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Note

Isolation of erythromycin E from commercial erythromycin*

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In a previous communication we reported the isolation of novel compounds from commercial erythromycin by thin-layer chromatography (TLC)[1]. In order to separate these compounds by high-performance liquid chromatography (HPLC) a previously described HPLC method was further investigated[2]. Using low concentrations of organic modifier, a new compound was separated from erythromycin A in commercial samples, see Fig. 1. The compound was found to be present at levels of 1–2%, expressed as erythromycin A.

The compound was isolated by liquid chromatography, and identified by nuclear magnetic resonance (NMR) and mass spectrometry (MS) as erythromycin E (EE). The structure of EE is shown in Fig. 2. EE has been described by Martin *et al.*[3]. The compound has a low antibiotic activity and is considered by Omura[4] to be obtained through transformation of erythromycin A (EA). Indeed, EE was obtained

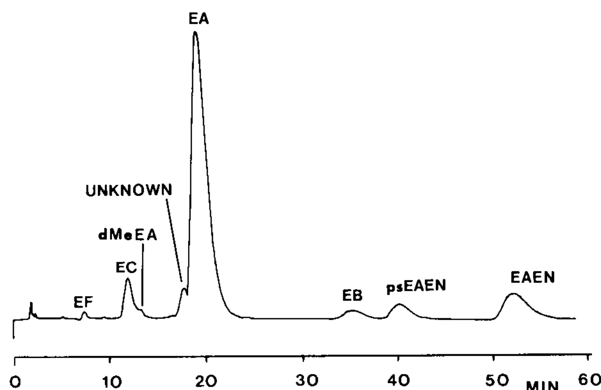

Fig. 1. Chromatogram of the commercial sample from which erythromycin E was isolated. Column: Partisil ODS, 10 μm, 25 cm × 4.6 mm I.D. Mobile phase: acetonitrile–0.2 *M* tetramethylammonium (TMA) phosphate at pH 6.5–0.2 *M* phosphate buffer pH 6.5–water (25:20:5:50). Temperature: 35°C. Flow-rate: 1.5 ml/min. Detection: UV at 215 nm. EF = Erythromycin F; EC = erythromycin C; dMeEA = demethylerythromycin A; EA = erythromycin A; EB = erythromycin B; psEAEN = pseudoerythromycin A enol ether; EAEN = erythromycin A enol ether.

* Dedicated to Professor H. Vanderhaeghe at the occasion of his 65th birthday.

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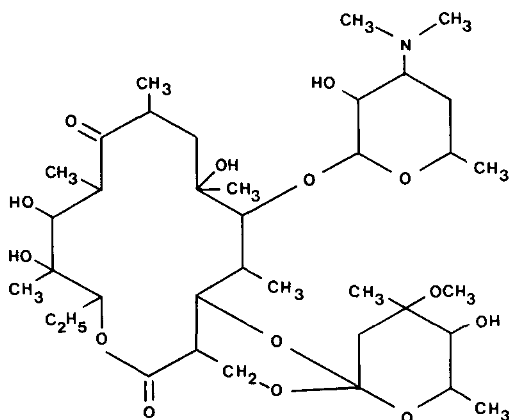

Fig. 2. Structure of erythromycin E.

by incubation of EA with a mutant of the erythromycin-producing Streptomyces erythreus. EE was purified from a chloroform extract of the fermentation broth. This extract contained about 30% of EE and preparative chromatography was performed on a Sephadex LH-20 column with acetone as the mobile phase[3].

EXPERIMENTAL

The origin of reference samples and the structure of different erythromycins and derivatives were mentioned in previous papers[1,2].

Ethyl acetate (Janssen Chimica, Beerse, Belgium) and methanol (Belgolabo, Overijse, Belgium) were distilled from glass apparatus. Dichloromethane (Janssen Chimica) was distilled over phosphorus pentoxide to remove the alcohol added as a stabilizer. Water was twice distilled from glass. Acetonitrile HPLC grade was obtained from Rathburn Chemicals (Walkerburn, U.K.). 25% Ammonia, diisopropyl ether, diethyl ether, ammonium dihydrogenphosphate and diammonium hydrogenphosphate were of pro analysi quality (E. Merck, Darmstadt, F.R.G.). Tetramethylammonium (TMA) hydroxide, 20% (w/v) in methanol, was obtained from Janssen Chimica. TMA solutions for HPLC were brought to the required pH by adding phosphoric acid.

Open-column chromatography was carried out on a glass column (3 cm I.D.) packed with 100 g of silica 60 H, 40–60 μm (E. Merck). Ethyl acetate–methanol–25% ammonia (85:5:1) was used as the mobile phase. For preparative HPLC the same equipment was used as that described previously[5]. The 25 cm × 22.7 mm I.D. HPLC column was packed in the laboratory with silica gel 60 H, 15 μm for TLC (E. Merck). The mobile phase was ethyl acetate–methanol–25% ammonia (100:3:0.5). Precoated TLC plates, silica gel 60 F_{254} (E. Merck), were used with four mobile phases (see Table I). A more thorough discussion of the TLC method has been reported previously[1,6]. Analytical HPLC was performed as described previously[2]. A 25 cm × 4.6 mm I.D. column, packed with Partisil ODS, 10 μm (Whatman, Clifton, NJ, U.S.A.), was used, see Fig. 1 for more details. Mass spectrometry was carried

TABLE I

R_{st} VALUES FOR DIFFERENT ERYTHROMYCINS

Mobile phases: I, ethyl acetate–methanol–25% ammonia (85:10:5); II, diethyl ether–methanol–25% ammonia (90:9:2); III; dichloromethane–methanol–25% ammonia (90:9:1,5); IV, diisopropyl ether–methanol–25% ammonia (75:35:2). Stationary phase: silica gel 60 F_{254} (E. Merck). Amount of each compound spotted: 5 µg. Detection by spraying with conc. sulphuric acid–4-methoxybenzaldehyde–ethanol (1:1:9) and heating at 120°C for 2 min.

	Mobile phase				*Colour of spot*
	I	*II*	*III*	*IV*	
EE	1.04	1.03	1.02	1.03	Brown
EB	1.02	1.04	1.01	1.04	Blue
EA	1 (R_F = 0.57)	1 (R_F = 0.43)	1 (R_F = 0.53)	1 (R_F = 0.44)	Grey-green
ED	0.87	0.82	0.90	0.93	Blue
EC	0.83	0.73	0.86	0.86	Grey-green
EF	0.73	0.57	0.64	0.76	Grey-green

out with an AEI MS-12 instrument by direct insertion at 70 eV and 190°C. NMR spectra were taken with a Jeol FX 90 Q spectrometer.

RESULTS AND DISCUSSION

In order to develop a chromatographic method for preparative separation of the new compound on silica gel, TLC of a commercial sample was performed with mobile phases I–IV. With mobile phase I a brown rim was observed above the spot of erythromycin B (EB). The upper part of the band corresponding to EB was scraped off and extracted with dichloromethane–methanol (1:1). The extract was evaporated to dryness and analysed by HPLC. This revealed the correspondence between the brown rim and the new compound originally detected by analytical HPLC. A commercial sample (10 g) exceptionally containing up to 3% of the compound was used for preparative isolation. A large amount (5 g) of EA was removed by crystallization on cooling for 1 day at −15°C of a solution in acetonitrile containing 0.1% ammonia (25%). Ammonia was added to prevent acid-catalysed conversion of EA into erythromycin A enol ether (EAEN). After evaporation of the filtrate, the residue was further purified by open-column chromatography. Fractions were monitored by TLC (mobile phase I). The fractions of interest, containing the new compound, EB and EA, were evaporated to dryness. By this procedure, the more polar components, *i.e.*, erythromycin F (EF), erythromycin C (EC) and demethylerythromycin A (dMeEA) and the major part of EA were removed. The residue (1 g) was dissolved in 40 ml of ethyl acetate and samples of 4 ml were chromatographed in the preparative HPLC system. A chromatogram thus obtained is shown in Fig. 3. The fractions were monitored by TLC (mobile phase I) and those corresponding to peak X, containing EB, EA and the new compound, were evaporated to give a residue of 300 mg. A second preparative HPLC experiment yielded 100 mg, containing 60% of the new compound with EB as the main impurity. Subsequent crystallization from diethyl ether at −15°C gave a sample with a purity better than 90%, as indicated by HPLC.

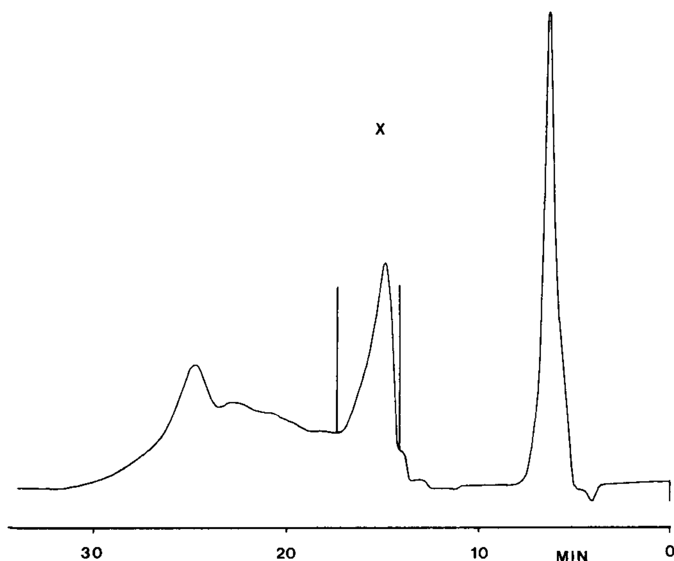

Fig. 3. Preparative high-performance liquid chromatogram. Column: silica gel H, 15 μm, 25 cm × 22.7 mm I.D. Mobile phase: ethyl acetate–methanol–25% ammonia (100:3:0.5). Flow rate: 15 ml/min. Detection: refractive index. Sample load: 100 mg. Peak X was collected for further purification.

The ^{13}C NMR spectrum (22.5 MHz, deuterochloroform solution) of the newly isolated compound clearly shows 35 carbon resonances, two of which have double intensity, and the shift pattern of these lines indicates the substance to be a carbon isomer of EA. The presence of a downfield shifted line at 109.1 ppm, which remains a singlet in the off-resonance decoupling (OFR) spectrum and thus can be attributed to a quaternary anomeric carbon, points to an *ortho* ester linkage in the molecule. Indeed, careful analysis of the spectrum reveals that the shift positions for all carbons are almost identical to within 0.2 ppm with the values already reported for erythromycin E[3]. The only substantial difference is observed for the lactone carbonyl C-1 which occurs at 170.3 ppm as compared to 166.9 ppm in the literature[3]. Such an effect however can easily be explained by considering intra- or intermolecular hydrogen bonding, the extent of which may be altered by variations in concentration or by the presence of very minor impurities.

The ^{1}H NMR spectrum (89.6 MHz, deuterochloroform solution), which clearly lacks the resonance of the anomeric proton of the neutral sugar moiety, cladinose (1″-H), also is in close agreement with the published spectrum[3] of erythromycin E and offers additional evidence of the proposed identity.

Final confirmation was obtained from the mass spectrum which shows the molecular ion at *m/z* (relative intensity in %) 747 (3.1), fragment ions at *m/z* 729 (3.4, M − H_2O), 662 (6.7, loss of the C12–C15 part, with gain of an hydrogen), 616 (7.2) and the desosamine fragment ions at *m/z* 174 (20.1), 140 (100) and 116 (30.5).

Table I lists the R_{st} values ($R_{st} = R_F/R_F$EA) for EA, EB, EC, erythromycin D (ED), EE and EF obtained with four mobile phases. This table demonstrates that only system I partly separates EE from EB. EE is distinguished only due to the

difference in colour. Previously described HPLC methods did not separate EE from EA[2,7]. Since EE is present in many commercial samples and since the antimicrobial activity of EE is low, a good chromatographic method for quantitative analysis of erythromycin necessitates the separation of EE from EA.

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