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Short Communication

High-performance liquid chromatography of heptaene polyenes: assay of heptaene produced by *Streptomyces griseoviridis*

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ABSTRACT

The antibiotic complex of a new heptaene polyene produced in *Streptomyces griseoviridis*, which is antagonistic to phytopathogenic fungi, was analysed by high-performance liquid chromatography (HPLC) and monitored with a diode-array detector. For reference six known heptaenes were also assayed. The individual components were separated on ODS Hypersil C_{18} using gradient or isocratic elution. Gradient elution was performed using 0.005 *M* EDTA buffer modified with methanol and acetonitrile. Isocratic separations were made with 0.05 *M* ammonium acetate modified with acetonitrile. Both elution systems separated the main components, but the gradient system eluted the long-retained components faster. The chromatogram of the heptaene isolated from *S. griseoviridis* was compared with those of aureofungin, candicidin, candimycin, hamycin and trichomycin. The heptaene synthesized by *S. griseoviridis* is a candicidin type, as indicated by HPLC.

INTRODUCTION

Polyenes are mixtures of compounds having a characteristic chromophore which contains 3–8 conjugated double bonds in the macrolide ring. The structures of various heptaenes possess seven conjugated double bonds and differ in the number of hydroxyl, keto and methyl groups attached to the ring. The absence or presence of an aromatic moiety indicates the aromatic nature of heptaenes, and typically they also contain an amino sugar moiety, mycosamine [1]. The aromatic character and sugar moiety can be evaluated by using modern chromatographic techniques [2]. Heptaenes and other polyenes are synthesized mainly in *Streptomyces* bacteria as multi-component complexes which can be separated by counter-current distribution or thin-layer or high-performance liquid chromatography (HPLC) [3,4].

Several column liquid chromatographic methods for qualitative and quantitative analyses of various polyene antibiotics have been published. Amphotericin B, a clinically used antifungal antibiotic against systemic fungal infections, has been determined quantitatively by an HPLC technique [5]. Other polyene antibiotics are generally analysed by HPLC [3,4].

In this paper I present an HPLC method suitable

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for the assay of heptaene polyenes. The method was used for the assay of the heptaene complex formed in suppressive *Streptomyces griseoviridis* strains isolated from Finnish sphagnum peat [6]. One of these strains was introduced as a biological pesticide against fungal plant diseases [7], and an assay method is necessary for residue analysis.

EXPERIMENTAL

Polyenes

Seven heptaene polyenes were analyzed (Table I). The antibiotics were dissolved in dimethyl sulphoxide (DMSO) (Merck, Darmstadt, Germany) at a concentration of 1 mg/ml for HPLC analysis and were used within 1 day because of the instability of the DMSO solution of polyenes. The heptaene from *S. griseoviridis* (obtained from Dr. R. Tahvonen, Agricultural Research Centre, Jokioinen, Finland) was extracted from mycelium grown for 50 h (at 28°C) in a medium containing yeast extract, malt extract and glucose (1% each). This extract was used for HPLC analysis.

HPLC analysis

The HPLC equipment for gradient elution was a Hewlett-Packard (Waldbron, Germany) Model 1090 equipped with a diode-array detector and autosampler, integrator (HP 3392A) and computer (HP 85B) combined with a double disk drive (HP 9121). All the solvents used were of HPLC purity and water was purified with a Milli-Q system (Millipore, Molsheim, France).

All analyses were done by using columns (125 × 4.0 mm I.D.) filled with Shandon ODS Hypersil (5-μm particles) (Bischoff Chromatography, Leonberg, Germany) reversed-phase packing material.

Two solutions were used in the gradient separations: (A) 80% (v/v) 0.005 *M* ethylenediaminetetraacetic acid (EDTA) (pH 7.0) and 20% (v/v) acetonitrile–methanol (30:70) and (B) acetonitrile. The gradient (Fig. 1) was as follows:

$$0\ \text{min}\ \xrightarrow{20\%}\ 3\ \xrightarrow{20\text{–}35\%}\ 3.1\ \xrightarrow{35\text{–}40\%}\ 15\ \xrightarrow{40\text{–}60\%}$$

$$20\ \xrightarrow{60\text{–}60\%}\ 25\ \xrightarrow{60\text{–}20\%}\ 25.1\ \xrightarrow{20\text{–}20\%}\ 30\ \text{min}$$

where the percentages represent the amount or linear change in acetonitrile (B) in the mobile phase (1 ml/min) during the indicated periods.

Isocratic assays (Fig. 1) were performed with the HP 1090 system or using a system equipped with a Model T 414 LC pump (Kontron, Zürich, Switzerland), a Uvicon 735 LC detector (Kontron) at 380 nm, and an Enica 21 integrator (Delsi Instruments, Suresnes, France) using a normal injection system (Rheodyne). The mobile phase (1 ml/min) used in the isocratic elution was usually acetonitrile–0.05 *M* ammonium acetate, (pH 3.8) (40:60).

TABLE I

DESCRIPTION AND SOURCES OF THE ANALYSED HEPTAENE POLYENES[a]

Heptaene polyene	Sugar moiety	Aromatic moiety	Source[b]
Amphotericin B	Mycosamine	None	1
Aureofungin	Mycosamine	*p*-Aminoacetophenone N-Methyl-*p*-aminoacetophenone[c]	2
Candicidin	Mycosamine	*p*-Aminoacetophenone	1
Candimycin	Mycosamine	*p*-Aminoacetophenone N-Methyl-*p*-aminoacetophenone[c]	3
Hamycin	Mycosamine	*p*-Aminoacetophenone	2
Trichomycin	Mycosamine	*p*-Aminoacetophenone	4
Heptaene	Not assayed	Not assayed	5

[a] Data from ref. 1.

[b] 1 = Dumex (Copenhagen, Denmark); 2 = Hindustan Antibiotics (Pimpri, India); 3 = Takeda Chemical Industries (Osaka, Japan); 4 = Fujisawa Pharmaceutical (Osaka, Japan); 5 = this study.

[c] Data from ref. 2.

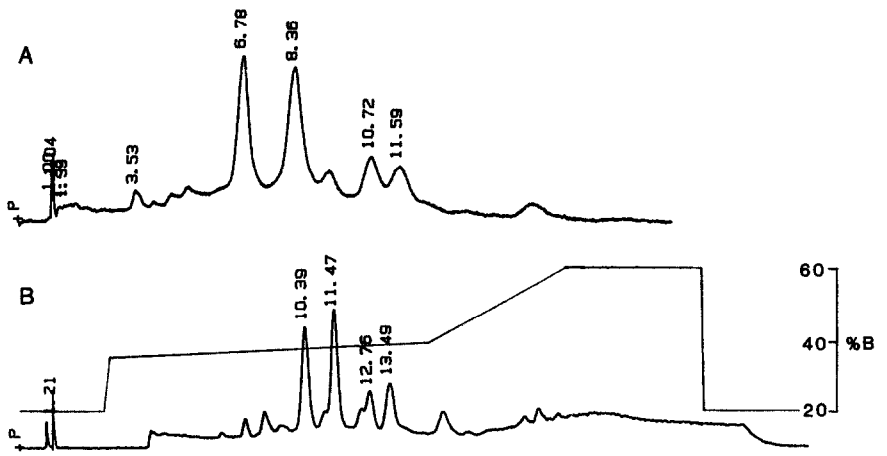

Fig. 1. HPLC separation of the individual components of candicidin complex (0.5 μg) with (A) isocratic or (B) gradient elution. The components were separated on ODS Hypersil C_{18}, monitored at 380 nm and eluted with the solvent compositions described in the text. Numbers at peaks indicate retention times in min.

RESULTS AND DISCUSSION

The chromatogram of a new heptaene isolated from *S. griseoviridis* was compared with those of the heptaene polyenes amphotericin B, aureofungin, candicidin, candimycin, hamycin and trichomycin. The chromatography of each (0.5 or 1.0 μg per injection) was performed using gradient or isocratic elution on 125 × 4 mm I.D. ODS Hypersil C_{18} columns and monitored at 380 nm (Fig. 1). The gradient system eluted all components in less than 30 min.

The heptaene produced by *S. griseoviridis* has one main component, but the pattern of the individual components is of the candicidin type. The relative intensities of the peaks differ from those of candicidin used as a reference standard (Fig. 2). This may be due to differences in the producer strains and in the composition of the culture media. The chromatogram shows also that there are many long-retained compounds dissimilar to candicidin. It is possible, however, that these long-retained components are produced by decomposition during

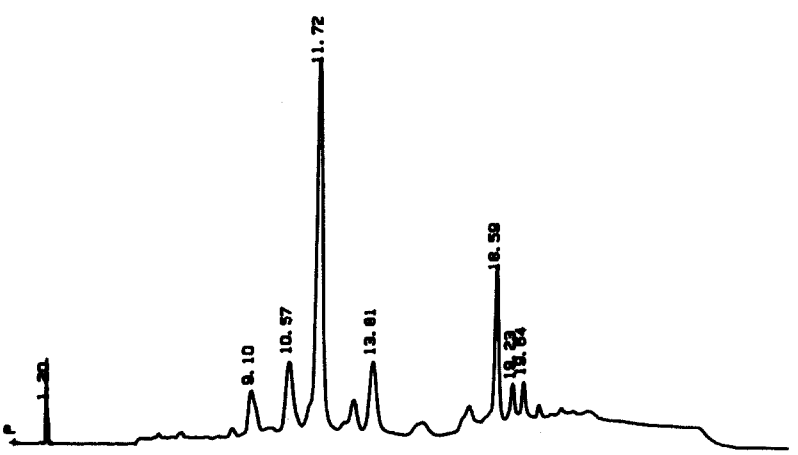

Fig. 2. HPLC of the heptaene polyene complex (1 μg) isolated from *S. griseoviridis*. Conditions as in Fig. 1B.

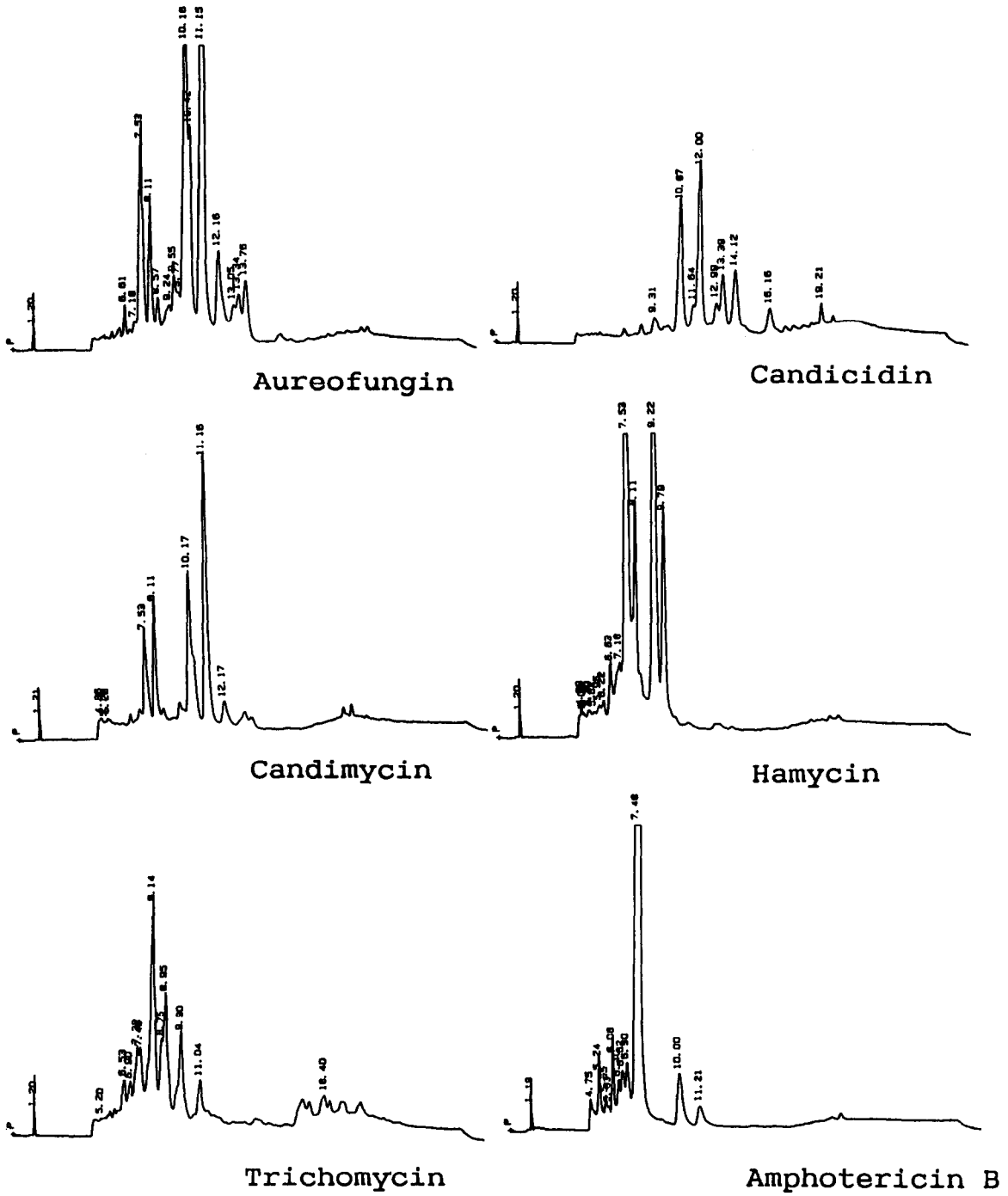

Fig. 3. HPLC of aureofungin, candicidin, candimycin, hamycin, trichomycin and amphotericin B (1.0 μg each). Conditions as in Fig. 1B.

the isolation of the polyene complex. By using the diode-array detector the heptaene character of these and the main peaks was also verified (data not shown).

Amphotericin B, a clinically used non-aromatic heptaene, consists of one major heptaene component and several other insignificant heptaene components (Fig. 3). Four aromatic heptaenes (hamycin, aureofungin, trichomycin, and candimycin) include 3–5 main components and all of them have many minor components (Fig. 3). The chromatograms of hamycin, aureofungin, trichomycin and candimycin were different from that of candicidin, but the HPLC data indicate that candimycin has the same individual components as aureofungin.

The further chemical characterization and biological effects of the heptaene produced by *S. griseoviridis* will be published separately.

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