CONGENERS OF ETAMYCIN PRODUCED BY *STREPTOMYCES GRISEOVIRIDUS*[*,†]

C. Chopra, D. J. Hook and L. C. Vining

Biology Department, Dalhousie University, Halifax, Nova Scotia, Canada

B. C. Das

Centre National de la Recherche Scientifique, Institut de Chimie
des Substances Naturelles, 91190, Gif-sur-Yvette, France

S. Shimizu, A. Taylor and J. L. C. Wright

National Research Council of Canada, Atlantic Regional Laboratory,
Halifax, Nova Scotia, Canada

Streptomyces griseoviridus produces in addition to etamycin several related compounds which can be separated by partition chromatography. One of these has been characterized by amino acid analysis and mass spectrometry and shown to have the same structure as etamycin except for replacement of the hydroxyproline residue by proline. Evidence was obtained for additional congeners similarly related to etamycin by amino acid exchange. The relative proportions of such congeners produced by *S. griseoviridus* depends upon the medium in which the culture is grown. Certain amino acids support good yields of the metabolites and the culture appears to be steered towards the synthesis of congeners containing such amino acids.

Etamycin (Fig. 1) is a member of the streptogramin B family of peptidolactone antibiotics[1] and has been isolated from cultures of *Streptomyces griseus*[2], *Streptomyces griseoviridus* (as viridogrisein)[3], *Actinomyces daghestanicus* (as antibiotic #6613)[4] and an unidentified species[5]. A common feature of the streptogramins as well as other groups of microbial peptides is the formation of closely related congeners, differing only in the exchange of an amino acid or hetero substituent. There is substantial evidence that this is due to broad substrate specificity in certain biosynthetic enzymes.[6] Etamycin is unusual in that it has been recovered from cultures as a homogeneous product. No extensive fractionation and purification of the metabolite was necessary. With the exception of a report by

Rozynov *et al.*[7] that a homologue containing one less methylene group could be detected by mass spectrometry of a sample from *A. daghestanicus*, the presence of congeners has not been described. To determine whether the co-synthesis of such compounds has hitherto been overlooked, or whether etamycin is formed by an exceptionally specific process of peptide assembly, we have reexamined the production of this antibiotic under a variety of physiological conditions.

Fig. 1. Structure of etamycin (Sar=sarcosine, Hopic =3-hydroxypicolinic acid, DiMeleu=N, β-di-methyl-L-leucine, HoPro=*allo*-4-hydroxy-D-proline, PheSar=2-phenylsarcosine).
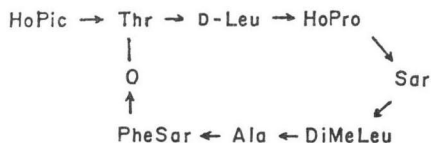
Materials and Methods

Culture

Streptomyces griseoviridus P-D 04955 was obtained from the Parke, Davis Company, Ann Arbor, Michigan. A substrain was selected for high etamycin production by plating a spore suspension. Progeny stored in the lyophilized state was used in the present study. For short-term maintenance the culture was grown to sporulation at 26°C on 0.4% D-glucose - 0.4% yeast extract - 1% malt extract (GYM) agar slants and then kept at 4°C. Spores and mycelium were transferred to a 250-ml Erlenmeyer flask containing 50 ml of GYM medium (without agar) and incubated for 24 hours at 26°C on a rotary shaker (220 r.p.m., 3.8-cm eccentricity) to produce a vegetative inoculum. The mycelium was washed twice with 0.9% saline and resuspended in saline at half its original volume before use.

Cultures for producing etamycin were grown under the same conditions as used for the vegetative inoculum except for variations in the medium and length of incubation. A 4% v/v inoculum was used.

Assay for Etamycin

Since *S. griseoviridus* is known to produce griseoviridin as well as etamycin and the two antibiotics act synergistically[1], a rapid assay based on chemical and spectral properties was chosen in preference to a bioassay. The procedure depended on the strong absorption maximum at 304 nm, due to the 3-hydroxypicolinyl residue in etamycin and to the efficient extraction of etamycin from the culture broth at pH values < 8.5. Sometimes acidic metabolites with strong absorption at 304 nm were produced by *S. griseoviridus*, but these remained in the aqueous raffinates. The assay method was as follows: cultures were filtered, the filtrate adjusted to pH 7.2 and extracted with one volume of ethyl acetate. The absorbance of the clarified extract was read at 304 nm and the amount of etamycin calculated from a calibration curve obtained by treating solutions of known concentration in the same manner.

Analyses

Growth was measured as the dry weight of cell material recovered by filtration. Total carbohydrates in culture filtrates was measured with the anthrone reagent of MORRIS[8] and total amino acids by a ninhydrin procedure based on those described by MOORE and STEIN[9] and YEMM and COCKING[10]. An amino acid analyser (JEOL model 5AH) was used to determine individual amino acids in hydrolysates.

Chromatography

Precoated plates of silica gel (HF 254; E. Merck, Darmstadt) were used for thin-layer chromatography (TLC). Etamycin and related compounds were detected by irradiation at 245 nm and at 366 nm where they gave quenching and intensely blue fluorescent zones respectively.

Partition chromatography was carried out in 2.5 × 90 cm columns packed with a cross-linked dextran gel (Sephadex G-25 fine beads, Pharmacia, Uppsala) equilibrated with the solvent mixture: benzene - petroleum ether (b.p. 68~80°C) - acetic acid - water (4: 4: 5: 3) as described by PITEL *et al.*[11] A sample of the culture extract, containing 100~200 mg of etamycin by assay and dissolved in 5 ml of each solvent phase was slurried in 2.5 g of dry gel and transferred to the top of the gel bed. The column was then percolated at a flow rate of 150 ml·h⁻¹ with upper phase while 10-ml fractions were collected. At fractions 86 and 160 the percolating solvent was changed to the upper phases of benzene - petroleum ether (b.p. 60~80°C) - acetic acid - water (6: 2: 5: 3) and benzene - acetic acid - water (8: 5: 3) mixtures respectively. At fraction 235 the lower phase of the initial solvent mixture was applied to clear the column for re-use.

Gas Chromatography—Mass Spectroscopy

Samples (approximately 10 mg) of etamycin components IV and V recovered from partition chromatography were freed of non-fluorescent impurities by fractional precipitation from ethyl acetate solution with petroleum ether (b.p. 60~80°C) and hydrolysed in 6 N hydrochloric acid at 110°C for

24 hours.   The solution was evaporated and the product esterified by heating at 100°C for 15 minutes in a sealed tube with either ethyl alcohol or n-butyl alcohol (1 ml) containing hydrogen chloride (3~4 N).   The reaction mixture was evaporated, the residue dissolved in methylene dichloride (1 ml) and trifluoroacetic anhydride (0.2 ml) and heated at 95°C in a sealed vessel for 5 minutes.   The reaction mixture was evaporated in a stream of dry nitrogen and the residue in methylene chloride (0.3 ml) was examined by gas chromatography.

The gas chromatograph (Hewlett Packard model 5750) was equipped with a 183 × 0.32 cm glass column packed with 3% OV-1 on Gas-chrom Q (100~200 mesh, Chromatographic Specialities, Brockville, Ontario).   Helium was used as the carrier gas; the injector block was at 270°C and the oven programmed to maintain 80°C for 8 minutes, and then rise at 4°C min⁻¹ to 250°C.   Products were detected with a hydrogen flame ionization detector.   For GC-MS analyses the chromatograph was equipped with the following stainless steel components: a 9: 1 jet splitter connected by a heated 4-port valve (Valco Instruments Co., Houston, Texas) and capillary tubing (55 cm) *via* a jet separator to the ion source of the mass spectrometer (Dupont model 491).

Reference specimens of amino acids were either purchased (Eastman Kodak, Rochester, N.Y.) or synthesised[12].   Electron impact mass spectra were obtained using a Dupont 21-110B mass spectrometer and electrical detection.   Samples were introduced directly into the source and precise mass measurements were made by the peak matching method using an ion in the spectrum of perfluorokerosene as a standard.   Chemical ionization mass spectra were obtained using an AEI MS-9 mass spectrometer with modification of the source as described by VARENNE *et al.*[13]; methane was used as the ionizing gas, and samples were introduced directly into the source at 230~240°C.

Results

Nutrient Effects on Production

Etamycin has previously been produced in complex media[1~5].   In preliminary tests we found titres in the 30~50 mg/liter range with cultures grown in the defined medium: 3% glucose, 0.34% potassium nitrate, 0.1% dipotassium hydrogen phosphate and appropriate inorganic salts.   Yields were insensitive to phosphate concentrations between 0.002% and 0.1%.   Production improved with substitution of soybean meal as a nitrogen source and exchange source of glucose for alternative carbon sources in this basal medium showed lactose to support the highest yields (Table 1).   Soybean meal could be replaced with varying success by single amino acids as sources of nitrogen (Table 2).   The course of such a fermentation on a lactose-threonine-salts medium is shown in Fig. 2.

Separation of Congeners and Influence of Media on Congener Composition

Partition chromatography of crude etamycin extracted from the filtrate of cultures grown in 3%

Fig. 2.   Growth, etamycin production, pH changes and nutrient utilization in cultures of *Streptomyces griseoviridus* grown in a medium containing 3% lactose, 0.4% L-threonine and the inorganic salts listed in the footnote to Table 1.
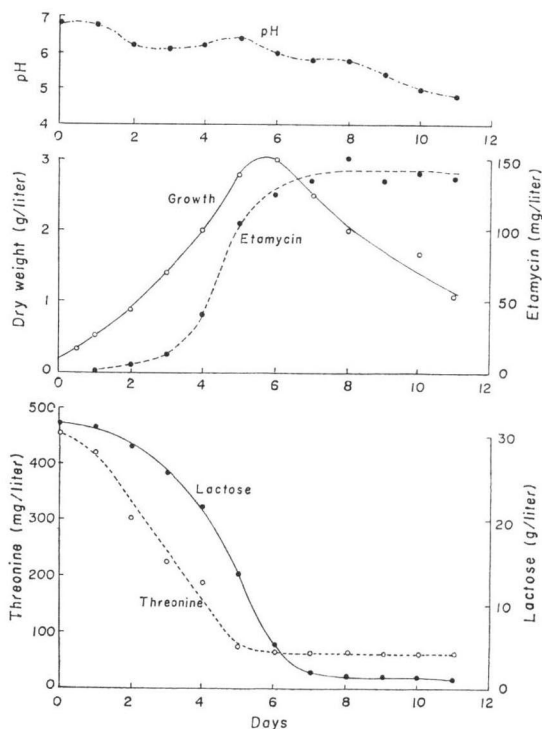
Table 1. Influence of carbon source on etamycin production*.

Carbon source	Etamycin (mg/liter)†
Lactose	237±16
D-Mannose	207± 9
D-Mannitol	207± 4
Glycerol	172± 1
D-Glucose	153± 6
Sucrose	123± 4
D-Galactose	81± 7

* The basal medium contained 1% soybean meal, 0.002% dipotassium phosphate, 0.5% sodium chloride, 0.1% magnesium sulfate heptahydrate and 1% v/v of a trace metals solution consisting of zinc sulfate heptahydrate (880 mg/liter), ferrous sulfate heptahydrate (100 mg/liter), cupric sulfate pentahydrate (40 mg/liter), manganese sulfate tetrahydrate (7.9 mg/liter), boric acid (6 mg/liter) and ammonium molybdate tetrahydrate (4 mg/liter). The carbon sources (12.6 g C/liter) were mixed with the magnesium sulfate and sterilized separately; the remaining ingredients were adjusted to pH 7.2 before sterilization.

† Cultures were analyzed 5 days after inoculation, when filtrate titres had reached a maximum; ± = standard deviation.

Table 2. Production of etamycin on single amino acid nitrogen sources*.

Nitrogen source	Etamycin (mg/liter)†
Soybean meal	253±11
L-Proline	182± 4
L-Threonine	126± 2
L-Histidine	121± 3
L-Alanine	119± 5
Glycine	95±11
DL-Serine	53±11
L-Tryptophan	23± 1
L-Arginine	19± 2
L-Valine	9± 1
L-Lysine	7± 1
L-Phenylalanine	7± 1
L-Leucine	3± 1
L-Methionine	2± 1

* The basal medium is that given in Table 1 with 3% lactose as carbon source and nitrogen sources replacing soybean meal at 0.47 g N/liter.

† Cultures were analyzed at 5 and 9 days of inoculation; values shown are for the higher titre, which was at the earlier sampling only for cultures grown on soybean meal, DL- serine and L-arginine.

Fig. 3. Partition chromatography of crude etamycin from cultures of *Streptomyces griseoviridus* grown on different media.

Samples were extracts from culture filtrates, adjusted to pH 7.2 and chromatographed on a column of cross-linked dextran equilibrated with benzene - petroleum ether - acetic acid - water as described in Materials and Methods.

Fractions (10 ml) were collected and their absorbance at 304 nm measured.

A: cultures grown in 3% glucose - 0.34% potassium nitrate medium.
B: cultures grown in 3% lactose - 1% soybean meal medium.
Both media also contained inorganic salts as listed in the footnotes to Table 1.
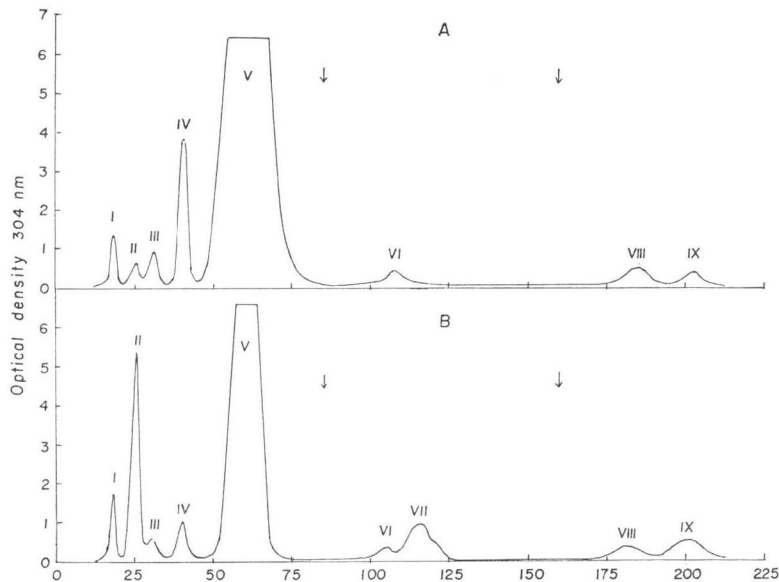
Table 3.   Relative amounts of metabolites with light absorption at 304 nm produced in different media*.

Component	Production media†				
	GluN	LS	LP	LT	LGly
I	1.1	3.1	3.9	3.3	1.1
II	0.1	11	2.2	4.5	3.5
III	0.6	0.6	2.8	0.8	0.7
IV	7.9	2.6	14	0.3	0.9
V	76	23	20	12	3.8
VI	2.3	0.7	0.2	1.3	0
VII	0.2	6.6	11	5.7	23
VIII	0.3	0.4	0	0.2	0
IX	0.8	1.7	0	0.2	0

* Components were separated by partition chromatography. Values represent the total integrated absorbance of a component as a percentage of the total absorbance of the sample applied to the column. The elution pattern of the components is shown in Fig. 3.

† The abbreviations used are: Glu=glucose, N=nitrate, L=lactose, S=soybean, P=L-proline, T= L-threonine, Gly=glycine. The composition of the media is described in the text and in Tables 1 and 2.

glucose - 0.34 % potassium nitrate - salts medium, separated 9 components that fluoresced when irradiated at 366 nm and absorbed light at 304 nm. In the extract from cultures grown in the lactose-soybean meal medium similar components were present but in different proportions (Fig. 3). Cultures grown in media with single amino acids as nitrogen sources gave metabolites with marked differences in composition (Table 3). In contrast, the products from media containing soybean meal with different carbon sources, showed little variation.

Properties of Congeners

All components except I, which was eluted at the void volume of the column, gave absorption maxima in the near u.v. region. They also gave blue-violet fluorescent zones, characteristic of compounds possessing a 3-hydroxypicolinic acid residue, on thin-layer chromatograms (Table 4). Analysis of the acid hydrolysates with an amino acid analyser, operated in the routine mode for protein hydrolysates, detected proline among the products of hydrolysis of components II, III and IV, allo-4-hydroxyproline in component V, and glycine as well as allo-4-hydroxy-proline in components VI and VII. However, this technique did not detect 3-hydroxypicolinic acid and failed to separate threonine from hydroxyproline, or leucine from N,β-dimethyl-leucine. We therefore investigated the combined use of gas chromatography and mass spectro-

Table 4.   Properties of components separated from crude etamycin by partition chromatography

Component	Light absorption*	Rf value and fluorescence by TLC†
I	λ infl. 225, 252, 300 nm	0.78 (yellow) 0.85 (white)
II	λ max. 240, 310 nm	0.63 (blue)
III	λ max. 268, 334; λ infl. 342 nm	0.35 (deep blue)
IV	λ max. 304 nm	0.65 (violet)
V	λ max. 304 nm	0.62 (violet)
VI	λ max. 304 nm	0.65 (violet)
VII	λ max. 310; λ infl. 243 nm	0.56 (blue)
VIII	λ max. 300; λ infl. 262, 290, 310 nm	0.26 (blue) 0.68 (white) 0.16 (white)
IX	λ max. 304 nm	0.65 (violet)

* in ethanolic solution

† in benzene - acetic acid - water (42: 24: 1) on silica gel; zones detected with light of 366 nm. Where more than one zone was present they are listed in order of decreasing intensity.

scopy (GC-MS)[14,15] to determine the relationship between components IV and V.

Amino acids were converted to their N-trifluoroacetyl n-butyl esters[16,17]. The corresponding ethyl esters were occasionally used to clarify fragmentation pathways.[14] In both derivatives loss of the carboxy ester group gave the principal and diagnostic fragment ion of the common protein amino acids (Table 5). A similar reaction occurred in the N-methylamino acids, but not in 2-phenylsarcosine which thereby differed from the protein aromatic amino acids. n-Butyl N-trifluoroacetyl-N,β-dimethyl leucinate was accompanied in the gas chromatographic effluent by two more volatile reaction products which did not interfere with the analysis and assisted in identifying this amino acid. The highest mass peak in the spectrum of 3-hydroxypicolinic acid corresponded to its butyl ester, lacking the trifluoroacetyl substituent. Under the chromatographic conditions used each of the hydrolysis products of components IV and V gave a separate peak and could be unequivocally identified from its mass spectrum.

Hydrolysates of component V contained 3-hydroxypicolinic acid, alanine, allo-4-hydroxyproline, leucine, N,β-dimethylleucine, 2-phenylsarcosine, sarcosine, and threonine. Chemical ionization mass spectroscopy of component V gave ions at m/e 877 $(M-1)^+$, 879 $(M+1)^+$, 907 $(M+29)^+$, and 919 $(M+41)^+$ of relative abundance 4:100:6:3, typical of molecular ions associated with the ionization of an ester or lactone group[18] when methane is used as the ionizing gas. In another paper[19] a mechanism for the reactions of the species $(M+H)^+$ in the depsipeptides series was presented. It was shown that fragmentation was attended by a proton transfer process that followed ring opening by two procedures at a lactone group. In the chemical ionization spectrum of component V (Fig. 4) it can be deduced that both types of ring opening reaction occur. The species pro-

Table 5. Principal fragment ions in the mass spectra of trifluoroacetyl-O-n-butyl derivatives of etamycin amino acids.

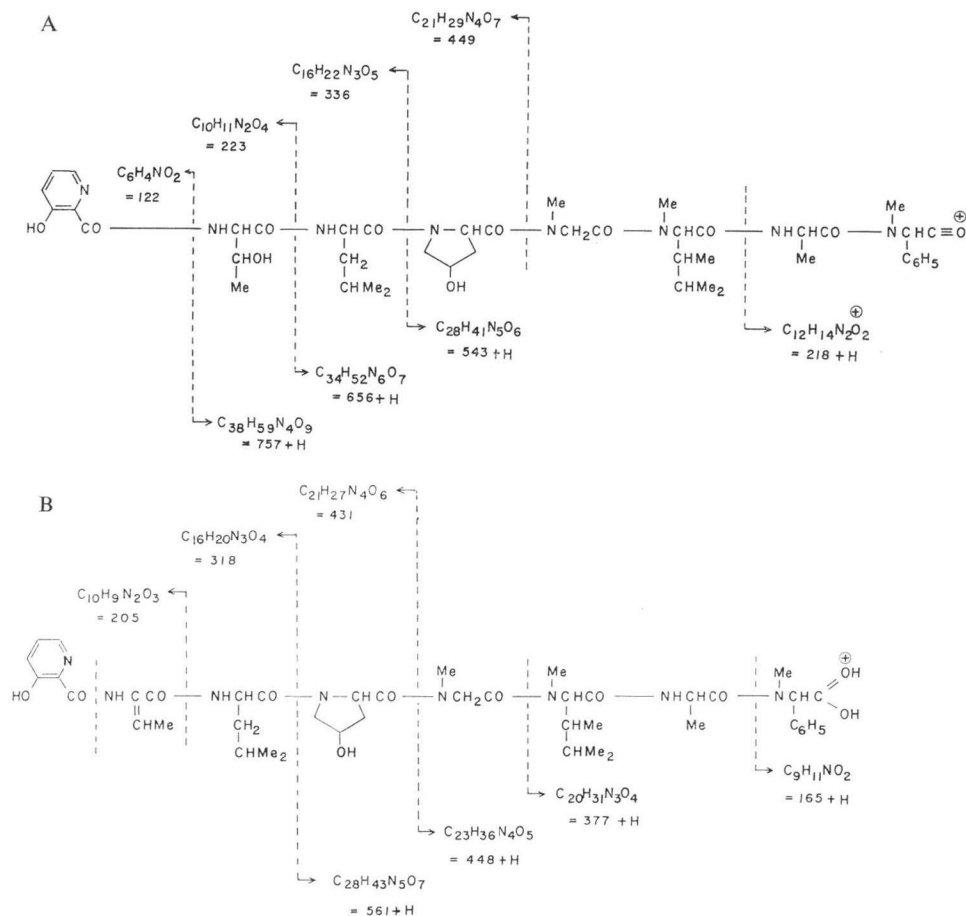| Assignment	Ala m/e (%)	Sar m/e (%)	Thr m/e (%)	Leu m/e (%)	HoPic m/e (%)	Pro m/e (%)	DiMeLeu m/e (%)	HoPro m/e (%)	PheSar m/e (%)
M^+		241(2.8)			195(5.0)	267(1.8)	311(1.1)		317(1.8)
$M^+ - C_4H_9$	184(0.8)	184(2.8)		226(3.0)					
$M^+ - C_4H_9O$	168(2.5)	168(3.5)		210(1.5)	121(40)	194(0.4)			
$M^+ - C_4H_{10}O$									243(42)
$M^+ - C_4H_9OOC$	140(100)	140(100)	266(1.8)	182(100)		166(100)	210(77)	278(7.0)	216(100)
$MH^+ - C_4H_9OOC$					95(100)				
$M^+ - CF_3COO$			254(0.4)					266(1.3)	
$M^+ - CF_3CO - C_4H_9O$			197(5.0)						
$M^+ - CF_3CO - C_4H_9O - H_2O$			179(100)						
$M^+ - CF_3COOH - C_4H_9OOC$								164(100)	
$M^+ - C_4H_9OOC - C_5H_{10}$							140(100)		

Fig. 4. Chemical ionization mass spectrum of etamycin. Source temperature 240°C; methane pressure in the source 0.5 torr.
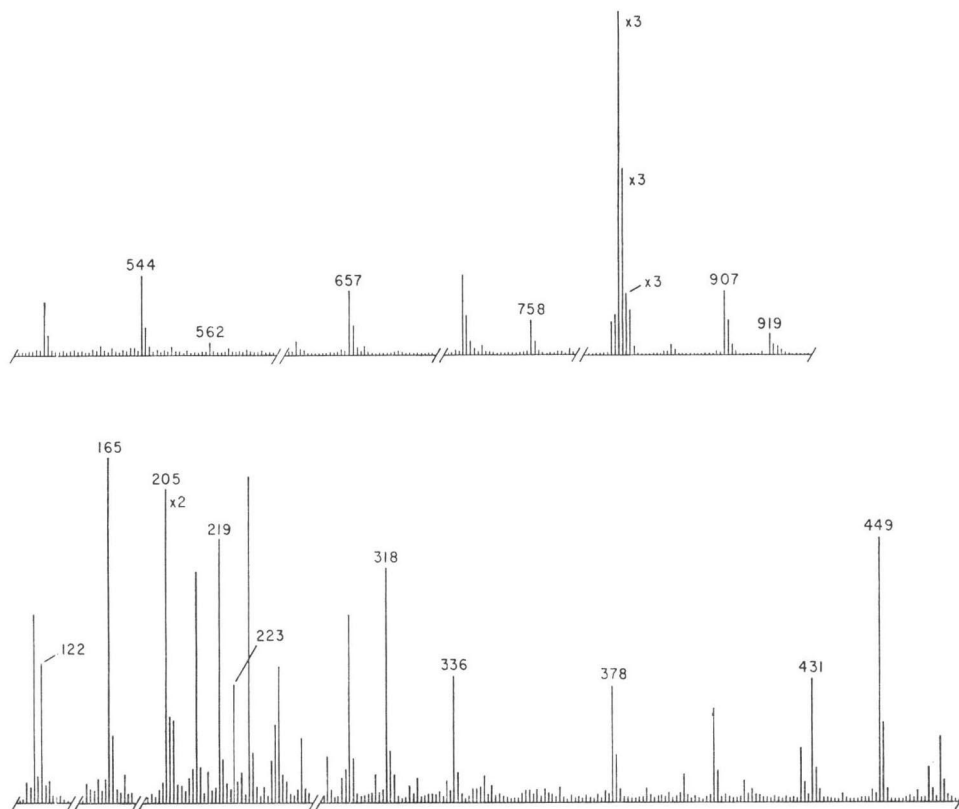
duced are shown in Fig. 5. Further fragmentation then occurs, with transfer of a proton as discussed in another paper[19]. Thus ions at m/e 219, 544, 657 and 758 in Fig. 5A indicate fission of 4 of the 7 peptide bonds, while a similar process for the other species, shown in Fig. 5B indicates fission of 5 of the peptide links, and the two processes combined, fortuitously provide evidence for the complete sequence. The ions m/e 122, 223, 336 and 449 in Fig. 5A and the corresponding ions at m/e 122, 205, 318 and 431 in Fig. 5B confirm the sequence of the amino acid residues, but in addition provide evidence for the proposed mechanism for the rupture of the lactone group. Presumably these ions are observed because of the easy ionization of the picolinyl residue. All of these results are therefore in accord with the structure proposed for etamycin[20,21]. Reference specimens of etamycin contained a fraction chromatographically and spectroscopically identical with component V, usually accompanied by components VI and VII (about 10% and 2%, respectively).

Component IV was separated from ethyl acetate as colorless plates, m.p. 150～154°, $[\alpha]_D^{22} + 20°$ (c 0.8, CHCl₃), λ_{max} (EtOAc) 304 nm (ϵ 7700). Electron impact mass spectroscopy gave an ion at m/e 862.4535 ($C_{44}H_{62}N_8O_{10}$ requires 862.4588). 3-Hydroxypicolinic acid, alanine, leucine, N,β-dimethylleucine, 2-phenylsarcosine, proline, sarcosine and threonine were found in acid hydrolysates of component IV. Chemical ionization mass spectroscopy gave ions at m/e 861 (M−1)⁺, 863 (M+1)⁺, 891 (M+29)⁺ and 903 (M+41)⁺ of relative abundance 4: 100: 5: 2, very similar to the ratios observed in the case of etamycin. Fragment ions at m/e 219 and at 122, 223 and 336, analogous

Fig. 5. Fragmentation of etamycin congeners by chemical ionization mass spectroscopy.
Elemental compositions of ions given are assumed; no precise mass measurements were made.

A

$C_{21}H_{29}N_4O_7$ = 449

$C_{16}H_{22}N_3O_5$ = 336

$C_{10}H_{11}N_2O_4$ = 223

$C_6H_4NO_2$ = 122

$C_{28}H_{41}N_5O_6$ = 543 + H

$C_{34}H_{52}N_6O_7$ = 656 + H

$C_{38}H_{59}N_4O_9$ = 757 + H

$C_{12}H_{14}N_2O_2$ = 218 + H

B

$C_{21}H_{27}N_4O_6$ = 431

$C_{16}H_{20}N_3O_4$ = 318

$C_{10}H_9N_2O_3$ = 205

$C_{20}H_{31}N_3O_4$ = 377 + H

$C_{23}H_{36}N_4O_5$ = 448 + H

$C_{28}H_{43}N_5O_7$ = 561 + H

$C_9H_{11}NO_2$ = 165 + H

to those in Fig. 5A and discussed above were observed in the spectrum, but the ions at m/e 758, 657, and 544 were not seen. In their place ions at m/e 742, 641 and 528, 16 mass units less, showed that the difference between component **IV** and etamycin lay in substitution of proline for hydroxyproline in the new depsipeptide. All other fragment ions supported this suggestion.

Component **VI** appears to be a mixture of at least 3 etamycin congeners. One component is possibly an isomer of etamycin, since the typical fragment ions at m/e 544, 431, 336, and 219 were found in the chemical ionization mass spectrum. Another component had molecular ions at 863 $(M-1)^+$, 865 $(M+1)^+$, 893 $(M+29)^+$, and 901 $(M+41)^+$ on the basis of their relative abundances. It is possible that this metabolite differs from etamycin by a glycine residue replacing either alanine or sarcosine, but the mass spectral data does not allow a decision to be made on this point. The data is in accord with the presence of glycine in hydrolysates of component **VI**.

Discussion

The evidence that *S. griseoviridus* produces a group of related metabolites, one of which is shown to be a congener of etamycin with proline replacing hydroxyproline, is in accord with the

common observation that peptide antibiotics are normally produced in families. Although we have characterised only one of the co-metabolites, others will likely prove to be related by substitution of one or two of the constituent amino acids with structurally similar compounds. Indeed, evidence from amino acid analysis and mass spectroscopy suggest that several congeners contain proline instead of hydroxyproline, and some glycine instead of alanine or sarcosine. The latter bears out the observation of ROZYNOV et al.[7] that etamycin samples contain a homologue with one less methylene group.

From the changes in component ratios when different amino acids are used as nitrogen sources for *S. griseoviridus* it is apparent that the fermentation can be steered to produce selected products. It has been shown that in the cases of actinomycins[22] and sporidesmolides[23] that precursor pressure from the amino acid pools influence the metabolite formed. It is noteworthy that a medium containing proline gave a high proportion of component **IV** and similarly a medium with glycine gave mainly component **VII** which probably contains that amino acid. The enzymes responsible for etamycin formation appear to have a degree of flexibility in accepting substrates and a range of modified antibiotics could probably be produced. Anticipating this we propose that etamycin henceforth be referred to as etamycin A and that component **IV** be named etamycin B.

Acknowledgements

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