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## Note

# Isolation of the aromatic heptaenic antibiotics trichomycin A–F by high-performance liquid chromatography

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Aromatic heptaene antibiotics possess a large lactone ring containing a hydroxylated portion, seven conjugated double bonds, an amino sugar and a characteristic aromatic moiety. Aureofungi, candicidin, DJ 400, hamycin, levorin, lucknomycin, partricin and trichomycin belong to this group. Trichomycin was the first member to be discovered[1,2].

Trichomycin, a potent and clinically useful antifungal drug, especially as the trichomonacide, is produced by *Streptomyces hachijoensis* from a soil on the Pacific Island, Hachijo Jima, Japan. Two compounds, trichomycin A and B, were first isolated from the *Streptomyces* by counter-current distribution methods[3]. The structure of the major constituent, trichomycin A, was tentatively deduced on the basis of chemical degradations[4]. It is surprising that the antibiotic has always been considered to be a mixture of only two components, trichomycin A and B.

However, it was subsequently reported that trichomycin is a mixture of more than ten components as determined by high-performance liquid chromatography (HPLC) by Helboe *et al.* in 1980[5], and that it is a complex mixture of ten components by thin-layer chromatography (TLC) and sixteen components by HPLC by Thomas and Newland in 1986[6]. None of these methods, however, appeared suitable for preparative-scale application. Our more recent detailed HPLC studies on the antibiotic indicated that it consists of more than seventeen closely related compounds and made possible the separation of the trichomycin complex into trichomycin A–F.

These studies were concerned with the isolation of six components, trichomycin A–F, representing about 65–75% of the total trichomycin.

The structure of trichomycin A, the major component, is shown in Fig. 1[7].

## EXPERIMENTAL

### *Materials*

A mixture of trichomycin was obtained from the extracts of the mycelial cake of *Streptomyces hachijoensis*.

### *Flash liquid chromatography*

Flash liquid chromatography (FLC) was carried out on a Yamazen FMI-C

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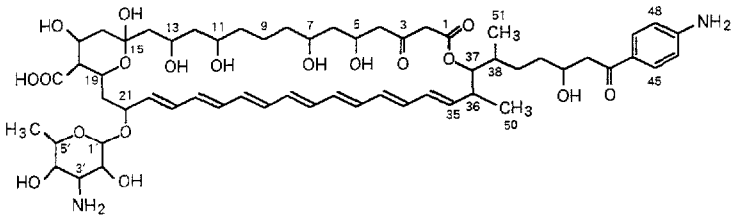

Fig. 1. Structure of trichomycin A.

instrument with a Yamazen Prep UV-1OV spectrophotometric detector. A silica gel column consisting of a 30 × 3 cm I.D. glass column packed with Fuji gel CQ-3 (particle size 30–50 μm) (Fuji Gel Hanbai) was used. The mobile phase consisted of the lower phase of a chloroform–methanol–water (2:2:1) mixture. Samples of 15 ml of solutions containing 80 mg of trichomycin in the same solvent were usually charged on to the column. Chromatography was carried out at a flow-rate of 4.0 ml/min with UV–VIS detection at 360 nm. Generally, two peaks were obtained, as shown in Fig. 3. The first fraction included trichomycin B and F and the second included trichomycin A, C, D and E. Each fraction was submitted to preparative HPLC.

*Analytical HPLC*

HPLC was carried out with a Hitachi 635 instrument with a Shimadzu SPD-1 spctrophotometric detector. A Nucleosil 5C8 column (particle size 5 μm; Macherey, Nagel & Co.) of dimensions 150 × 4 mm I.D. packed with $C_8$ reversed-phase silica was used. A 3-μl volume of solution containing 1 mg/ml of sample in dimethylformamide was usually injected. The mobile phase was acetonitrile–(phosphate–citrate buffer) (32.5:67.5, pH 4.6). The buffer was prepared from 33.47 g of $Na_2HPO_4 \cdot 12H_2O$ and 11.18 g of citric acid in 1 l of water. Chromatography was carried out at a flow-rate of 1.0 ml/min with UV–VIS detection at 360 nm.

*Preparative HPLC*

HPLC was carried out on a Hitachi 635 instrument with a Shimadzu SPD-1 spectrophotometric detector. A μBondasphere $C_{18}$ column (particle size 5 μm; Waters Assoc.) of dimensions 150 × 19 mm I.D. was used. The mobile phase was acetonitrile–(phosphate–citrate buffer) (34.5:65.5, pH 4.75). A 0.2-ml volume of DMF solution containing 2 mg of trichomycin obtained from FLC was usually injected. Chromatography was carried out at a flow-rate of 4.0 ml/min and the column pressure ranged from 95 to 98 kg/cm$^2$, with detection at 355 nm. The peaks corresponding to trichomycin A–F were cut out manually in the following order: 76–80 ml (trichomycin C), 92–96 ml (D), 100–107 ml (E), 110–149 ml (A), 150–175 ml (B) and 215–255 ml (F). Each fraction was concentrated *in vacuo* in the dark until there was no odour of acetonitrile. The concentrate was extracted twice with 20% of its volume of *n*-butanol. The *n*-butanol extracts were washed twice with water and then concentrated *in vacuo*, adding water, to given an opaque solution, in the dark. The precipitated fine yellow powder was filtered off, washed throughly with water in order to desalt it and dried *in vacuo* over $P_2O_5$. Rechromatography was carried out to increase the purity as required.

The operating conditions are given in Figs. 2 and 3.

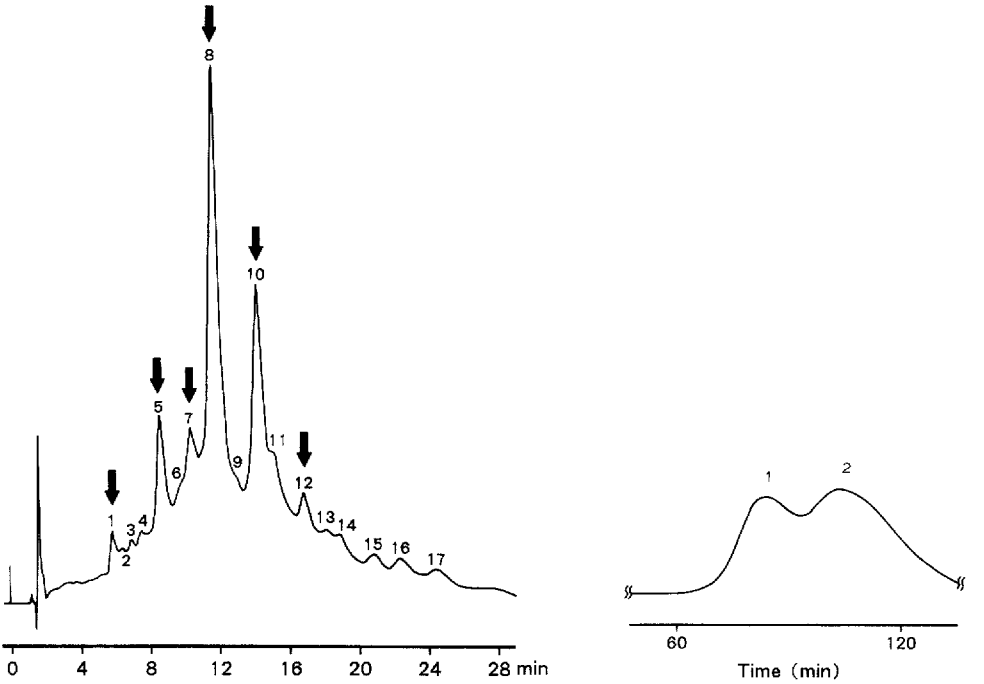


Fig. 2. Chromatogram of trichomycins by HPLC. Column: Nucleosil 5C8, 5 μm (150 × 4 mm I.D.). Mobile phase: acetonitrile–(phosphate–citrate buffer) (32.5:67.5, pH 4.6). Detection: 360 nm. Flow-rate: 1.0 ml/min. The isolated components are shown by arrows. Peaks: 1 = trichomycin C; 5 = trichomycin D; 7 = trichomycin E; 8 = trichomycin A; 10 = trichomycin B; 12 = trichomycin F.

Fig. 3. Chromatogram of trichomycins by FLC. Column: silica gel (Fuji gel CQ-3). Mobile phase: the lower phase of a chloroform–methanol–water (2:2:1) mixture. Detection: 360 nm. Flow-rate: 4.0 ml/min. Peaks: 1 = trichomycin B and F; 2 = trichomycin A, C, D and E.

*Assay and testing procedures*

Antibiotic potency was measured by a microbiological cylinder-plate method[8].

## RESULTS AND DISCUSSION

Fig. 2 shows a chromatogram of a mixture of trichomycins. Seventeen peaks were completely resolved on the Nucleosil 5C8 column. Good resolution of the antibiotic was obtained with acetonitrile–(phosphate–citrate buffer) (32.5:67.5, pH 4.6) as the solvent at a flow-rate of 1.0 ml/min.

These results prompted us to use a similar solvent system for the preparative isolation of the main components from a mixture of trichomycins. The desired main components were concentrated by chromatography on alumina and silica gel, followed by FLC on silica gel with the lower phase of a chloroform–methanol–water (2:2:1) mixture. The components studied divided into two groups. One group

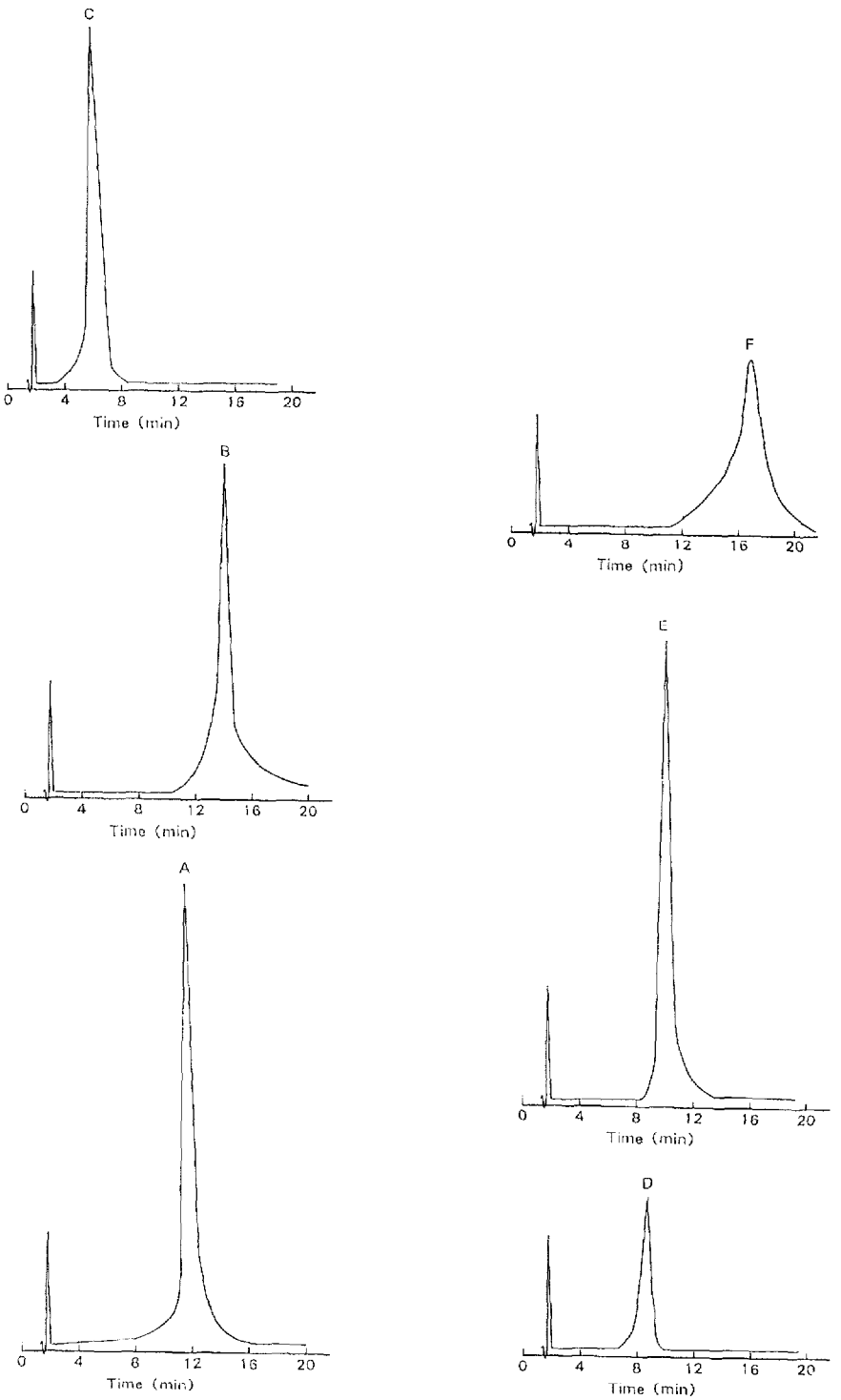


Fig. 4. Chromatogram of trichomycin A–F after isolation by preparative HPLC. Chromatographic conditions as in Fig. 2.

contained trichomycin B and F, and the other trichomycin A, C, D and E. Each fraction was submitted to preparative HPLC on μBondasphere $C_{18}$ with acetonitrile–(phosphate citrate buffer) (34.5:65.5, pH 4.75).

In conclusion, six main components of trichomycin, shown by arrows in Fig. 2, were isolated simultaneously by preparative HPLC. Although trichomycin A–F were effectively separated by only one procedure, a more satisfactory result was obtained by rechromatography under the same conditions. A chromatogram of trichomycin A–F after preparative HPLC is shown in Fig. 4.

A major advantage of this system is therefore that it has made it possible to clarify the structures and characterization of trichomycin A–F and other closely related compounds. The HPLC method reported here is also very suitable for the analysis and preparation of polyene macrolides.

The structure determination of trichomycin A[7] and B[9] and the properties of trichomycin A–F will be described elsewhere[9].

Trichomycin A, the major component, possesses the greatest potency against diverse fungi and yeasts, and exhibits a lower minimum inhibitory concentration against many kinds of candidas and trichomonas than amphotericin B and ketoconazole, a well known antifungal drug.

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