given (Fig. 2) can be obtained routinely provided suitable precautions are taken to prevent polymerisation on the wire. We have found that the best carbon emitters for the examination of such fragile molecules are those with relatively short (15-25 μ) and non-dendritic microneedles. Slow heating of the emitter facilitates decomposition of the sample and best results are obtained using the rapid heating procedures described in Materials and Methods. Under these conditions desorption occurs with very little damage to the carbon whiskers; for example we have obtained excellent results from an emitter after more than 50 loadings of bleomycins and their derivatives.

Phleomycins are structurally very similar to the bleomycins (see Fig. 1), differing only in the absence of a double bond in one of the bithiazole rings. The consequent loss in aromaticity results in a greater degree of chemical reactivity. The phleomycins are sensitive to oxidising agents because of the enhanced stability achieved by re-aromatisation of the heterocyclic nucleus and FDMS is a useful tool for determining the

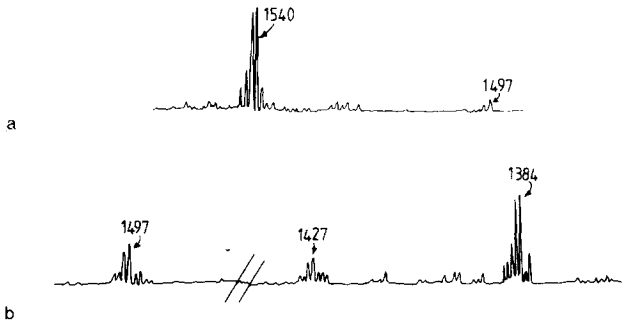

Fig. 3 Field desorption mass spectrum of phleomycins D_1 and E (a) scanning from m/e 1570 to 1450 and (b) scanning from m/e 1520 to 1350. Emitter current was 23-25 mA.

extent of contamination by the corresponding bleomycins. FD mass spectra were obtained on a phleomycin sample and typical spectra are shown in Fig.3. Pseudomolecular ions for D_1 and E are present at m/e 1427 and 1540 respectively with their diagnostic fragments at m/e 1384 and 1497. Note the presence of small but significant signals 2 m.u. below each of the major peaks. These are assigned to the corresponding oxidised molecules.

A number of acetylation experiments were carried out on bleomycin B_2 with the following aims: - (a) to improve the desorbing properties of the molecule so that collision induced decomposition (CID) (18) studies could be performed on the parent ion in order to obtain structural information on new members of the family, (b) to specifically introduce into the basic bleomycin skeleton a "handle" in the form of a deuterium label which would facilitate the interpretation of spectra of derivatives and complex mixtures, and (c) to explore the applicability of FD for characterising bleomycin derivatives and synthetic or semi-synthetic analogues. Based upon our experience in the peptide area (19), acetylation conditions (see "Short Acetylation" in Materials and Methods) were chosen which were expected to selectively modify only the α-amino group of the terminal diamino propionamide residue. Acetylation was performed using a 1 : 1 mixture of $(CH_3CO)_2O/(C^2H_3CO)_2O$; hence the progress of the reaction

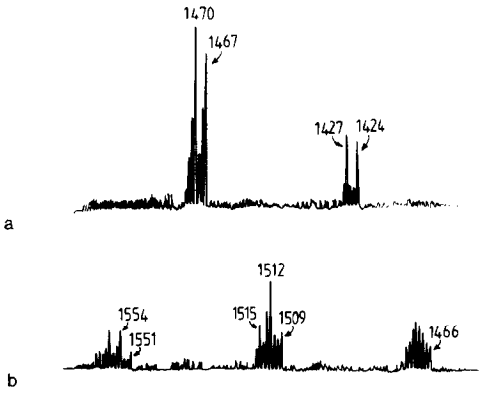

Fig. 4 (a) Field desorption mass spectrum of the product of short acetylation of bleomycin B_2; the doublet at m/e 1467, 1470 is the pseudomolecular ion of the mono-acetyl derivative; emitter current 20 mA.
(b) Field desorption mass spectrum of the product of base catalysed acetylation of bleomycin B_2 ; pseudomolecular ions for di- and tri-acetyl derivatives occur at m/e 1509, 1512, 1515 and 1551, 1554, 1557, 1560 respectively; emitter current 20 mA.

could be readily determined from labelling patterns in the FD spectra. Acetyl derivatives of bleomycin B_2 yielded excellent spectra, ion beams being both more intense and longer lived than those of the parent molecule. The FD spectrum of the product of short acetylation which is given in Fig. 4a demonstrates unambiguously that complete acetylation of a single functional group has taken place. The new pseudomolecular ion occurs at m/e 1467 and 1470 (1 : 1) with m/e 1382 shifting to m/e 1424 and 1427 (1 : 1). If the reaction is allowed to proceed for a further 3 h under the same conditions very little additional modification takes place. However, in the presence of the basic catalyst triethylamine, reaction proceeds rapidly to the di-acetyl and tri-acetyl derivatives with a small quantity of the tetra-acetyl product also being formed. Fig. 4b shows a typical spectrum of the products of base catalysed acetylation. Note the characteristic 1 : 2 : 1 and 1 : 3 : 3 : 1 intensity ratios enabling the assignment of di- and tri-acetyl derivatives respectively (20).

The results of the short acetylation experiment are important for two reasons. Firstly, we have demonstrated that it is possible to produce in high yield a mono-acetyl

derivative which yields an intense and fairly long-lived FD ion beam. This derivative will be useful in structure investigations of other bleomycins. Secondly, the N-acetyl derivative has also proven to be an important species for studying the mechanism of action of bleomycin; the derivative has no activity in strand scission of DNA and has been shown to have altered metal binding properties relative to bleomycin (21).

In conclusion, we have shown that FD mass spectrometry is a valuable tool for characterising bleomycins, phleomycins and their derivatives. In view of the high degree of toxicity of some members of this family, the availability of an analytical method for the assessment of the purity of commercial preparations of bleomycin A_2 and B_2 is clearly an important advance. We are currently extending our studies to other bleomycins and are investigating the use of CID procedures for determining structures of these complex molecules. The utility of FDMS for the characterisation of products of relevance to studies on the biomolecular mechanism of the bleomycins has also been demonstrated here.

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