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Note

Preparative high-performance liquid chromatography of macrotetrolides

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The macrotetrolides nonactin (I), monactin (II), dinactin (III) and trinactin (IV) are cyclopolylactone antibiotics (Fig. 1), and have been identified in several streptomycetes species[1–4].

The compounds have so far been isolated by means of classical column chromatography on silica gel[2] or by counter-current distribution[3]. However, these procedures have poor selectivity[5] and are very time consuming. Thin-layer chromatography (TLC) on silica gel has only been used to identify individual macrotetrolides[2,3] and preparative TLC is impossible owing to the very low capacity of the plate (0.5–1.0 μg per spot)[6,7].

It was the aim of this study to isolate I–IV from their crystalline mixture by means of preparative high-performance liquid chromatography (HPLC) on silica gel using UV detection for physico-chemical investigations and for biological experiments.

The method is fast, one preparative run requiring about 1 h, components are detected directly in the column effluent and it is possible to inject about 16 mg of the macrotetrolide mixture for one chromatographic run.

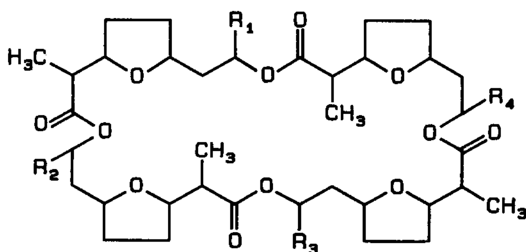

Fig. 1. Structural formulae of microtetrolides. (I) $R_1 = R_2 = R_3 = R_4 = C_2H_5$, nonactin; (II) $R_1 = R_2 = R_3 = CH_3$, $R_4 = C_2H_5$, monactin; (III) $R_1 = R_2 = CH_3$, $R_3 = R_4 = C_2H_5$, dinactin; (IV) $R_1 = CH_3$, $R_2 = R_3 = R_4 = C_2H_5$, trinactin (see ref. 7).

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EXPERIMENTAL

Sample

A crystalline complex of macrotetrolides I–IV was obtained by extracting the lyophilized mycelium of *Streptomyces griseus* LKS-1 with *n*-hexane. The extracted material was repeatedly crystallized and further purified by means of classical column chromatography on silica gel in chloroform–ethyl acetate (2:1, v/v). Prior to the injection the crystalline macrotetrolide mixture was dissolved in degassed mobile phase.

Chemicals

n-Hexane and isopropyl alcohol for UV spectrophotometry (Lachema, Brno, Czechoslovakia) and distilled water were used.

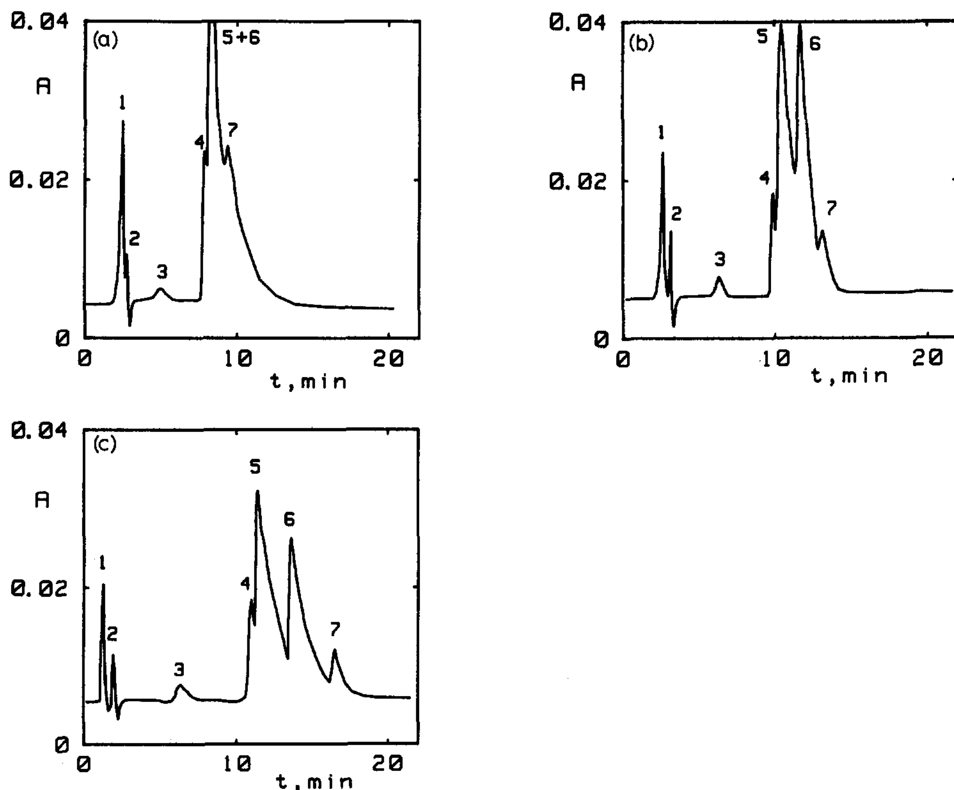

Fig. 2. Analytical separations of a mixture of macrotetrolides I–IV. (a) Column, 250 × 4 mm I.D., packed with Separon SGX, 7 μm; mobile phase, *n*-hexane–isopropyl alcohol containing 4% (v/v) of water (92:8, v/v); temperature, 45°C; flow-rate, 1 ml min^{-1}; detection, UV, 215 nm. Peaks: 1,2 = mobile phase components; 3 = unidentified mixture; 4 = trinactin (IV); 5 = dinactin (III); 6 = monactin (II); 7 = nonactin (I). (b) Mobile phase, *n*-hexane–isopropyl alcohol containing 4% (v/v) of water (95:5, v/v). Other conditions as in (a). (c) Mobile phase, *n*-hexane–isopropyl alcohol containing 4% (v/v) of water (97:3, v/v). Other conditions as in (a).

Analytical and preparative chromatography

An SP 8000B high-performance liquid chromatograph (Spectra-Physics, Santa Clara, CA, U.S.A.) with an SP-770 variable-wavelength detector with an 8-μl flow cell at 215 nm (Schoeffel Instruments, Westwood, NJ, U.S.A.) was used. The absorbance was set to 0.04 and 0.2 for the analytical and preparative separations, respectively. Chromatograms were recorded on a strip-chart recorder at a chart speed of 0.25 cm min^{-1} and a setting of 10 mV.

For analytical purposes an SP 8780 XR automatic injector equipped with a 20-μl injection loop was applied. A stainless-steel column (250 $\times$ 4 mm I.D.) was packed with Separon SGX silica gel, 7 μm (Laboratorni pristroje, Prague, Czechoslovakia).

For preparative purposes a stainless-steel column (250 $\times$ 23 mm I.D.) packed with LiChrosorb Si 60-5 silica gel (Chrompack, Middelburg, The Netherlands) was used. A 100-μl injection loop was used.

RESULTS AND DISCUSSION

Analytical chromatography

Fig. 2 shows chromatograms of the macrotetrolide mixture at a concentration of 2.8 mg ml^{-1} for various compositions of the mobile phase [*n*-hexane–isopropyl alcohol containing 4% (v/v) of water] at 45°C and a flow-rate of 1 ml min^{-1}. When the content of isopropyl alcohol in the mobile phase was higher than 8% (v/v), individual components of the macrotetrolide did not separate, and when its content was lower than 3% (v/v) the components were not eluted in 120 min.

Fig. 3 shows chromatograms of the same mixture with the mobile phase *n*-hexane–isopropyl alcohol containing 4% (v/v) of water (95:5, v/v) at 20, 35 and 45°C. The separation improves with increasing temperature, owing to the decreased viscosity of the mobile phase and, consequently, to improved mass transfer between the stationary and mobile phases, resulting in improved column efficiency.

Optimal conditions for the analytical separation were 45°C, *n*-hexane–isopropyl alcohol containing with 4% (v/v) of water (95:5, v/v) as the mobile phase and flow-rate 1 ml min^{-1}, and the time of analysis did not exceed 20 min.

NMR spectroscopy and spiking studies confirmed that peaks 4, 5, 6 and 7 correspond to compounds IV, III, II and I, respectively. Peaks 1 and 2 correspond to the mobile phase components and peak 3 is an unidentified mixture.

Preparative chromatography

When the conditions used in the analytical separation were applied to the preparative column, it was not possible to elute the components of the chromatographed mixture even after 120 min. By means of empirical optimization the following conditions were determined: *n*-hexane–isopropyl alcohol containing 4% (v/v) of water (80:20, v/v) as the mobile phase, 35°C, flow-rate 10 ml min^{-1} and concentration of macrotetrolide mixture, 16 mg per 100 μl of the mobile phase; the time of one preparative run was less than 1 h. The above conditions made it possible to achieve a resolution between neighbouring components of better than unity.

According to the detector response, fractions 3–7 were collected; peaks 1 and 2 corresponded to the mobile phase components (Fig. 4). On the basis of NMR and

Fig. 3. Analytical separations of a mixture of macrotetrolides I–IV. (a) Column as in Fig. 2a; mobile phase, *n*-hexane–isopropyl alcohol containing 4% (v/v) of water (95:5, v/v); temperature, 20°C. Other conditions as in Fig. 2a. (b) Temperature, 35°C. Other conditions as in Fig. 2a. (c) Temperature, 45°C. Other conditions in Fig. 2a.

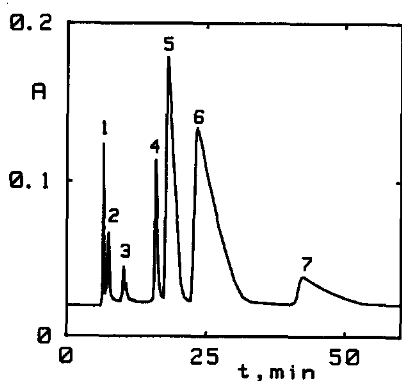

Fig. 4. Preparative separation of a mixture of macrotetrolides I–IV. Column, 250 × 23 mm I.D., packed with LiChrosorb Si 60-5; mobile phase, *n*-hexane–isopropyl alcohol containing 4% (v/v) of water (80:20, v/v; temperature, 35°C; flow-rate, 10 ml min^{-1}; detection, UV, 215 nm. Peaks: 1,2 = mobile phase components; 3 = unidentified mixture; 4 = nonactin (I); 5 = monactin (II); 6 = dinactin (III); 7 = trinactin (IV).

mass spectra, it was found that peaks 4, 5, 6 and 7 are identical with compounds I, II, III and IV, respectively, indicating that in the preparative column the order elution of individual compounds is the reverse of that in the analytical column. Peak 3 is an unidentified mixture.

The retention time of peak 1 in Fig. 4 after *n*-hexane injection was taken as the hold-up elution time (390 s). The capacity ratios of peaks 4, 5, 6 and 7 were 1.4, 1.7, 2.5 and 5.4, respectively.

From a single preparative run (16 mg of mixture), 1.0 mg of I, 4.2 mg of II, 8.1 mg of III, 1.5 mg of IV and 0.3 mg of the mixture designated 3 were obtained. An amount of 0.9 mg was lost. The above mass balance corresponds roughly to the proportions of the individual components calculated from the peak areas.

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