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Note

Electrophoretic, chromatographic and mass spectrometric procedures for the identification and isotopic assay of amino acid constituents in etamycin

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Etamycin is a peptidolactone antibiotic produced by *Streptomyces griseoviridus*, *S. griseoroseus* and other Streptomyces species[1,2]. The complete structure has been elucidated[3] and the antimicrobial activity described[1,4]. In the earlier studies, the amino acid constituents were identified by classical techniques involving isolation of crystalline hydrolysis products[3,5]. These procedures are unsuitable for biosynthetic investigations and, therefore, appropriate methodology was sought. The present paper describes the use of electrophoretic, paper, thin-layer, ion-exchange and gas chromatographic–mass spectrometric (GC–MS) procedures which provide rapid and effective techniques for the separation of the amino acid constituents in etamycin hydrolysates.

EXPERIMENTAL

Apparatus

High-voltage paper electrophoresis was effected with an apparatus from Gilson Medical Electronics, Middleton, Wisc., U.S.A., using 4% formate buffer, pH 1.9, at 200 mA and 3600 V for 3 h. The amino acid analyzer was a Beckman-Spinco Model 120C, with 0.2 *M* sodium citrate buffer, pH 3.05 and 4.25, flow-rate 34 ml/h. For gas–liquid chromatography (GLC), a Shimadzu Model 4BM gas chromatograph equipped with flame ionization detectors was employed with argon (40 ml/min) as carrier gas. The column was glass (2 m × 3 mm I.D.), packed with 3% OV-17 on Gas-Chrom Q (100–120 mesh). Combined GC–MS was performed on a LKB 9000 instrument equipped with a 6 ft. column of 1% OV-17. A refrigerated liquid scintillation spectrometer (Mark I; Nuclear-Chicago, Des Plaines, Ill., U.S.A.) was employed for radioactivity measurements.

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Materials and methods

Etamycin was obtained by fermentation with *S. griseoviridus* in a chemically defined medium[6] and it was also supplied by Dr. H. Dion (Parke, Davis Co., Detroit, Mich., U.S.A.). L-Alanine, L-threonine, sarcosine, D-leucine and *allo*-hydroxy-D-proline were obtained from commercial sources. Phenylsarcosine, N,β-dimethyl-leucine and 3-hydroxypicolinic acid were kindly supplied by Drs. J. Sheehan and D. Ponzi (Massachusetts Institute of Technology, Cambridge, Mass., U.S.A.) and Dr. L. C. Vining (Dalhousie University, Halifax, Nova Scotia, Canada).

Etamycin samples were hydrolyzed in 6 *N* HCl for 3 h at 15 p.s.i., 121° in a PTFE-lined, screw-capped test-tube. After evaporation *in vacuo*, the residual amino acids were treated as described below. For two-dimensional paper electrophoresis–paper chromatography, amino acids were first separated by high-voltage electrophoresis, as noted above, followed by ascending chromatography with the solvent systems: (a) 1-butanol–acetic acid–water (4:1:5) and (b) methanol–pyridine–water (20:1:5). The thin-layer (Sil Gel 1B-Baker flex, J. T. Baker, Phillipsburgh, N.J., U.S.A.; or silica gel 60 F-254, Merck, Darmstadt, G.F.R.) chromatography (TLC) systems were (1) for *allo*-hydroxyproline, 1-propanol–water–methanol (7:3:2) and (2) for 3-hydroxypicolinic acid, ethanol–water–ammonia (90:5:5). Amino acids were rendered visible with ninhydrin and isatin reagents. 3-Hydroxypicolinic acid was detected under UV light.

For gas–liquid chromatography the authentic amino acids and the dried etamycin hydrolysate were derivatized by conversion to their trifluoroacetylated (TFA) methyl esters[7] and N-formyl methyl esters[8] as described previously. The column temperature was initially 80° with a 4°/min temperature program (for TFA methyl esters) or isothermally at 150° (for N-formyl methyl esters). In the GC–MS apparatus similar methods were employed except for an initial temperature of 60° (for TFA methyl esters) and 115° (for N-formyl methyl esters).

In the investigation of the distribution of radiolabel from [$^{14}CH_3$]L-methionine during biosynthesis of etamycin, *S. griseoviridus* was cultivated first in glucose–yeast extract–malt extract liquid medium and then in a chemically defined medium for 24 h (each) prior to addition of the ^{14}C-labeled precursor[9]. Incubation was continued for 60 min and the etamycin was recovered by extraction of the culture filtrate with benzene. Details of this procedure will be published elsewhere. The etamycin was hydrolyzed as described above, and after paper electrophoresis in parallel with standard amino acids (located with ninhydrin reagent), the individual components were cut out, placed in counting vials, moistened with water (0.1 ml) and counted in Bray's scintillation fluid[10] (10 ml).

RESULTS AND DISCUSSION

The results of two-dimensional paper electrophoresis (PE)–paper chromatography (PC 1 and PC 2) are shown in Table I, together with data from the amino acid analyzer (AAA) and thin-layer chromatography (TLC). Difficulties were encountered in the visualization of 3-hydroxypicolinic acid, which is weakly fluorescent, after PC (second dimension). However, it could be identified on PE (1 or 3 h) or TLC. The latter technique also served to distinguish *allo*-hydroxyproline (R_F = 0.26) from its diastereoisomer (hydroxyproline, R_F = 0.34). The two-dimensional

TABLE I

ELECTROPHORETIC AND CHROMATOGRAPHIC PROPERTIES OF HYDROLYSATE AMINO ACIDS OBTAINED FROM ETAMYCIN

Amino acid	*PE (ref. to sarcosine = 1.00)*	*PC1* (R_F)*	*PC2** (R_F)*	*AAA (min)*	*TLC*** (R_F)*	*GC (min)*	*GC–MS (min)*
L-Alanine	1.08	0.30	0.60	278	—	2.5	1.1
Sarcosine	1.00	0.25	0.60	177	—	5.1	2.3
D-Leucine	0.84	0.57	0.81	362	—	6.2	3.3
L-Threonine	0.82	0.25	0.59	150	—	3.4	2.0
allo-Hydroxy-D-proline	0.76	0.19	0.55	150	0.26	15.7	11 3
N,β-Dimethyl-L-leucine	0 60	0.67	0.91	296	—	6.4[§]	3.0[§]
Phenylsarcosine	0.45	0.55	0.80	320	—	20.2	13.9
3-Hydroxypicolinic acid	0.19[§§]	—	—	—	0.64	—	—

* Solvent = butanol–acetic acid–water (4:1:5), upper phase.

** Solvent = methanol–pyridine–water (20:1:5), 6 h.

*** For *allo*-hydroxyproline, solvent = 1-propanol–water–methanol (7:3:2); for 3-hydroxypicolinic acid, solvent = ethanol–water–ammonia (90:5:5).

[§] Retention times for N,β-dimethylleucine refer to the N-formyl methyl ester; all others refer to trifluoroacetylated methyl esters.

[§§] Electrophoresis for 1 h.

paper electrophoresis–chromatography procedure was necessitated by the overlap of leucine and threonine (PE) and of threonine and *allo*-hydroxyproline (AAA).

GC retention times (min) for the TFA amino acid methyl esters are also presented in Table I. N,β-Dimethylleucine was not detected by this procedure due to steric hindrance during esterification, and was identified instead as the N-formyl methyl ester (prepared with diazomethane). GC–MS data (not shown) of the deriva-

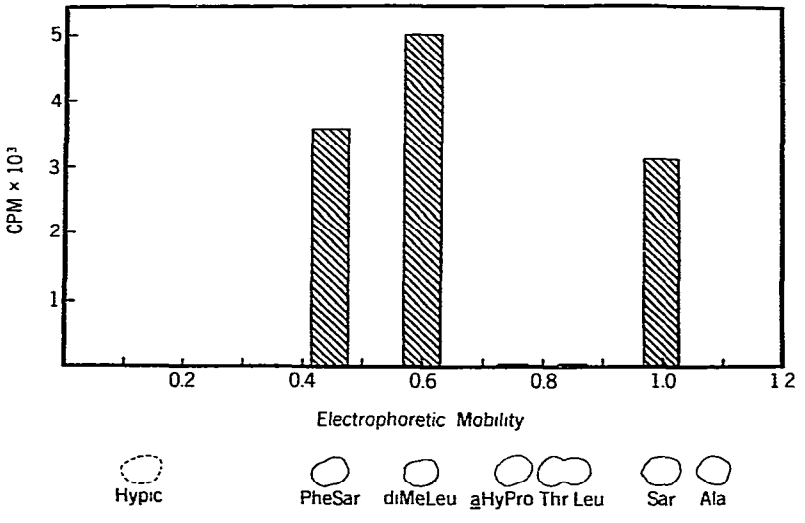

Fig. 1. Paper electrophoretic demonstration of distribution of radiolabel from [$^{14}CH_3$]L-methionine in etamycin hydrolysate.

tized amino acids from an etamycin hydrolysate are virtually identical with those obtained with standard amino acids. In the GC studies, the *allo*-hydroxyproline derivative is the N,O-bis(trifluoroacetyl) methyl ester.

As an example of the application of one of the above separation techniques to biosynthetic studies on etamycin, the distribution of radiolabel from [$^{14}CH_3$]L-methionine into the antibiotic components[6,9] was demonstrated after hydrolysis by means of PE (Fig. 1). The data reveal that the methyl group of methionine is incorporated selectively into sarcosine, N,β-dimethylleucine and phenylsarcosine.

In addition to the use of radioisotopes in conjunction with PE and PC, the GC–MS procedures outlined above could be applied with stable isotopes to biosynthetic labeling studies. The latter approach has been utilized in an investigation of the biogenesis of sarcosine in several actinomycins[8]. Due to the separations achieved, these procedures would also be suitable for studies of directed biosynthesis[11] using analogs of the amino acids present in etamycin.

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