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Isolation of erythromycin A N-oxide and pseudoerythromycin A hemiketal from fermentation broth of *Saccharopolyspora erythraea* by thin-layer and high-performance liquid chromatography

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ABSTRACT

Erythromycin A N-oxide and pseudoerythromycin A hemiketal, accompanying erythromycin A and erythronolide B, were isolated from the fermentation broth of *Saccharopolyspora erythraea* by combination of thin-layer chromatography on silica gel and reversed-phase high-performance liquid chromatography. Their identification is based on the mass and ^{1}H and ^{13}C NMR spectrometric results.

INTRODUCTION

During the monitoring of erythromycin A (EA) production in a cultivation of mixed culture of *Saccharopolyspora erythraea* mutants 5002 and 5005 blocked at different steps of erythromycin biosynthesis, two biologically inactive substances were produced in large amounts, simultaneously with the production of biologically active EA and erythronolid B (ELB) [1]. Before suppressing their formation, it was necessary to identify them. These substances were isolated from the liquid part of fermentation broth separated by centrifugation. Extraction and a combination of thin-layer chromatography (TLC) on silica gel and reversed-phase high-performance liquid chromatography (HPLC) were used for their isolation (Fig. 1). The substances were identified as erythromycin A N-oxide (EANO) and pseudoerythromycin A hemiketal (psEAHK) by spectroscopic means. ^{1}H and ^{13}C NMR spectra of EANO and psEAHK are reported here to facilitate the future identification of these compounds. ELB and EA were also isolated from the same source. The structures are depicted in Fig. 2.

EANO has already been isolated from commercial EA by classical column chromatography on silica gel with TLC monitoring [2] and psEAHK was described as a product of the reaction of EA with diethylamine and glacial acetic acid [3].

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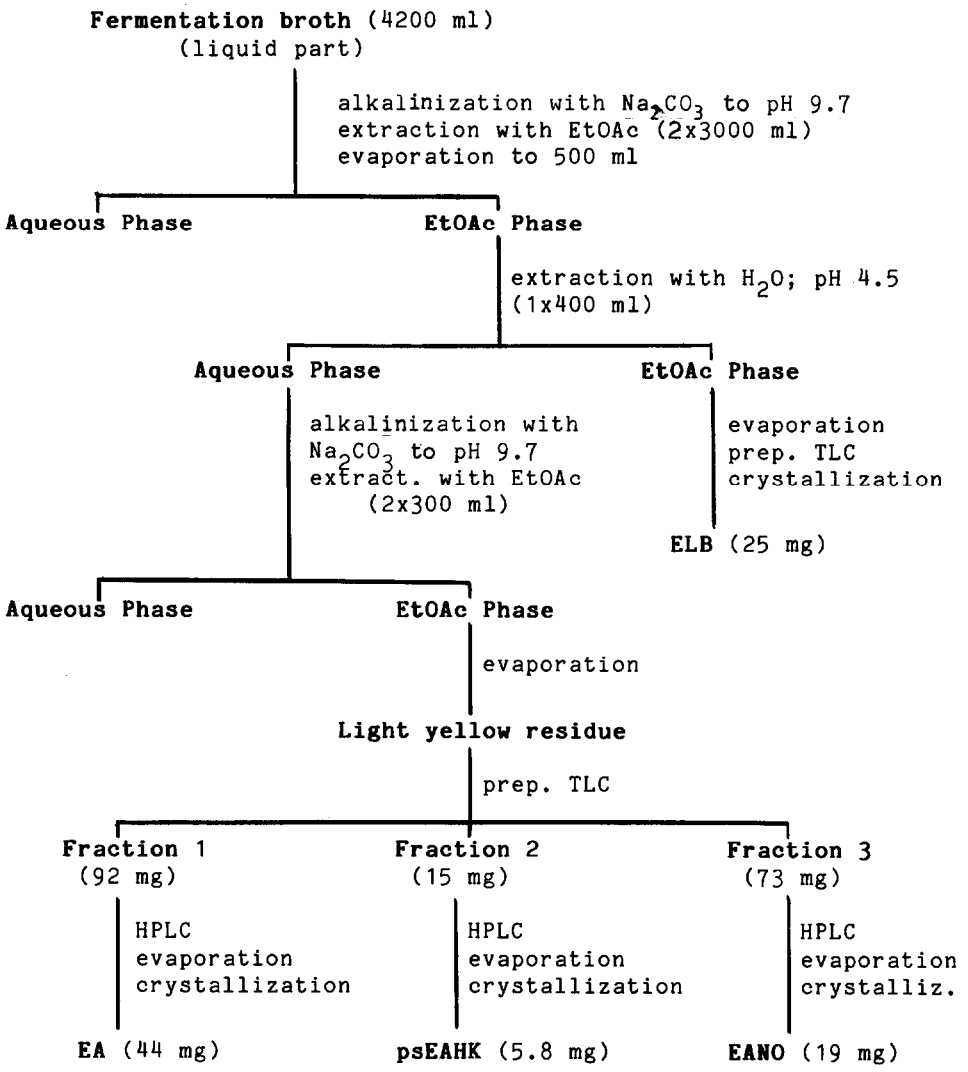

Fig. 1. Isolation scheme. EtOAc = Ethyl acetate.

EXPERIMENTAL

Chemicals

Ethyl acetate, chloroform, methanol, ammonia solution and sodium carbonate were of analytical-reagent grade (Lachema, Brno, Czechoslovakia). All solvents were distilled before use. A standard of EA was obtained from Upjohn (Kalamazoo, MI, USA).

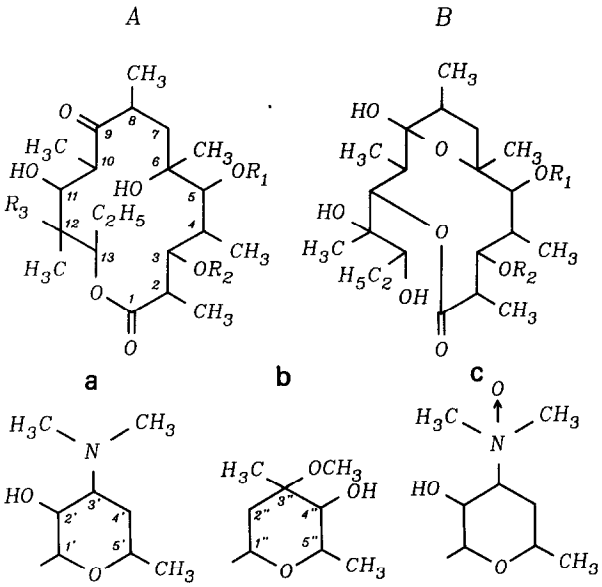

Fig. 2. Structure of isolated erythromycins: erythronolide B (ELB), erythromycin A (EA), erythromycin A N-oxide (EANO) and pseudoerythromycin A hemiketal (psEAHK).

Compound	Aglycone	R_1	R_2	R_3
ELB	A	H	H	H
EA	A	a	b	OH
EANO	A	c	b	OH
psEAHK	B	a	b	–

Samples

Samples suitable for TLC and HPLC analysis were prepared from fermentation broth according to the procedure described in Fig. 1.

Preparative TLC

Preparative TLC was performed on DC-Alufolien Kieselgel 60 ready-made silica gel plates (Merck, Darmstadt, Germany) without prior activation. The mobile phase was chloroform–methanol–ammonia solution (90:10:1, v/v/v). The plates were developed at room temperature over a distance of 17 cm in chromatographic tanks lined with filterpaper, which had been saturated for at least 2 h. The plates were then dried at room temperature. The left and right margins of the TLC plates were cut off and sprayed with a solution of anisaldehyde–sulphuric acid–95% ethanol (1:1:9, v/v/v) and heated in hot air (100°C). Positions of substances of interest were interpolated in the middle part of the preparative TLC plates using marginal strips. Three fraction (1, 2, and 3, R_F ranges 0.1–0.2, 0.2–0.3 and 0.3–0.5, respectively) were obtained after scraping, eluting with chloroform–methanol (2:1, v/v), filtration and evaporation (Fig. 1).

TABLE I

EXPERIMENTAL CONDITIONS FOR PREPARATIVE HPLC

Column	250 × 8 mm I.D.
Stationary phase[a]	Separon SGX C_{18}, 7 μm
Mobile phase	Methanol–water–ammonia solution 850:150:0.6 (pH = 10)
Flow-rate	3 ml min^{-1}
Injection volume	100 μl
Sample amount per run[b]	8 mg
Detection	Refractive index

[a] Tessek, Prague, Czechoslovakia.
[b] Sample dissolved in methanol.

Preparative HPLC

A Model SP 8000B high-performance liquid chromatograph (Spectra-Physics, Santa Clara, CA, USA) with an RIDK 101 differential refractometer (Laboratorní přístroje, Prague, Czechoslovakia) was used. Chromatograms were recorded on a strip-chart recorder at a chart speed of 0.25 cm min^{-1} and a setting of 10 mV. Experimental conditions described in ref. 4 were modified to suit our purpose using EA standard and are summarized in Table I.

Nuclear magnetic resonance

^{1}H and ^{13}C NMR spectra (400 and 100 MHz, respectively) were measured in deuterochloroform at 25°C with a Varian VXR-400 spectrometer. Reported assignments are based on various two-dimensional NMR experiments.

Mass spectrometry

Mass spectral data were measured on a MAT 90 double-focusing mass spectrometer (Finnigan MAT). Samples were studied in the electron impact ionization mode under the following conditions: direct inlet, 150–250°C; electron energy, 70 eV; ion-source temperature, 225°C; total pressure, $2 \cdot 10^{-7}$ Torr; and emission current, 1 mA. Raw spectral data were recorded with a scan speed of 10 s per decade.

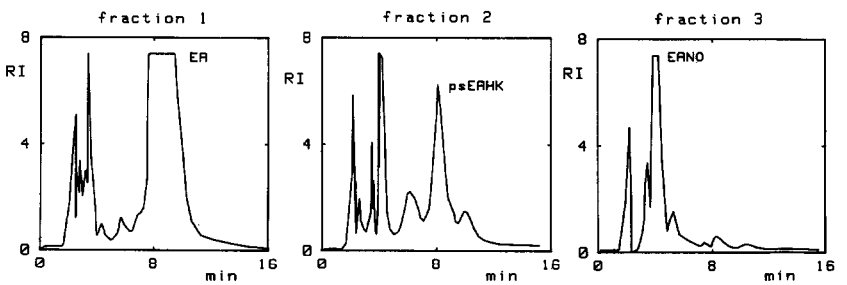

Fig. 3. Typical chromatograms of fractions 1–3 obtained by preparative HPLC. EA = erythromycin A; EANO = erythromycin A N-oxide; psEAHK = pseudoerythromycin A hemiketal. RI = Refractive index.

RESULTS AND DISCUSSION

Extraction, preparative TLC and HPLC

Fermentation broth was separated into liquid and mycelium part by centrifugation. Mycelium was discarded and the fermentation broth filtrate (liquid part) was

TABLE II

^{13}C NMR DATA (CHEMICAL SHIFT, ppm) FOR ERYTHROMYCIN A (EA), ERYTHROMYCIN A N-OXIDE (EANO) AND PSEUDOERTHROMYCIN A HEMIKETAL (psEAHK)

Carbon	EA [7]	EANO	psEAHK [3]	psEAHK
Aglycone				
1	176.30	175.87	176.70	176.95
2	45.02	45.19	46.90	47.04
3	80.30	80.02	80.30	80.94
4	39.50	39.43	39.90	38.33
5	84.00	83.85	85.90	85.78
6	74.78	74.78	84.10	84.39
7	38.54	38.54	40.90	41.09
8	44.93	44.81	37.50	37.76
9	221.90	222.09	106.60	106.84
10	38.10	37.33	38.10	38.31
11	68.76	68.92	72.90	72.92
12	74.45	74.62	76.50	77.20
13	77.10	76.82	77.70	78.00
14	21.16	21.06	22.90	23.03
15	10.70	10.63	11.60	11.90
16	15.90	15.97	14.00	14.22
17	9.20	9.05	10.30	10.50
18	26.50	26.92	29.70	29.95
19	18.40	18.28	11.90	11.90
20	12.00	12.03	10.00	10.25
21	16.20	16.14	17.10	17.09
Desosamine				
1′	103.30	102.68		105.35
2′	71.10	72.62		70.30
3′	65.30	67.08		65.20
4′	29.20	34.80		28.93
5′	68.81	76.20		69.26
6′	21.35	21.17		21.10
7′	40.30[a]	52.13[a]		40.38[a]
8′	40.30[a]	58.54[a]		40.38[a]
Cladinose				
1″	96.50	96.25		96.69
2″	35.00	34.99		35.32
3″	72.70	76.62		72.42
4″	77.90	77.95		78.21
5″	65.70	65.64		65.56
6″	18.46	18.64		17.49
7″	21.43	21.84		21.46
8″	49.50	45.59		49.15

[a] Signals in the same column can be interchanged.

TABLE III

^{1}H NMR DATA FOR ERYTHROMYCIN A (EA), ERYTHROMYCIN A N-OXIDE (EANO) AND PSEUDOERYTHROMYCIN A HEMIKETAL (psEAHK)[a]

Atom	Multiplicity[b]	Chemical shift (ppm)			Coupling constant (Hz)		
		EA [7]	EANO	psEAHK	EA [7]	EANO	psEAHK
Aglycone							
H-2	dq	2.87	2.900	2.734	9.5,7.1	9.3,7.1	10.1,6.3
H-3	dd	3.99	3.988	3.950	9.4,1.4	9.3,1.5	10.1,0.7
H-4	ddq	1.97	1.914	2.180	7.5,1.5,7.5	n.d.	10.7,11.1,1.7
OH-5	d	3.56	3.559	3.472	7.8	7.8	11.1
H-7^{ax}	dd	1.93	1.957	2.310	14.9,11.9	n.d.	n.d
H-7^{eq}	ddd	1.74	1.654	2.383	14.9,8.4,1.0	14.8,2.0,0.6	n.d.
H-8	ddq	2.68	2.684	2.289	11.4,2.3,7.0	11.5,2.2,7.1	n.d.
H-10	dq	3.08	3.087	2.044	1.4,6.9	1.4,6.9	2.3,7.0
H-11	dbr	3.82	3.842	5.863	1.2	1.4	2.3
11-OH	sbr	3.95	1.281	–	–	–	–
12-OH	sbr	3.13	n.o.	n.o.	–	–	–
H-13	dd	5.03	5.026	2.376	11.0,2.3	10.0,2.3	n.d.
H-14^{eq}	ddq	1.91	1.910	1.683	14.2,2.4,7.5	14.2,2.3,7.4	n.d.
H-14^{ax}	ddq	1.475	1.478	1.356	14.3,11.0,7.3	14.2,11.2,7.4	n.d.
H-15	t	0.84	0.841	0.995	7.4	7.4	7.3
H-16	dbr	1.175	1.192	1.216	7.8	7.1	6.3
H-17	d	1.10	1.108	1.092	7.4	7.4	7.0
H-18	sbr	1.46	1.129	1.378	–	–	–
H-19	d	1.15	1.170	0.952	7.1	7.1	6.3
H-20	d	1.13	1.147	1.146	6.9	6.9	7.0
H-21	sbr	1.12	1.453	1.224	–	–	–
Desosamine							
H-1′	d	4.40	4.531	4.173	7.2	7.0	7.3
H-2′	dq	3.21	3.752	3.304	10.3,7.3	10.1,7.0	10.3,7.3
H-3′	ddd	2.43	3.462	2.545	12.3,10.2,3.9	10.1,12.7,7.4	12.2,10.3,3.7
H-4′^{eq}	ddd	1.66	2.010	1.661	2.2,3.9	n.d.	n.d
H-4′^{ax}	ddd	1.22	1.573	1.278	n.d.	n.d.	n.d.
H-5′	ddq	3.48	3.630	3.482	10.8,2.1,6.1	10.4,1.7,6.3	10.8,2.1,6.2
H-6′	d	1.22	1.267	1.210	6.1	6.2	6.2
H-7′	s	2.29	3.213	2.306	–	–	–
H-8′	s	2.29	3.347	2.306	–	–	–
Cladinose							
H-1″	dbr	4.88	4.888	4.765	4.5	4.9,0.8	4.2
H-2″^{eq}	dd	2.35	2.375	2.354	15.2,0.8	15.0,0.8	15.4,0.7
H-2″^{ax}	dd	1.56	1.567	1.531	15.1,5.0	15.0,4.9	15.4,4.6
4″-OH	d	2.23	n.o.	–	–	–	–
H-4″	dd	3.00	3.025[c]	3.002[c]	9.7,9.7	9.3	9.4
H-5″	dq	3.99	4.012	4.061	9.7,6.2	9.3,6.6	9.4,6.3
H-6″	d	1.27	1.282	1.260	6.2	6.6	6.3
H-7″	s	1.23	1.247	1.155	–	–	–
H-8″	s	3.31	3.213	3.239	–	–	–

[a] n.o. = Not observed; n.d. = not determined.
[b] Multiplicity of ^{1}H resonance. s = Singlet; d = doublet; t = triplet; q = quartet; br = broad.
[c] Additional signal: 10-OH,3.168.

made alkaline (pH 9.7) with sodium carbonate and extracted with ethyl acetate. The extract was reduced in volume and washed with acidified water (pH 4.5). After evaporation of the ethyl acetate ELB was isolated by preparative TLC. The aqueous phase was made alkaline (pH 9.7) with sodium carbonate and again extracted with ethyl acetate. The solvent was evaporated *in vacuo* and the light-yellow residue was separated by preparative TLC.

The three fractions obtained were further purified by preparative HPLC. The respective chromatograms are shown in Fig. 3. Peaks were collected according to the detector response, the solvent was evaporated and the compounds were crystallized. The yields are given in Fig. 1.

Mass spectrometry and nuclear magnetic resonance

ELB, isolated by preparative TLC, was identified using mass and NMR spectra (Tables II–IV). Later we found that fractions 1, 2 and 3 contained as the major components EA, EANO and psEAHK, respectively. Other peaks were not identified owing to the lack of sufficient material or its insufficient purity.

The mass spectrum of EANO closely resembles that of EA, and it was identical with that of authentic EANO prepared as described [5]. The absence of a molecular peak in the mass spectrum agrees with the known behaviour of N-oxides [6]. The similarity of the ^{13}C and ^{1}H NMR spectra (Tables II and III) confirms the presence of erythronolide A and cladinose in the molecule. The 4,6-dideoxyhexose attached to C-5 of the aglycone has the same configuration at C-2′ and C-3′ as desosamine (Table III), but is clearly different. The six-proton singlet of the NMe_2 group is replaced by

TABLE IV

MASS SPECTROMETRIC DATA FOR ERYTHROMYCIN A (EA), ERYTHROMYCIN A N-OXIDE (EANO) AND PSEUDOERYTHROMYCIN A HEMIKETAL (psEAHK)

m/z	Relative intensity (%)		
	EA	EANO	psEAHK
733	1.5	0.3	0.4
716	9.6	–	–
715	1.8	5.1	7.2
657	–	–	1.8
656	–	–	1.8
559	3.6	–	–
558	11.9	1.3	1.1
557	–	–	1.0
382	–	–	1.7
365	1.3	–	–
175	3.0	12.0	15.2
174	3.8	4.0	6.6
159	21.9	26.5	28.8
158	100.0	100.0	100.0
127	11.3	10.7	11.5
115	34.9	25.6	21.0
109	3.3	5.7	5.0
100	3.3	6.6	9.8

two methyls (δ_H 3.213, 3.347; δ_C 52.13, 58.54 ppm). These signals deceptively suggest the presence of the methoxyl groups, but this hypothesis has to be rejected on the basis of results of elemental analysis (1.82% N) and high-resolution measurement of the *m/z* 158 ion ($C_8H_{16}NO_2$). Another explanation of these signals requires a charge on the nitrogen atom. NMR spectra of both canditates, EA hydrochloride and EANO, were compared with those of our compound and the latter was found to be identical with EANO.

Comparison of the NMR spectra of psEAHK and EA confirms the presence of desosamine and cladinose in the molecule. The most downfield proton in ^1H NMR spectrum belongs to an $OCHCH(CH_3)$ spin system and was therefore assigned to H-11. This finding, together with chemical shifts of H-13 and -14 (Table III), indicates a lactone ring closed to C-11. The signal of the C-9 carbonyl in the ^{13}C NMR spectrum is missing and a new signal appearing at 106.81 ppm points to ketal formation. As the distribution of methyl types is unchanged, the ketal is formed between C-6 and C-9. The ^{13}C NMR (Table II) and mass spectrometric data (Table IV) agree well with those published for psEAHK [3].

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