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Identification of aromatic moieties and mycosamine in antifungal heptaenes with high-performance liquid chromatography, high-performance liquid chromatography–mass spectrometry and gas chromatography–mass spectrometry

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method for the determination of the aromaticity of heptaene polyene antibiotics has been developed. The released aromatic moiety of the heptaene polyenes aureofungin, candicidin, candimycin, hamycin and trichomycin was assayed after alkaline hydrolysis. The presence of *p*-aminoacetophenone (PAAP) and N-methyl-*p*-aminoacetophenone (N-methyl-PAAP) in the hydrolysates was determined by HPLC, HPLC–mass spectrometry (HPLC–MS) and gas chromatography–MS (GC–MS). Candicidin and hamycin contained only the PAAP residue; aureofungin contained both PAAP and N-methyl-PAAP. Trichomycin contained PAAP and also some unknown component of molecular weight 179. The aromatic nature of the individual components of the heptaene complex was demonstrated using radioactivity flow detection for the determination of the incorporation of [^{14}C]-*p*-aminobenzoic acid to individual candicidin components. Ammonia chemical ionization MS was successfully used for the GC–MS identification of the acetylated mycosamine moiety of heptaenes.

INTRODUCTION

The antifungal heptaene antibiotics belong to the group of polyenes and are produced dominantly in various *Streptomyces* strains. The heptaenes are divided into two subgroups on the basis of their aromaticity: aromatic and non-aromatic heptaenes. Heptaenes and other polyenes usually contain mycosamine as a sugar moiety [1]. The complete structure has been identified for only a few individual heptaenes such as amphotericin B, candicidin D (levorin A) and partricins A and D [1]. Most heptaene complexes, such as aureofungin (Fig. 1), are only partially characterized based on their UV spectra and the presence and structure of their aromatic and sugar moieties.

Aromatic heptaenes usually contain the *p*-aminoacetophenone (PAAP) moiety but some hep-

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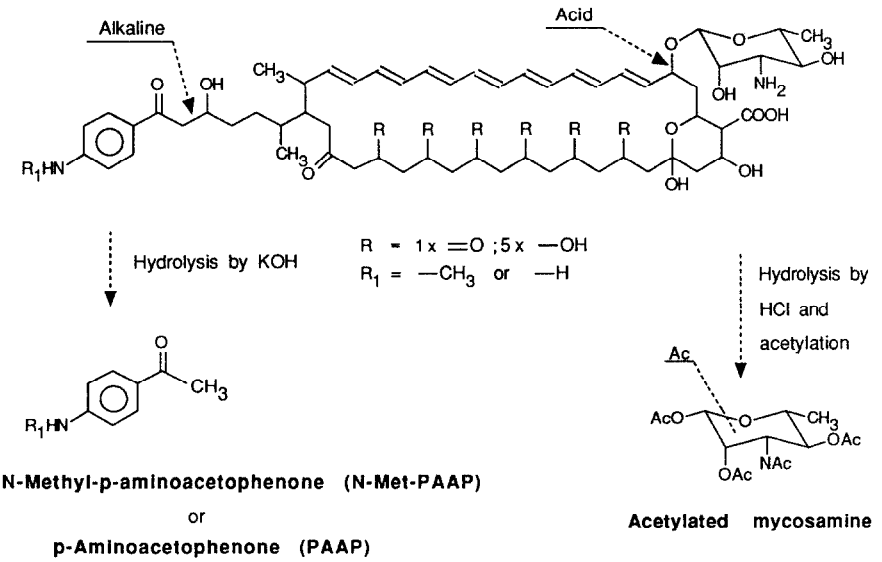

Fig. 1. Structures of the aromatic moieties (PAAP and N-methyl-PAAP) and acetylated sugar moiety mycosamin found in aureofungin. The structure of the aureofungin molecule is according to ref. 1.

taenes also contain N-methyl-*p*-aminoacetophenone (N-methyl-PAAP) moiety [1,2]. The aromatic moiety apparently originates from *p*-aminobenzoic acid (PABA), which has been postulated to be the starter unit for the biosynthesis of aromatic heptaenes [2]. Aromatic heptaenes are always produced as a complex of several individual heptaenic components [3,4]. The aromatic nature of heptaenes can be determined by hydrolysing the molecule with alkaline solution and identifying the released aromatic group by thin-layer chromatography (TLC) or gas chromatography–mass spectrometry (GC–MS) [5,6]. There are no previous data on the aromatic nature of the individual components in the heptaene complex. On the basis of the postulation that PABA is needed as a starter unit for the biosynthesis of the macrolide ring in candicidin [2] it is plausible, however, that all the components in candicidin and probably in other heptaene complexes are aromatic.

The aminosugar mycosamine (3-amino-3,6-dideoxy-D-mannose) is found in most glycosylated polyene macrolide antibiotics but its isomer perosamine (4-amino-4,6-dideoxy-D-mannose) has been found only in perimycin [1]. These aminosugars are attached to the macrolide ring with a β-glycosidic linkage which can be hydrolysed by treatment with acid [5,6]. The released mycosamine has been separated by TLC and identified using GC–MS [5,6]. The technique of ammonia chemical ionization within GC–MS has been successfully used for the determination of O-acetyl [7] and O-trimethylsilyl [8] derivatives of various carbohydrates, but not used for the determination of aminosugars of various polyenes.

In this work chromatographic methods for the determination of the aromaticity and mycosamine contents of some heptaenes were developed. The aromatic group in some heptaenes was determined by high-performance liquid chromatography (HPLC), HPLC–MS and GC–MS. The aromatic character of individual components of the candicidin complex was verified by determining the [^{14}C]PABA incorporation into the candicidin complex. The analysis of acetylated mycosamine was performed by GC–MS. The methods presented here are applicable to the characterization of new heptaenes. These methods are also useful for biosynthetic studies and for the development of analysis techniques for polyene residues in biological samples.

EXPERIMENTAL

Reagents

Heptaene antibiotics were supplied from several sources (Table I)) and they were dissolved in dimethylsulphoxide before use at a concentration of 1 mg/ml. For the [^{14}C]PABA incorporation experiments candicidin was synthesized in *Streptomyces griseus* (ATCC strain 11746).

Ammonium acetate (Merck, Darmstadt, Germany), PAAP (98%, Aldrich-Chemie, Steinheim, Germany), potassium hydroxide (Merck), methyl-α-D-mannoside (Sigma, St. Louis, MO, USA), radioactive [^{14}C]-*p*-aminobenzoic acid (2.04 GBq/mmol, Amersham, UK), acetonitrile (HPLC grade, Rathburn, Walkerburn, UK) and chloroform (Merck) were the main reagents and solvents. Water was purified using the Milli Q system (Millipore, Molsheim, France) and was sonicated before use. N-methyl-PAAP was synthesized from PAAP by methylation using methyliodide under basic conditions. Briefly, alkaline PAAP was shaken with chloroform containing methyl iodide. The synthesized *N*-methyl-PAAP was identified in the organic layer by GC–MS and used as a reference standard.

Assay of aromatic groups in heptaene

A small amount (1.5–8 mg) of heptaene and 300 μl of potassium hydroxide (10%, w/v) per milligram of polyene was boiled for 30 min in a water-bath (90 ± 5°C). After cooling the mixture was diluted about five fold with water, saturated with sodium chloride and extracted three times with about equal amounts of acetonitrile to achieve more than 99% recovery. As acetonitrile is miscible with water, sodium chloride was used to salt out the acetonitrile layer [9]. Before analysis the acetonitrile-containing phase was evaporated to dryness, redissolved in 200 μl of dichloromethane and filtered.

An aliquot (5–20 μl) of filtrates was analysed in an isocratic HPLC system (LC pump T 414, Kontron, Zürich, Switzerland) equipped with an UV detector (Uvicon 735 LC, Kontron) and an integrator (Enica 21, Delsi Instruments, Suresnes, France). The aromatic moieties were separated at ambient room temperature on a column (125 × 4 mm) filled with ODS Hypersil 5-μm reversed-phase (Bischoff Chromatography, Leonberg, Germany) using 0.05 *M* ammonium acetate buffer (pH 3.8)–acetonitrile (30:70, v/v) as the mobile phase. The flow-rate was 1.0 ml/min and the eluate was monitored at 314 nm. The determination of PAAP was calibrated by using PAAP instead of the antibiotic in the hydrolysis process.

A quadrupole VG Trio 2 mass spectrometer (VG Analytical, Manchester, UK) with the VG thermospray–plasmaspray probe was used to analyse the acetonitrile extracts of the hydrolysates. The samples (20 μl containing 2–10 μg of aromatic moieties) were pumped with a Kontron 420 dual-piston pump to the thermospray probe at a flow-rate of 1 ml/min using 0.10 *M* ammonium acetate buffer (pH 3.5)–acetonitrile (75:25) as the mobile phase and a 125 × 4 mm column filled with ODS Hypersil 5-μm phase (at ambient room temperature). The temperatures of the thermospray probe (210°C) and ion source (200°C) were adjusted so that a maximum solvent ion signal was achieved. The protonated

TABLE I

CLASSIFICATION OF HEPTAENES ANALYSED

Classification data are from ref. 1.

Heptaene	Aromatic moiety	Sugar moiety	Supplier
Amphotericin B	None	Mycosamine	Dumex, Copenhagen, Denmark
Aureofungin	PAAP	Mycosamine	Hindustan Antibiotics, Pimpri, India
Hamycin	PAAP	Mycosamine	Hindustan Antibiotics, Pimpri, India
Candicidin	PAAP	Mycosamine	Dumex, Copenhagen, Denmark
Thricomycin	PAAP	Mycosamine	Fujisawa Pharmaceutical, Osaka, Japan
Candimycin	None	No information	Takeda Chemical Industries, Osaka, Japan

molecular ions $(M+H)^+$ of PAAP (*m/z* 136) and N-methyl-PAAP (*m/z* 150) were monitored and their mass spectra recorded.

The presence of PAAP and N-methyl-PAAP in the acetonitrile extracts was also verified by GC–MS using electron ionization (EI). The mass fragmentation of PAAP and N-methyl-PAAP was performed with a JEOL JMS-D 300 (Jeol, Tokyo, Japan) mass spectrometer equipped with a JMA 2000 mass data analysis system. The samples were separated on a Hewlett-Packard gas chromatograph fitted with a 25 m × 0.2 mm I.D. fused-silica capillary column (0.1-μm NB-1 phase, HNU-Nordion, Helsinki, Finland). The EI fragmentation at 70 eV was investigated for all components possessing the fragment ions at *m/z* 135 (PAAP) or at *m/z* 149 (N-methyl-PAAP) and for other possible prominent ions which were found with HPLC–MS system.

Biosynthesis and HPLC of ^{14}C-labelled candicidin

The original candicidin-producing strain, *Streptomyces griseus* ATCC 11746, was grown on yeast–malt–glucose (YMG; 1, 2.5 and 1 g/l, respectively) agar (1%, w/v) plates. The primary culture was shaken in liquid YMG medium for 15–18 h. Studies of the [^{14}C]PABA incorporation in candicidin were made in YMG medium containing 750 000 dpm/ml [14C]PABA in labelling experiments. The medium (2 ml) was inoculated with 100–150 mg (wet weight) of primary culture shaken in glass tubes at a rate of 250 rev/min rpm at 28°C for 50 h. The cells were centrifuged at 1000 *g* and extracted with acetonitrile–water (300 μl per 100 mg wet cell mass).

The components of candicidin complex were separated with the same HPLC system as used for the aromatic groups and a 0.05 *M* ammonium acetate buffer (pH 3.8)–acetonitrile solution (38:62) was used as the mobile phase at a flow-rate of 1 ml/min (ambient room temperature). The components were monitored by UV absorption at 380 nm. A radioactivity flow detector (Radiomatic FlowOne/β CR, Radiomatic Instruments, Tampa, FL, USA) was used for ^{14}C-activity monitoring. Homogeneous detection in 2.5-ml flow cell was performed by mixing the 1 ml/min eluate with a 4 ml/min scintillant (Ecoscint, National Diagnostics, Manville, NJ, USA). Counts (print-out of average counts every 6 s) between energies of 5 and 100 keV were accepted and the integrated background was subtracted manually. The counting was about 68% effective, as determined by the injection of different amounts of pure [^{14}C]PABA into the counting cell.

Assay of mycosamine residue in heptaenes

To achieve the release of aminosugar from the polyene molecule, small amounts (10 mg) of candicidin or amphotericin B were incubated in 2 *M* hydrochloric acid at room temperature for 2 h [5]. Hydrolysis was confirmed with TLC on silica gel TLC plates (Kieselgel 60 F254, Merck) using a chloroform–methanol (1:1) solvent system. The sugars were identified by spraying the plates with a 1:1 mixture of 1% (v/v) ninhydrin and 3% iodoplatinate solution. The carbohydrates in the hydrolysates were acetylated after reduction [10] and the acetylated aminosugar (Fig. 1) was identified by ammonia chemical ionization MS after separation by GC in an OV-1 capillary column.

RESULTS

Aromatic groups

The chromatogram of aureofungin hydrolysate shows the separation of PAAP and N-methyl-PAAP (Fig. 2) on ODS Hypersil with UV detection at 315 nm. The recovery of the PAAP moiety in the extraction was more than 99% after three repeated extractions. Owing to the lack of aromatic groups, amphotericin B was assayed as a non-aromatic heptaene control. No interfering substances were seen in the hydrolysate of amphotericin B monitored at 315 nm.

The HPLC–MS profile of the hydrolysate of au-

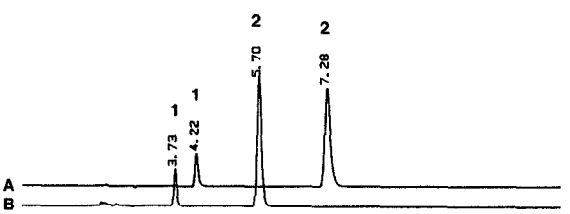

Fig. 2. HPLC separation of the aromatic components (about 4 μg total) (1) PAAP and (2) N-methyl-PAAP on ODS Hypersil C_{18} phase with acetonitrile–0.05 *M* ammonium acetate, pH 3.8. UV detection at 314 nm. The components were released from aureofungin by alkaline hydrolysis. The percentage of acetonitrile in mobile phase was (A) 25% or (B) 30%.

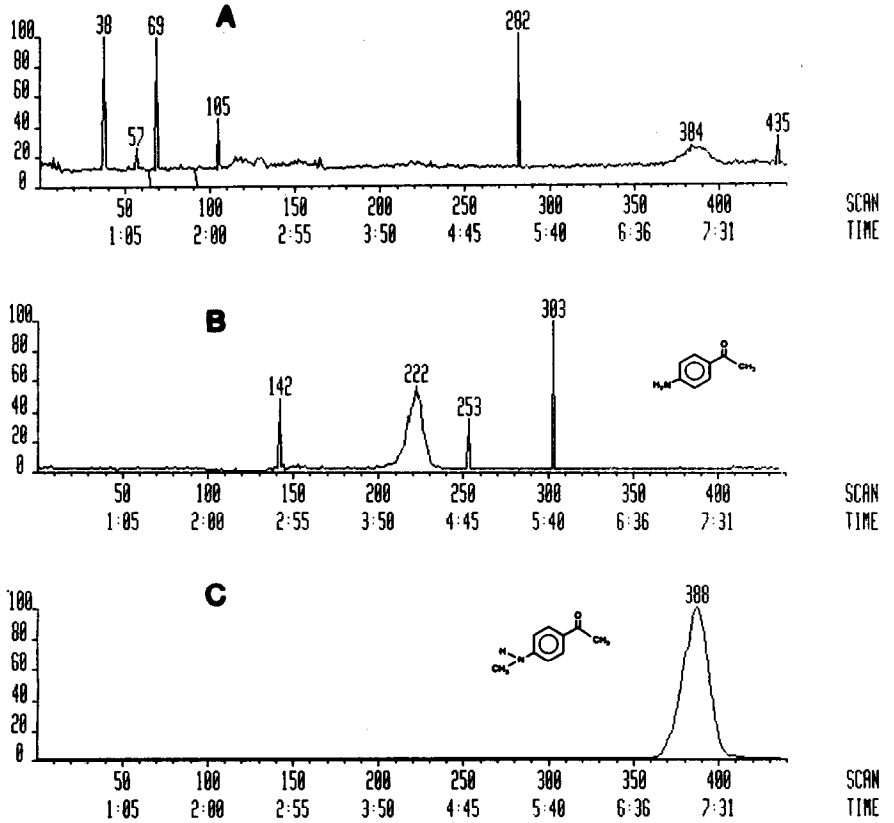

Fig. 3. HPLC–MS total ion current chromatogram (A), and HPLC–MS selected ion current profiles of *m/z* 136 (B) and *m/z* 150 (C) of aureofungin hydrolysate. The sharp peaks are due to the lack of background subtraction.

reofungin shows the separation of the peaks which were seen with HPLC at 315 nm (Fig. 3). Notice the noise peaks, which lowered the relative intensity of PAAP and N-methyl-PAAP (Figs. 3A and B). The peak had *m/z* value (M + 1) 136 for PAAP in aureofungin (Fig. 4A). Candicidin, hamycin and trichomycin also contained the PAAP signal (chromatograms not shown). In aureofungin the main peak was *m/z* 150 (Fig. 4B), indicating the presence of the N-methyl-PAAP moiety which was identified from the fragmentation data (Fig. 4B, inset). In trichomycin there was one minor peak in HPLC with an *m/z* value of 180 in the HPLC–MS system (data not shown). No fragmentation of PAAP and N-methyl-PAAP occurred in the HPLC–MS analysis but several ions originating from the solvent were found (Fig. 4A and B).

The peaks separated by HPLC were also verified by GC–MS. The most prominent ions of PAAP were the fragments *m/z* 92 and 120 (base peak), accompanied by the molecular ion *m/z* 135 (Fig. 4A, inset). The respective ions of N-methyl-PAAP were *m/z* 106, *m/z* 134 (base peak) and molecular ion *m/z* 149 (Fig. 4B, inset). The fragmentation of the ions *m/z* 135 and *m/z* 149 were similar to the PAAP and N-methyl-PAAP standards (spectra not shown).

According to the HPLC profiles, candicidin and hamycin contain only PAAP. Aureofungin and candimycin were identical, each containing about 30% PAAP and 70% N-methyl-PAAP. The trichomycin sample contained about 85% PAAP and 15% of some unknown component of molecular weight 179 which absorbed at 314 nm. The structure of this compound was not identified on the mass spectrum. The relative amounts of the peaks were calculated from the integrated areas.

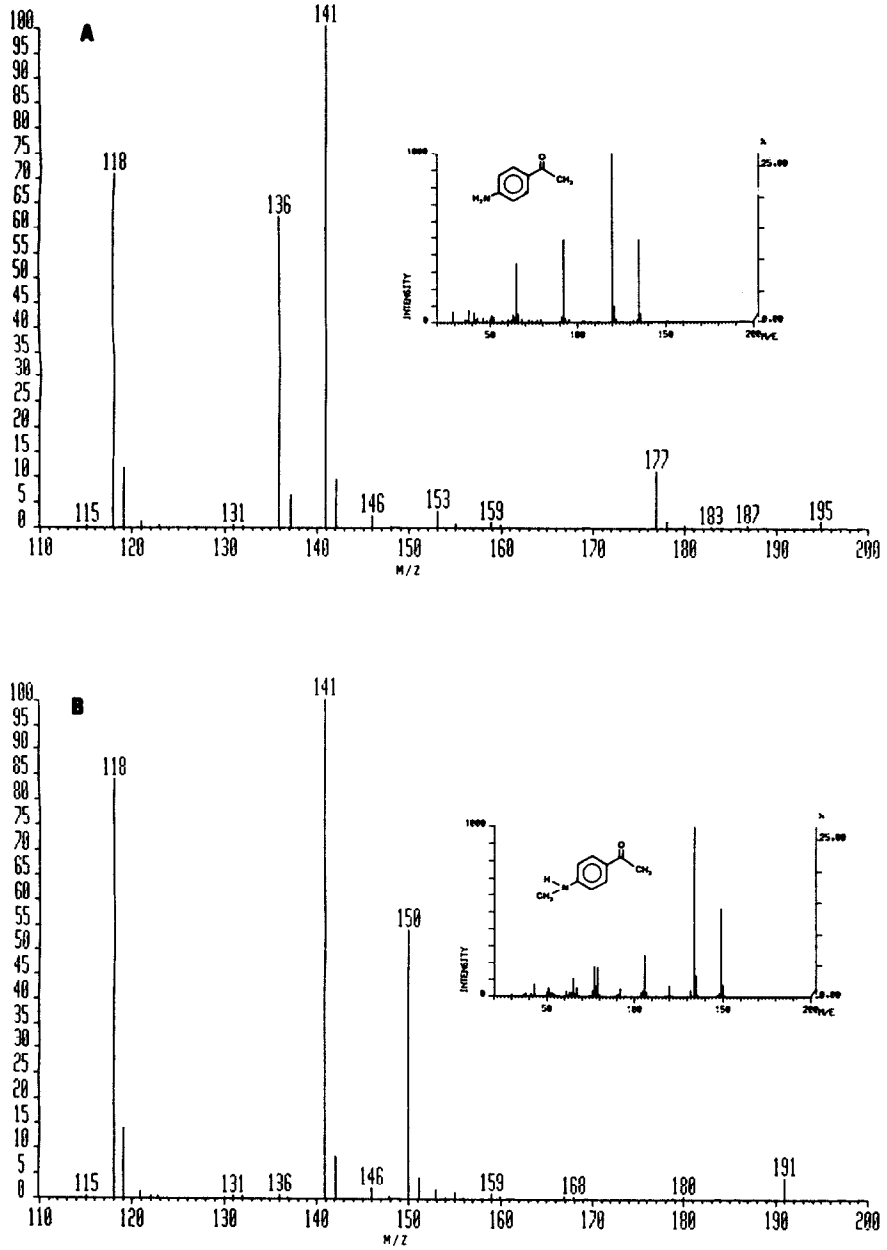

Fig. 4. Mass spectrum of PAAP (A; *m/z* 136) and N-methyl-PAAP (B; *m/z* 150) after HPLC separation. Ions 118, 141 and 177 originated from the eluent. The EI fragmentation spectrum (inset) of the aromatic components of aureofungin indicating the presence of PAAP (A) and N-methyl-PAAP (B) are shown also.

[^{14}C]PABA incorporation

The UV chromatogram of candicidin shows that synthesized heptaene includes three to four main and several other minor components (Fig. 5A). The presence of seven double bonds of the heptaene chromophore was investigated from the UV spectrum of the complex. Absorption maxima found at about 360, 380 and 404 nm are typical for heptaene

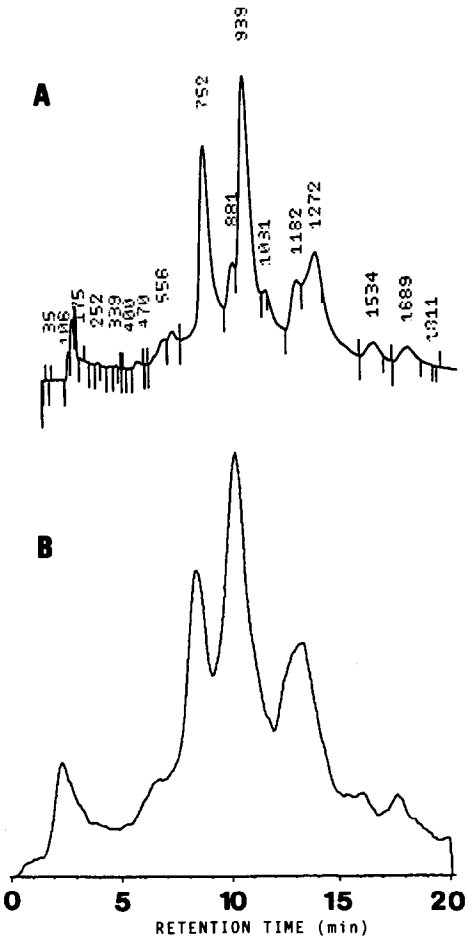

Fig. 5. HPLC separation of candicidin produced in *Streptomyces griseus* ATCC 11746. The UV-absorbance at 314 nm (A) or ^{14}C radioactivity (B) of candicidin was monitored.

polyenes [1]. The ^{14}C chromatogram shows the ^{14}C radioactivity of the candicidin complex (Fig. 5B). The first minor peak before the candicidin peaks probably comes from unused [^{14}C]PABA and may also include small amounts of labelled phenylalanine, tryptophan and tyrosine, which are known by products and suppressors of candicidin biosynthesis [2]. A comparison of the chromatograms presenting UV absorption and ^{14}C activity shows that the individual candicidin components are labelled, suggesting the incorporation of [^{14}C]PABA into the molecule. About 40% of the total activity added was incorporated into candicidin.

Mycosamine

The separation of the acetylated derivatives of mycosamine and α-methylmannoside was good using these GC conditions used (Fig. 6A). The retention time of the acetylated saccharide from the hydrolysate of candicidin (Fig. 6) was identical to the acetylated derivative of mycosamine found in the amphotericin B hydrolysate. The fragmentation was also similar in both candicidin and amphotericin. The ammonia chemical ionization mass spectrum of the acetylated mycosamine of candicidin exhibits strong proton-capture ($[M+H]^+$, m/z 376) and ammonium adduct ($[M+NH_4^+]^+$, m/z 393) ions (Fig. 6B). With these ions the molecular weight of the acetylated derivatives of the saccharide from polyene hydrolysates can be easily determined. The spectrum also exhibits a strong $[M+H]^+$–acetic acid ion (m/z 316). This ion was the base peak in the

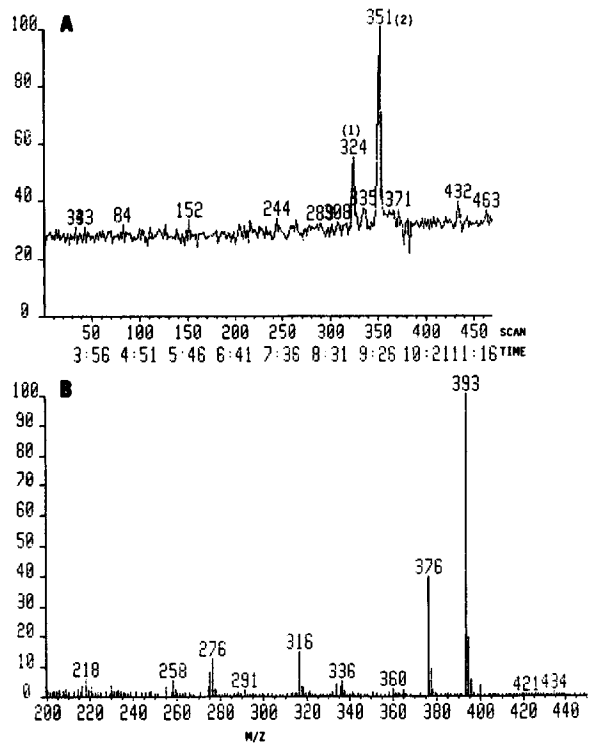

Fig. 6. Separation of internal standard α-methylmannoside (1) and mycosamine (2) with GC–MS using ammonia chemical ionization (A): total ion chromatogram of the acetyl derivatives from the hydrolysate of candicidin (A) and fragmentation of mycosamine from candicidin (B).

methane chemical ionization spectrum, whereas the molecular ion M + H and adduct ion $[M + C_2H_5]^+$ (*m/z* 404) were of much lower abundance. Ammonia was used for the ionization because of the total fragmentation of the molecular ion with methane ionization. The ammonia chemical ionization mass spectra of the internal standard α-methylmannoside derivative showed abundant $[M + H]^+$ ions with less fragmentation (data not shown).

DISCUSSION

The results indicate that this sample of aureofungin contained PAAP and N-methyl-PAAP (Fig. 1). It has been reported that aureofungin contains N-methyl-PAAP, but the PAAP moiety has not been mentioned [1]. Candicidin and hamycin contained only the PAAP residue, and candimycin contained PAAP and N-methyl-PAAP. The trichomycin extract contained PAAP and some unknown component of molecular weight 179 which was not characterized. [^{14}C]PABA incorporated into all the heptaene components found in the candicidin complex (Fig. 5.) and the percentage of incorporation was the same as described previously [2]. These results support the notion that all of the candicidin components contain an aromatic group, and generally all the individual components in aromatic heptaene complexes probably contain aromatic groups. This is in accordance with previous data that *p*-aminobenzoic acid (released as PAAP when hydrolysed with alkaline) is needed as the starter unit for the biosynthesis of the aglycone of aromatic heptaenes [2].

The methyl group in N-methyl-PAAP may originate from L-methionine as has been proposed previously for the biosynthesis of fungimycin [11]. Thus it is possible that the presence of both PAAP and N-methyl-PAAP in aureofungin could be due to limited methylation of PAAP during the biosynthesis of the antibiotic.

The identification of PAAP was confirmed by comparing the UV-spectra of the eluted peak and PAAP standard (data not shown). The UV spectra of the HPLC effluent at the PAAP peak seemed to be identical with the standard, supporting the similarity of the eluate and PAAP. Chlorination of PAAP occurred during extraction if chloroform was used (data not shown); this chlorination was not seen in the UV spectrum. To avoid false results from unwanted chlorinated aromatic groups, acetonitrile was used for the extraction of the released aromatic groups instead of chloroform.

The results of the mycosamine analysis show that the acetylation of mycosamine and subsequent GC–MS analysis with ammonia chemical ionization is the method of choice for the assay of the sugar moieties of all kind of polyene antibiotics.

Only a few milligrams of a heptaene were needed for the determination of the aromatic moiety and mycosamine. These methods are very convenient for the characterization and classification of new polyene antibiotics. The incorporation of radioactivity into the individual polyene components can be determined after HPLC separation with a radioactive flow detector and may be useful in studies of polyene biosynthesis.

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