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# ISOLATION OF ERYTHROMYCINS AND RELATED SUBSTANCES FROM FERMENTATION RESIDUES OF *STREPTOMYCES ERYTHREUS* BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON SILICA GEL

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## SUMMARY

Preparative high-performance liquid chromatography on silica gel allows the isolation of erythromycins and related substances from mother-liquor concentrates. Three mobile phases were used consecutively: A, ethyl acetate–methanol–25% ammonia (100:8:1, v/v); B, diethyl ether–methanol–25% ammonia (100:7:1, v/v) and C, dichloromethane–methanol–25% ammonia (100:5:0.5, v/v). The separation and purification was confirmed by thin-layer chromatography. Thirteen pure substances were isolated among which are erythromycins A, B, C and D, 8,9-anhydroerythromycin A-6,9-hemiketal, erythromycins A and C-6,9;6,12-spiroketals and N-demethylerythromycin A-6,9;9,12-spiroketal.

## INTRODUCTION

It has been shown by high-performance liquid chromatography (HPLC)[1] and also by thin-layer chromatography (TLC)[2,3] that commercially available erythromycin contains erythromycins B and C (EB, EC) besides the main component erythromycin A (EA). We wished to isolate these related substances in order to determine their response in the microbiological assay for this antibiotic. For this study we decided to isolate them from the mother-liquor concentrate from an industrial process.

Numerous methods have been employed to isolate erythromycin and related substances from the fermentation liquors of *Streptomyces erythreus*. The major metabolite, EA, is isolated by extraction and crystallization[4,5]. From the filtrate, EB[6] and EC[5] have been obtained by use of countercurrent distribution. EA and EB have been separated on a column packed with cellulose[6]. Oleinick and Corcoran[7] used consecutive columns of Amberlite IRC-50 resin and silica gel to separate labelled EA from mycelium-free fermentation beer. ED was isolated by chromatography on a column packed with Sephadex LH-20[8]. Columns packed with similar materials have been used to separate human EA metabolites[9,10] and to fractionate erythromycin E from a chloroform extract of a *S. erythreus* mutant[11]. Recently, preparative HPLC

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has been used to isolate erythromycin F from a mother-liquor concentrate[12]. Several other erythromycin-related glycosides and biosynthetic intermediates have been isolated from fermentation broths of blocked mutants[13–18] of *S. erythreus*.

In this paper we report on the separation and purification of EA, EB, EC and ED from mother-liquor concentrates, using preparative HPLC on silica gel. A number of other substances were also isolated. Some were identified, the structures of others are still under investigation.

## EXPERIMENTAL

### *Preparative HPLC*

*Instrumentation.* The laboratory-assembled chromatograph was the same as that described for analytical work by Wouters *et al.*[19]. It has now been equipped with two Milton Roy Minipumps (Laboratory Data Control, Riviera Beach, FL, U.S.A.), a 10-ml loop and a Waters differential refractometer R403 (Waters Assoc., Milford, MA, U.S.A.). The detector attenuation was set at either X64 or X32 depending on the sample concentration.

*Column and packing material.* The column, 25 cm × 22.7 mm I.D. (Chrompack, Middelburg, The Netherlands), was packed in the laboratory with silica gel 60H, 15 μm, for TLC (E. Merck, Darmstadt, F.R.G.). Fine particles were removed by flotation in water as follows. Water (500 ml) was added to silica gel (75 g) in a 600-ml beaker (diameter 7.5 cm). The suspension was mechanically stirred for 5 min to obtain an homogeneous suspension. After standing for 2 h the supernatant was decanted. The procedure was repeated twice. The silica gel residue was filtered on a sintered glass filter (D4) and dried in an oven, first at 80°C for 1 day then at 120°C for 1 day. The dried silica was cooled in a desiccator, over phosphorus pentoxide, under vacuum.

*Packing procedure.* Silica (65 g) was slurried in 250 ml of carbon tetrachloride. The slurry was sonicated for 4 min and quickly introduced into the slurry reservoir, a 50 × 2.0 cm I.D. stainless-steel tube, fixed to the column. The reservoir was filled with carbon tetrachloride and the slurry was immediately packed into the column using a Haskel Pump Model DSTV-122 at a pressure of about 250 kg/cm² and with hexane as the pressurizing liquid. The reagent grade solvents were distilled from glass apparatus.

The column was tested by chromatography of a mixture of *o*-, *m*- and *p*-nitroanilines with dichloromethane, distilled over phosphorus pentoxide, as the mobile phase. The flow-rate was set at 15 ml/min. A column efficiency of 12 000 plates per metre was obtained, and a selectivity of 1.3 and a resolution of 2.33 was calculated for the last two peaks (*m*- and *p*-nitroanilines).

*Solvents, mobile phases and operating conditions.* Ethyl acetate (>99.5%), methanol (>99%), dichloromethane (>99%) (Janssen Chimica, Beerse, Belgium), and diethyl ether (Belgolabo, Overijse, Belgium) were distilled from glass apparatus. Dichloromethane was distilled over phosphorus pentoxide. Ammonia (25% pro analysi, E. Merck) was used. Three mobile phases were used: A, ethyl acetate–methanol–25% ammonia (100:8:1, v/v); B, diethyl ether–methanol–25% ammonia (100:7:1, v/v) and C, dichloromethane–methanol–25% ammonia (100:5:0.5, v/v). They were degassed for 10–15 min in an ultrasonic bath. When purifying smaller

fractions, the composition of the mobile phase, with respect to methanol and ammonia, was sometimes slightly modified to obtain better separation of close eluting impurities.

Samples were dissolved in the mobile phase to a concentration of not more than 500 mg per 10 ml and filtered through paper before injection. The flow-rate was set at 15 ml/min and the chart speed at 5 mm/min. The chromatograph was operated at room temperature.

*Sample.* Mother-liquor concentrates from the industrial production of erythromycin, obtained after crystallization of the majority of erythromycin, were donated by Roussel-Uclaf (Paris, France) and are herein referred to as MLC. Part (11 g) of the anhydroerythromycin A (erythromycin A-6,9;9,12-spiroketal) present was removed by twice crystallizing a 50-g sample of MLC from dichloromethane–hexane.

*Thin-layer chromatography (TLC)*

Pre-coated TLC plates, Stratochrom $SIF_{254}$ (Carlo Erba, Milan, Italy), and Silica gel $60F_{254}$ (E. Merck) were used throughout. Three mobile phases were used: A, ethyl acetate–methanol–25% ammonia (85:10:5, v/v); B, diethyl ether–methanol–25% ammonia (90:9:2, v/v) and C, dichloromethane–methanol–25% ammonia (90:9:1.5, v/v). Samples in dichloromethane solutions were spotted in 10-$\mu$g quantities for qualitative analysis and comparative evaluation, while 100-$\mu$g quantities were used for purity tests. The spots were visualized by spraying with conc. sulphuric acid–2-methoxybenzaldehyde–ethanol (1:1:9, v/v) and heating at 110°C for 1 min. Details of these TLC methods have been described[3].

*Analytical HPLC*

*Instrumentation.* The chromatographic system consisted of a Waters Model M-45 solvent-delivery system, a Pye Unicam LC 3 UV variable wavelength detector (Pye Unicam, Cambridge, U.K.) set at 215 nm and a Kipp and Zonen BD40 recorder (Kipp and Zonen, Delft, The Netherlands). The column was maintained at 40°C by means of a glass water-jacket connected to a water-circulating bath[20]. The column, 25 cm × 4.6 mm I.D. was packed in the laboratory with RoSiL $C_{18}$, 5 $\mu$m (RSL-Alltech Europe, Eke, Belgium), following the packing procedure previously described[21].

*Solvent, mobile phase and operating conditions.* Acetonitrile and methanol HPLC grade S were purchased from Rathburn Chemicals (Walkerburn, Scotland, U.K.). Analytical grade ammonium dihydrogen phosphate and diammonium hydrogen phosphate (E. Merck) were used to prepare 0.2 *M* phosphate buffer pH 7.0. Tetramethylammonium (TMA) hydroxide (20%, w/w in methanol) (Janssen Chimica) was diluted in water to prepare a 0.2 *M* TMA solution, and adjusted to pH 7.0 with phosphoric acid 85% (E. Merck). The mobile phase was acetonitrile–methanol–0.2 *M* TMA pH 7.0–0.2 *M* phosphate buffer pH 7.0–water (30:30:25:5:10, v/v) and was degassed by sonication. Samples were prepared by dissolving about 10 mg in 1 ml of acetonitrile–water (1:1). They were introduced onto the column using a Valco Model CV-6-uHPa-N60 sampling valve (Valco, Houston, TX, U.S.A.) equipped with a 20-$\mu$l loop. The flow-rate was set at 1.0 ml/min and the chart speed at 5 mm/min. The retention times were measured manually.

*Other analytical methods*

Melting points (m.p) were determined in open capillary tubes using a Büchi Model SMP 20 apparatus (Büchi, Flawil, Switzerland). Ultraviolet (UV) absorption spectra of the compounds in methanol were recorded with a Beckman Model 25 spectrophotometer (Beckman Instruments, Fullerton, CA, U.S.A.). Optical rotations, $[\alpha]_D$, were obtained in methanol solutions using a Thorn-NPL automatic polarimeter Type 243 (Thorn Automation, Nottingham, U.K.). Infrared (IR) absorption spectra of potassium bromide pellets containing 1% (w/w) of sample were recorded on a Perkin-Elmer Model 197 IR spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.).

Mass spectra were recorded on a single-focusing AEI MS-12 mass spectrometer (Kratos, Manchester, U.K.) operated at an accelerating voltage of 8 kV, trap current 100 $\mu$A, ionization energy 70 eV and ion-source temperature 150–190°C. Samples were introduced by the direct insertion probe. Exact mass measurements were performed by computer processing at a resolving power (10% valley) of 9000 using perfluorokerosene as the mass standard and a double-focusing AEI MS-902 S mass spectrometer (Kratos) equipped with a VG-2020 data system. The experimental conditions were the same as for the low resolution mass spectrometry (MS).

Proton magnetic resonance (PMR) spectra were obtained on a Varian XL 100 15 spectrometer (Varian, Palo Alto, CA, U.S.A.) using solutions in deuterochloroform.

*Reference samples*

Erythromycin A was purified by five consecutive crystallizations of a commercial sample, twice from a 10% (w/v) solution in dichloromethane then three times in acetone–water (1:1). The crystals were left to dry in contact with the atmosphere, to obtain a pure dihydrate (m.p. 132–136°C). Erythromycin B was donated by Proter (Milan, Italy) and crystallized (m.p. 200–201.5°C) from acetone. Small quantities of impure samples of erythromycins C and D were donated by Abbott (North Chicago, IL, U.S.A.). The following reference compounds were prepared following procedures described in the literature: erythromycin A-6,9;9,12-spiroketal or anhydroerythromycin A (AEA)[22], yield 64% (m.p. 146.5–149.5°C and at 161.5–162.5°C); 8,9-anhydroerythromycin A-6,9-hemiketal or erythromycin enol ether (EAEN)[23], yield 68% (m.p. 138–141.5°C); erythromycin B enol ether (EBEN)[23], yield 62% (m.p. 139–142°C); N-demethylerythromycin A (dMeEA)[24], yield, overall from EA, 73% (m.p. 144.5–147.5°C).

Anhydro-N-demethylerythromycin A (AdMeEA) was prepared following the procedure for the preparation of AEA. dMeEA (2 g) was suspended in 100 ml of water and dissolved by adding concentrated hydrochloric acid to pH 2.5. The solution was left at room temperature for 30 min. An excess of sodium carbonate was added and the mixture was extracted four times, each with 50 ml of dichloromethane. The organic layer was dried over anhydrous sodium sulphate and evaporated to dryness under reduced pressure, yielding 1.6 g of residue. The residue was dissolved in 5 ml of dichloromethane and 10 ml of *n*-pentane were added. The crystals which formed overnight at −15°C were collected to yield 1.1 g (yield 56%) of AdMeEA; $R_F$ 0.27 (TLC, mobile phase, C); $[\alpha]_D^{21}$ − 43° ($c = 1$, methanol); m.p. 142.5–146.5°C.

Erythromycin C-6,9;9,12-spiroketal (AEC) was obtained following the pro-

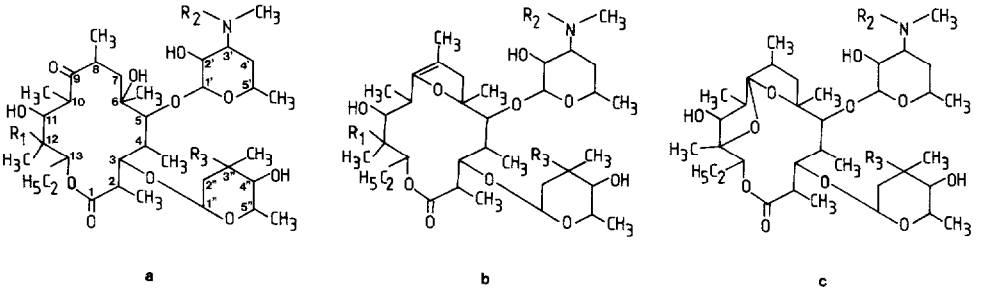
cedure for preparing AdMeEA. EC (200 mg), obtained by preparative HPLC, was suspended in 10 ml of water and dissolved by adding concentrated hydrochloric acid to pH 2.5. The solution was left at room temperature for 30 min. The solution was diluted in 40 ml of water, an excess of sodium carbonate was then added and the mixture was extracted three times, each time with 25 ml of dichloromethane. The organic layer was dried over anhydrous sodium sulphate and evaporated to dryness under reduced pressure. AEC in the residue was purified by preparative HPLC with solvent system C, yielding 120 mg (yield 61.5%); $R_F$ 0.28 (TLC, mobile phase C); $[\alpha]_D^{22}$ − 54° ($c = 1$, methanol).

The structures of the examined reference compounds are shown in Fig. 1.

## RESULTS AND DISCUSSION

### *Chromatography*

Three different packing materials were evaluated for preparative HPLC. The silanized silica column had low capacity (≈50 mg of a simple mixture of erythromycins per injection) and gave inadequate separation of components. Similar problems were observed for macroporous poly(styrene–divinylbenzene) copolymer (XAD-2) used in the reversed-phase mode. The usefulness of silica gel in classical column chromatography[6,7] and in TLC[3] of erythromycins prompted us to investigate silica packed columns. The development of mobile phase systems for such columns



a b c

Fig. 1.

| | | | | |
|---|---|---|---|---|
| (a) | Structures of erythromycins | $R_1$ | $R_2$ | $R_3$ |
| | Erythromycin A (EA) | OH | $CH_3$ | $OCH_3$ |
| | Erythromycin B (EB) | H | $CH_3$ | $OCH_3$ |
| | Erythromycin C (EC) | OH | $CH_3$ | OH |
| | Erythromycin D (ED) | H | $CH_3$ | OH |
| | N-Demethylerythromycin A (dMeEA) | OH | H | $OCH_3$ |
| b) | Structure of enol ether | | | |
| | 8,9-Anhydroerythromycin A-6,9-hemiketal (EAEN) | OH | $CH_3$ | $OCH_3$ |
| (c) | Structures of spiroketals | | | |
| | Erythromycin A-6,9;9,12-spiroketal (AEA) | – | $CH_3$ | $OCH_3$ |
| | N-Demethylerythromycin A-6,9;9,12-spiroketal (AdMeEA) | – | H | $OCH_3$ |
| | Erythromycin C-6,9;9,12-spiroketal (AEC) | – | $CH_3$ | OH |

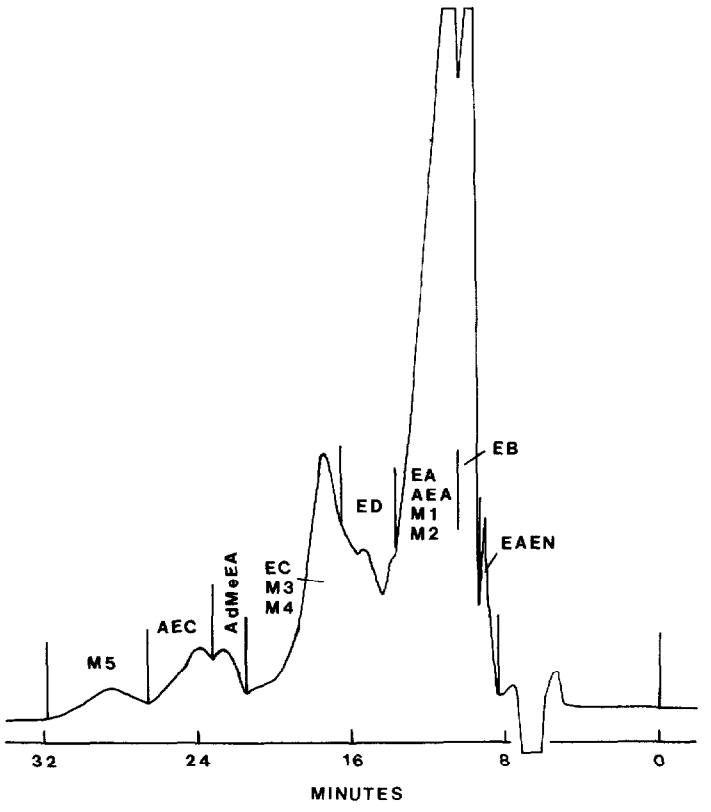



Fig. 2. Typical preparative high-performance liquid chromatogram of mother-liquor concentrates (MLC) of *S. Erythreus* on silica gel, 15 μm (25 cm × 22.7 mm I.D.). Mobile phase: ethyl acetate–methanol–25% ammonia (100:8:1, v/v). Flow-rate: 15 ml/min. Detection: refractive index. Sample load: 500 mg. EAEN = 8,9-Anhydroerythromycin A-6;9-hemiketal; EB = erythromycin B; EA = erythromycin A; AEA = erythromycin A-6,9;9,12-spiroketal; ED = erythromycin D; EC = erythromycin C; AdMeEA = N-demethylerythromycin A-6,9;12-spiroketal; AEC = erythromycin C-6,9;9,12-spiroketal; M1–M5 = compounds of unknown structure.

was done using TLC plates. The details of the TLC investigations have been published[3].

Small changes in the methanol and ammonia contents of some of the mobile phases gave suitable mobile phases for preparative HPLC. Details are given in the Experimental section. TLC was used in the follow up of the purification process. The capacity of silica gel packed columns (500 mg of erythromycins per injection) was ten times higher than that of reversed-phase materials, and afforded better separations. Furthermore the use of organic solvents in the mobile phases facilitates easier recovery of the isolated products by removal of solvents under reduced pressure. During the separation of substances from MLC, the mobile phases were used in the order in which they are listed in the Experimental section. With the first mobile phase the fractions were collected according to the chromatogram shown in Fig. 2. Similar fractions from about 80 runs (40 g of MLC) were pooled and chromatographed using the second mobile phase and so on. From the purer fractions, the major compound

was isolated by crystallization and the filtrate was discarded or used for further purification.

The order of use of the mobile phases has little affect on the final results. This was confirmed by carrying out an experiment with another fermentation residue. In this second experiment, the mobile phases were used in reverse order with some changes in methanol and ammonia content. The same substances as those obtained in the first separation were isolated but in different yields.

The columns used were found to be stable over 6 months of nearly continuous use.

In all, thirteen substances were isolated. Some other substances were detected but not isolated. The yields, chromatographic and some physical characteristics are shown in Table I. Those substances identified are listed with their names. Details of the identifications are presented below. An analytical HPLC chromatogram of a mixture of purified substances is shown in Fig. 3. M4 was not chromatographed because of the small amount of material available.

*Identification of isolated substances*

The identity of the eight compounds shown in Table I was established by comparison of their chromatographic and spectroscopic behaviour with those of reference substances. The data presented in the table and in the discussion below were obtained with the isolated compounds only. These data were identical to those obtained for the reference compounds. Mixtures of EAEN, AEA and AdMeEA and the corresponding reference substances gave no depression of the m.p.

The isolated substances can be divided into three groups: (a) the primary metabolic compounds EA, EB, EC and ED (Fig. 1a); (b) compounds formed by acid-catalysed transformation, EAEN, AEA, AEC and AdMeEA (Fig. 1b, c) and (c) compounds isolated but not identified (M1–M5) the identification of this latter group is currently being pursued.

In the group of erythromycins, identity was first determined by comparing TLC $R_F$ values and HPLC retention times with those of reference samples. A very diagnostic feature of TLC is the colour reaction of the compounds with the spray reagent 2-methoxybenzaldehyde–sulphuric acid. EA and EC give greyish green spots whereas EB and ED give violet-blue spots. The UV spectra of methanolic solutions of the compounds show a maximum at about 280 nm; $A^{1\%}_{1cm}$ = 0.4 (EA), 0.5 (EB), 0.4 (EC) and 0.6 (ED). The IR spectra of the compounds show bands at 3500–3400 (OH), 3000–2700 ($CH_2$, $CH_3$), 1720 (lactone), 1700 (ketone), 1450 and 1375 $cm^{-1}$. The identity was confirmed by MS which gave prominent ions: molecular ion, $m/z$ 733 (EA), 717 (EB), 719 (EC) and 703 (ED); desosamine, $m/z$ 158 (EA, EB, EC and ED); cladinose, $m/z$ 159 (EA and EB) and mycarose, $m/z$ 145 (EC and ED). Owing to the composite nature of the ion at $m/z$ 159[25], the presence of cladinose was verified by the presence of the ions at $m/z$ 127[26] and 115, both related to the parent ion by appropriate metastable peaks.

In the group of compounds formed by acid-catalysed transformation, two types must be distinguished, the 8,9-anhydro 6,9-hemiketals (enol ethers) and the anhydro 6,9;9,12-spiroketals (spiroketals). The structures are shown in Fig. 1b and c respectively. Only one enol ether was isolated. TLC and HPLC showed it to be identical to the reference sample EAEN. The UV spectrum is transparent in the

TABLE I

SUBSTANCES ISOLATED FROM MOTHER-LIQUOR CONCENTRATES OF *S. ERYTHREUS* BY PREPARATIVE HPLC

M1–M5 = Products of unknown structure. NC = Not crystallized; ND = not determined.

| *Substance* | *Crystallizing solvent* | *Yield** *(g)* | *M.p. (°C)* | $[\alpha]_D^{20-22}$ *(°)* | *TLC* | | | | *HPLC*§ |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | $R_F$** | | | *Colour of spot**** | |
| | | | | | *A* | *B* | *C* | | |
| Erythromycin A | Acetone–water | ND | 132–136 | −72 | 0.46 | 0.40 | 0.43 | Greyish green | 1.00 |
| B | Acetone | 2.9 | 200–201 | −94§§ | 0.50 | 0.45 | 0.44 | Violet-blue | 1.67 |
| C | Acetone–water | 0.95 | 200–202 | ND | 0.36 | 0.27 | 0.34 | Greyish green | 0.54 |
| D | Acetone | 0.26 | ND | ND | 0.43 | 0.32 | 0.37 | Violet-blue | 0.87 |
| 8,9-Anhydroerythromycin A-6,9-hemiketal (EAEN) | Ethanol | 0.47 | 138–141.5 | −43 | 0.53 | 0.60 | 0.53 | Greyish green | 2.99 |
| Erythromycin A-6,9;9,12-spiroketal (AEA) | Dichloromethane–hexane | 7.60 | 146.5–149.5 and 161.5–162.5 | −55 | 0.43 | 0.43 | 0.38 | Greyish green | 1.23 |
| N-Demethylerythromycin A-6,9;9,12-spiroketal (AdMeEA) | Dichloromethane–pentane | 0.32 | 142.5–146.5 | −13 | 0.34 | 0.32 | 0.27 | Greyish green | 0.70 |
| Erythromycin C-6,9;12-spiroketal (AEC) | Acetonitrile | 0.47 | 140.5–144.5 | −54§§ | 0.31 | 0.27 | 0.28 | Greyish green | 0.76 |
| M1 | Acetonitrile | 0.25 | 135–136.5 and 185–187 | −24 | 0.44 | 0.40 | 0.39 | Greyish yellow | 0.44 |
| M2 | Acetonitrile | 0.90 | 131–136 | −45 | 0.44 | 0.37 | 0.44 | Yellowish brown | 1.43 |
| M3 | Acetonitrile | 0.30 | 222–223 | −14 | 0.36 | 0.28 | 0.26 | Yellowish brown | 1.35 |
| M4 | NC | 0.02 | ND | ND | 0.34 | 0.23 | 0.33 | Grey | ND |
| M5 | NC | 0.04 | ND | ND | 0.31 | 0.21 | 0.22 | Yellowish brown | 0.81 |

* Yields are from 40 g of MLC.

** Mobile phases: A, ethyl acetate–methanol–25% ammonia (85:10:5, v/v); B, diethyl ether–methanol–25% ammonia (90:9:2, v/v); C, dichloromethane–methanol–25% ammonia (90:9:1.5, v/v).

*** Spray reagent: concentrated sulphuric acid–2-methoxybenzaldehyde–ethanol (1:1:9, v/v).

§ Column RoSiL $C_{18}$, 5 μm (25 cm × 4.-6 mm I.D.), at 40°C. Mobile phase: acetonitrile–methanol–0.2 *M* tetramethylammonium phosphate pH 7.0–0.2 *M* phosphate buffer pH 7.0–water (30:30:25:5:10, v/v). Relative retentions are given.

§§ Concentration is 0.5% (w/w).

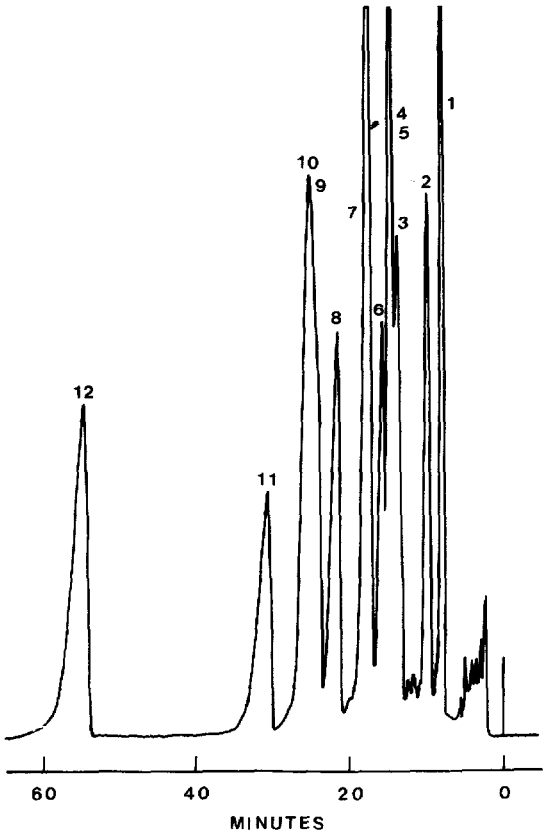



Fig. 3. Reversed-phase HPLC chromatogram of a mixture of compounds isolated from mother-liquor concentrates (MCL) of *S. erythreus* on RoSiL $C_{18}$, 5 μm (25 cm × 4.6 mm I.D.), at 40°C. Mobile phase: acetonitrile–methanol–0.2 *M* tetramethylammonium phosphate pH 7.0–0.2 *M* phosphate buffer pH 7.0–water (30:30:25:5:10, v/v). Flow-rate: 1 ml/min. Detection at 215 nm. Peaks: 1 = M1; 2 = EC; 3 = AdMeEA; 4 = AEC; 5 = M5; 6 = ED; 7 = EA; 8 = AEA; 9 = M3; 10 = M2; 11 = EB; 12 = EAEN. M4 was not chromatographed because of the lack of material.

ketone-absorbing region (280 nm) but shows a maximum at 212 nm ($A^{1\%}_{1cm}$ = 102) which is about six times more intense than that obtained with EA (207 nm, $A^{1\%}_{1cm}$ = 15.5). The IR spectrum shows absorption bands at 3500–3400 (OH), 3000–2700 ($CH_2$, $CH_3$), 1720 (lactone), 1450, 1375, 1275 and 1160 $cm^{-1}$. The mass spectrum exhibits the molecular ion at *m/z* 715, $C_{37}H_{65}NO_{12}$ (calculated: 715.4506, found: 715.4438), with other peaks at *m/z* 159 (cladinose) and *m/z* 158 (desosamine). A prominent fragment ion, *m/z* 434, $C_{21}H_{40}NO_8$ (calculated: 434.2753, found: 434.2750), is formed by loss of the C6–C10 part of the macrolide ring from the molecular ion with back transfer of a hydrogen to yield *m/z* 592, $C_{29}H_{54}NO_{11}$ (calculated: 592.3696, found: 592.3684), followed by loss of the cladinosyl moiety and gain of a hydrogen. Both fragmentations are supported by the presence of appropriate metastable peaks. This fragmentation is characteristic of compounds containing the 8,9-anhydroerythronolide-6,9-hemiketal structure[27].

Although EBEN could have been formed under the same conditions as for EAEN, none was isolated.

Three spiroketals were isolated, *i.e.*, AEA, AEC and AdMeEA. AEA is present in the greatest amount, although it was partly removed from the MLC by crystallization. EB and ED cannot form spiroketals because they lack a hydroxyl group at the C12 position. The identity of the three compounds was first established by TLC and HPLC in comparison with reference samples. The UV spectra of the compounds are transparent in the ketone-absorbing region (280 nm). The IR spectra show absorption bands ast 3500–3400 (OH), 3000–2700 ($CH_2$, $CH_3$), 1720 (lactone), 1450, 1375, 1160 and 905 $cm^{-1}$. The band at 905 $cm^{-1}$ has been attributed to the spiroketal structure[9]. The mass spectra show the molecular ion at *m/z* 715 (AEA), $C_{37}H_{65}NO_{12}$ (calculated: 715.4506, found: 715.4466), 701 (AEC) and 701 (AdMeEA), $C_{36}H_{63}NO_{12}$ (calculated: 701.4350, found: 701.4315). AEA gives fragments for desosamine (*m/z* 158) and cladinose (*m/z* 159). AEC and AdMeEA are differentiated by fragments assigned to the sugar moieties: AEC gives *m/z* 145 (mycarose) and *m/z* 158 (desosamine), whereas AdMeEA gives *m/z* 144 (N-demethyldesosamine) and *m/z* 159 (cladinose). A prominent characteristic fragment ion at *m/z* 341, $C_{18}H_{29}O_6$ (calculated: 341.1964, found 341.1970 for AEA, 341.1964 for AdMeEA), is common to the three compounds. It consists of the C1–C12 part of the aglycone and is formed by loss of the desosaminyloxy group and of propene ($C_{13}$–$C_{15}$) yielding the ion at *m/z* 499, $C_{26}H_{43}O_9$ (AEA and AdMeEA) (calculated: 499.2907, found: 499.2975 for AEA, 499.2904 for AdMeEA) and *m/z* 485 (AEC), followed by loss of the cladinosyl group and back transfer of a hydrogen as established by the presence of metastable peaks. An ion at *m/z* 341 also occurs in the mass spectrum of the 6,9;9,12-spiroketal of N-demethyl-N-propionylerythromycin A[9]. The identity of these compounds was further confirmed by PMR spectroscopy. The spectrum of AEA gives signals at 2.3 ppm [6H, $N(CH_3)_2$] and 3.3 ppm [3H, $O-CH_3$]. The spectrum of AEC lacks the latter signal, confirming that the neutral sugar is mycarose, whereas for AdMeEA the signal is present but the signal at 2.3 ppm [3H, $N-CH_3$] has half the intensity recorded for AEA. Additional proof of the AdMEA structure was obtained by its methylation to AEA using formaldehyde and cyanoborohydride[28].

The isolation of numerous erythromycin-related glycosides has been mentioned in the literature. The presence of large amounts of AEA and AEC in filtrate residues has been reported[8], but no systematic separation of these compounds has been published. To our knowledge, this is also the first time that AdMeEA has been detected and isolated from *S. erythreus* culture media.

The isolation of AdMeEA from MLC infers the presence of dMeEA in *S. erythreus* culture media. However, no dMeEA was isolated. The presence of dMeEA in commercial samples of erythromycin has been shown previously[3]. The rôle of dMeEA in culture media could be two-fold, either as a biosynthetic intermediate or as a product of EA metabolism by *S. erythreus*.

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