Preparative Separation and Analysis of Complex Mixtures of Leucomycins and Desmycarosyl Leucomycins Using HPLC and Mass Spectrometry

P. Gebhardt, A. Perner, U. Gräfe
Hans-Knoell-Institute for Natural Products Research, Beutenbergstrasse 11a, 07745 Jena, Germany; E-Mail: Peter.Gebhardt@hki-jena.de

Received: 4 November 2003 / Revised: 19 April 2004 / Accepted: 30 April 2004
Online publication: 9 July 2004

Abstract

Composition and hydrolysis products of a biotechnical available complex of macrolides were analyzed by HPLC and ESI-CID-MS. Major components are leucomycin-type antibiotics (leucomycin A1, A7, A9, 9-desoxy-9-oxo-turimycin H3 and niddamycin B). Hydrolysis of the complex mixture yielded 9α-, 13β-isoforocidins (7, 8, 9, 11) and 9-oxo-forocidin (10). A preparative separation procedure was elaborated furnishing compounds 3-11 for semisynthetic experiments.

Keywords

Column liquid chromatography
ESI-CID-MS
Leucomycin complex
Preparative separation of leucomycins and desmycarosyl leucomycins

Introduction

Non-polyene macrolide antibiotics have widely been used in the treatment of bacterial infections. Previously, new attention was paid to the macrolides as antibacterial agents due to the development of resistant Gram-positive bacteria such methicillin-resistant Staphylococcus aureus and others [1–3]. Azalides and ketolides [4–6], derivatives of the 14-membered macrolide antibiotic erythromycin opened new horizons for the treatment of infectious diseases. Comparably few semisynthetic work was dedicated to 16-membered macrolides such as leucomycins [7, 8] and spiramycins [9–11]. Recent papers on derivatives of tylosin [12–14] suggested an increasing interest in the 16-membered macrolides and improvement of their biological activities, too. However, a major problem of semisynthetic modification of 16-membered macrolide antibiotics such as leucomycins was the occurrence of complex mixtures of homologous and isomeric structures amongst the biotechnical available products. Hence, first steps towards new derivatives, e.g. of the leucomycins, should involve analysis of the naturally occurring products and elaboration of suitable separation procedures for purified components. Analytical HPLC of leucomycins (such as e.g. turimycin) was reported earlier [15]. However, the advent of electrospray MS and diagnostic fragment formations under soft conditions (CID-MS-MS and CID-MSn) enable a more detailed characterization of the individual components, even of the macrolide antibiotics [16–19].

Here we report the analytical characterization of leucomycins (turimycins) from Streptomyces hygroscopicus JA 6595 R27 by HPLC-ESI+CID-MSn via their fragmentation behavior. Moreover, a preparative separation procedure for a naturally occurring mixture of leucomycins and their hydrolysis products (desmycarosyl leucomycins) was elaborated.

Experimental

Materials

The leucomycin complex (turimycins) was obtained from the fermentation broth of Streptomyces hygroscopicus as described elsewhere [20].

Mass Spectrometry

Routinely ESI-MS of lyophilized HPLC fractions were recorded on a VG Quattro of Fisons Instruments equipped with electrospray ion source and a 10 μL sample loop. The flow rate was 20 μL min−1 MeOH/H2O 99/1 (v/v). A Finnigan LCQ benchtop mass spectrometer equipped with electrospray ion source and ion-trap mass analyzer was used for ESI-CID-MSn.
Fragmentation (CID-MS) of pseudomolecular ions ([M+H]⁺, [M+Na]⁺) was done by collision-induced dissociation using helium as collision gas. Spray voltage was 5 kV, capillary temperature 200°C, and the flow rate 40 μL/min. 1 MeOH/H₂O 99/1 (v/v).

HREI-MS was recorded on a Finnigan MAT 95XL sector field mass spectrometer equipped with a direct inlet system (ionization energy 70 eV).

NMR spectra were measured on Bruker Avance DRX 300 and DRX 500 instruments. The concentration was about 0.08 mol L⁻¹ or 30 mg sample in 0.5 mL CDCl₃.

Preparative HPLC

The HPLC system was a product of Gilson (France) and consisted of master and slave pumps, UV-VIS 156 detector, a manometric modul and gradient mixer. Data were recorded on PC using Gilson software. Preparative scale separations were by a Knauer, (Berlin, Germany) vertex HPLC-column (250 mm × 32 mm, Nucleosil Eurosphere RP18, 7 μm) and by a Rheodyne valve with 10 μL loop. The detector wave length was 232 nm. The crude ethyl acetate extract from Streptomyces hygroscopicus HKI-strain RH27 respectively the acid hydrolysis products were dissolved in 5 mL of ACN/water 87/13% (v/v/w) and evaporated. 1.62 g of an oily residue of acyl mycaroses was obtained. The pH of the weak acidic aqueous phase was adjusted to 8–9 with NaOH (20%, w/v) and extracted 8 times with 50 mL of chloroform. The combined organic solutions were dried over Na₂SO₄ and evaporated under high vacuum. 1.96 g of demycarosylproducts as solid yellowish foam was obtained. The conditions of preparative HPLC and the results are given in Table 1b.

Results and Discussion

A complex mixture of leucomycin-type antibiotics as products of Streptomyces hygroscopicus JA 6595-R27 was separated preparative by HPLC using a binary gradient of ACN/H₂O containing 0.05% diethylamine to adjust the pH at 8.5. Five major peaks or six components (1-6) appeared subsequently (Fig. 1(a)). The isolated fractions were collected and

| Table 1a. Retention time and yields for preparative HPLC of leucomycins, Conditions: 400 mg crude leucomycin complex, flow 20 mL/min⁻¹, eluent starts with 31% ACN, 69% water, 0.05% Et₂NH (v/v/v), after 5 min. unto 25 min. gradient flow 83% ACN, 17% water, 0.05% Et₂NH (v/v/v) |
|---|---|---|---|---|---|
| Retention time (min) | 1/2 | 3 | 4 | 5 | 6 |
| Yields (mg) | 23.1 | 25.0 | 27.2 | 28.9 | 30.9 |

| Table 1b. Retention time and yields for preparative HPLC of forocidins, Conditions: 500 mg forocidine mixture, flow 20 mL/min⁻¹, eluent starts with 5% ACN, 95% water, 0.05% Et₂NH (v/v/v), after 5 min. unto 35 min. gradient flow 83% ACN, 17% water, 0.05% Et₂NH (v/v/v) |
|---|---|---|---|---|---|
| Retention time (min) | 7 | 8 | 9 | 10 | 11 |
| Yields (mg) | 25.5 | 26.3 | 27.4 | 28.6 | 30.6 |

Acidic Hydrolysis of Turimycin

10 g of turimycin base, batch number 181, was dissolved in 150 mL of 0.3 M HCl. The resulting yellow solution was kept for 20 h at ambient temperature. After carefully increasing of pH to 3–4 with aqueous NaOH (20%, w/v) it was extracted 10 times with 50 mL chloroform each. The combined organic extracts were dried over Na₂SO₄ and evaporated. 1.62 g of an oily residue of acyl mycaroses was obtained. The pH of the weak acidic aqueous phase was adjusted to 8–9 with NaOH (20%, w/v) and extracted 8 times with 50 mL of chloroform. The combined organic solutions were dried over Na₂SO₄ and evaporated under high vacuum. 1.96 g of demycarosylproducts as solid yellowish foam was obtained. The conditions of preparative HPLC and the results are given in Table 1b.

Fig. 1. HPLC-chromatograms of the (a) leucomycin-complex and (b) hydrolysis products of the leucomycin-complex