IDENTIFICATION OF CIS-5-METHYLPROLINE IN HYDROLYSATES OF ACTINOMYCIN Z_5

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SUMMARY

Actinomycins of the Z series, synthesized by Streptomyces fradiae, contain the unusual amino acid, N-methylalanine, but no proline. Hydrolysates of actinomycin Z_5 were investigated using paper, gas and ion-exchange chromatographic procedures. Identification of an unknown amino acid in actinomycin Z_5 as 5-methylproline was confirmed by mass spectrometry. Configuration of the imino acid was defined as cis.

INTRODUCTION

The actinomycins represent a family of chromopeptide antibiotics which differ solely in the peptide portion of the molecule (1). Those synthesized by Streptomyces chrysomallus differ at the D-valine-D-alloisoleucine site, whereas those produced by S. antibioticus vary at the adjacent site which may be occupied by proline, hydroxyproline, oxoproline or sarcosine. Bössi et al. (2) described a novel series of actinomycins, (designated Z_0 to Z_5) produced by S. fradiae, which are highly unusual in that they contain N-methylalanine but no proline. Brockmann and Manegold reported that actinomycin Z_1 hydrolysates contain valine, threonine, sarcosine, N-methylalanine, N-methylvaline and three previously unknown amino acids, one of which was tentatively identified as 4-oxo-5-methylproline (3,4). We have now identified the related cis-5-methylproline in hydrolysates of actinomycin Z_5, which was kindly supplied by Prof. H. Brockmann. Cis-3-methylproline was found in bottromycin (5), cis-4-

methylproline is present in antibiotic ICI 13,959 (6) and trans-4-methylproline occurs in the monamycins (7). By contrast, 5-methylproline has not previously been reported as a component of a naturally occurring substance.

MATERIALS AND METHODS

Actinomycin preparations: Samples of actinomycins Z_1 and Z_5 were provided by Prof. H. Brockmann, Göttingen University, West Germany. Actinomycin IV was isolated from actinomycin mixtures synthesized by S. antibioticus.

Methylproline isomers: 5-Methylproline of unspecified stereochemistry was supplied by Dr. H. Gershon, Boyce Thompson Institute, Yonkers, New York. This material was found to be a single diastereoisomer (see Results and Discussion), and was epimerized in 25% Ac_2O/AcOH (15 min at 150°) followed by hydrolysis with 2N HCl (6 hr at 110°) to furnish a cis/trans mixture. Cis and trans-3-methylprolines (8) and 4-methylproline (9) (cis/trans mixture) were synthesized as reported previously.

Paper electrophoretic and chromatographic procedures: Actinomycins were hydrolyzed in 6N HCl as described previously (10). The amino acids were separated by high-voltage electrophoresis (Gilson Mfg. Co., 4% formate buffer, pH 1.9, 3 hr, 4800 volts) in one dimension and chromatography in the second dimension with n-BuOH/AcOH/H_2O (4:1:5) on Whatman 3MM paper. After drying, spots were visualized with 0.2% ninhydrin/acetone, 0.2% isatin/acetone and Ehrlich's reagent.

Amino acid analyses: Analytical separations of amino acids were effected with a Beckman Spinco automatic amino acid analyzer, model 120 C, using 0.2 M sodium citrate buffer, pH 3.05 and 4.25. The flow rate of the buffer and ninhydrin solution were both 34 ml/hr.

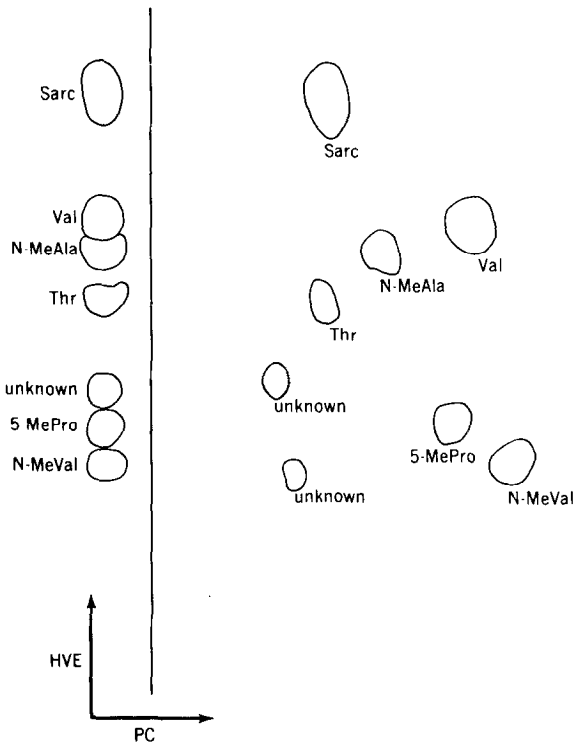

Fig. 1. Separation of amino acids in hydrolysates of actinomycin Z_5 by the two-dimensional paper electrophoretic-chromatographic procedure. Sarcosine = Sarc, Valine = Val, N-Methylalanine = N-MeAla, Threonine = Thr, 5-Methylproline = 5-MePro, N-Methylvaline = N-MeVal.

Gas chromatography: Amino acid derivatives were separated by gas chromatography using a Shimadzu model 4BM equipped with flame ionization detectors. The glass columns were 6 ft. x 3 mm. I. D. Column A: 3% EGSP-Z on Gas Chrom Q (100-120 mesh). Column B: 0.5% EGA on Chromosorb W (60-80 mesh). Carrier gas: argon at 40 ml/min.

Derivatization of amino acids for gas chromatography: An amino acid (1 mg.) or actinomycin hydrolysate (0.5 umol.) was treated with 10% $SOCl_2/CH_3OH$ (0.6 ml) at 80° for 1 hr (sealed tube). To obtain N-trifluoroacetyl methyl esters, one-half of this solution was evaporated *in vacuo* and the residue treated with 10% trifluoroacetic anhydride/CH_2Cl_2 (0.05 ml) at 55° for

Table 1. High-voltage electrophoretic (HVE), paper chromatographic (PC) and amino acid analyzer (AAA) comparisons of amino acids from actinomycins with standards (std.)

Amino acid	HVE		PC (R_f)		AAA (min)	
	Z_5	std.	Z_5	std.	Z_5	std.
Sarcosine	1.00	1.00	0.26	0.25	160	158
Valine	0.89	0.89	0.47	0.49	313	311
N-Methylalanine	0.86	0.86	0.34	0.38	160	158
Threonine	0.82	0.82	0.25	0.27	135	133
Unknown	0.75	-	0.18	-	107*	-
Cis-5-methylproline	0.72	0.72	0.45	0.44	177	176
N-Methylvaline	0.68	0.69	0.54	0.56	171	168
Unknown	0.68	-	0.21	-	111*	-

*Interchangeable

1 hr. To obtain N-acetyl methyl esters, the other half was evaporated and the residue treated with 20% Ac_2O/pyridine at 25° for 2 hr.

Combined gas chromatography-mass spectrometry: An LKB 9000 combined gas chromatograph-mass spectrometer was used with a 6 ft. glass column of 3% EGSP-Z at 120°.

RESULTS AND DISCUSSION

Hydrolysates of actinomycin Z_5 were compared with those of actinomycins IV and Z_1, and with standard amino acids, by a variety of techniques. The two-dimensional paper electrophoresis-chromatogram of actinomycin Z_5 hydrolysate is shown in Fig. 1, and the data comparing this result with the standard amino acids are given in Table 1. Sarcosine, valine, threonine, N-methylvaline and proline are present in actinomycin IV

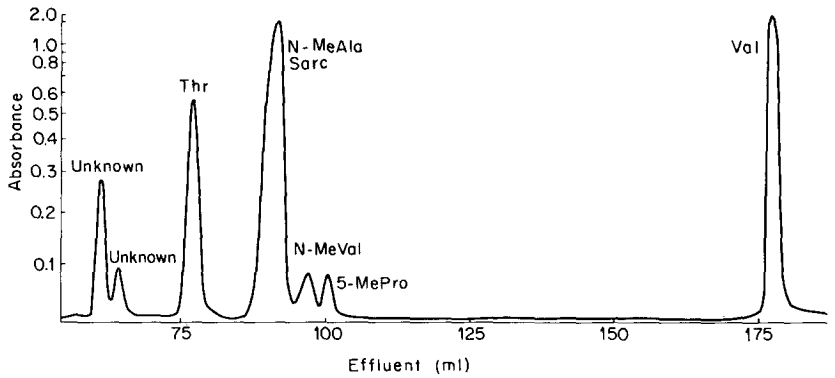

Fig. 2. Separation of amino acids in hydrolysates of actinomycin Z_5 with the amino acid analyzer (Beckman Spinco model 120C) using 0.2 M sodium citrate buffer, pH 3.05 and 4.25.

hydrolysates. In common with actinomycin Z_1, Z_5 contains sarcosine, valine, N-methylalanine, threonine, N-methylvaline and two unknown amino acids which give purple spots with ninhydrin. Proline is absent, but a spot of higher R_f with the same color reactions (ninhydrin, yellow; isatin, blue) was observed. Parallel results were obtained with the amino acid analyzer (Fig. 2). The unknown peak at 177 min had the same retention time as one of the 5-methylproline diastereoisomers (the other having a retention time of 195 min), whereas 3- and 4-methylprolines had longer retention times. This result was confirmed by co-chromatography. 5-Methylproline also occupied the same position on a two-dimensional paper electrophoresis-chromatogram as the ninhydrin-yellow spot from actinomycin Z_5. These comparisons are shown in Table 1.

The conclusion that actinomycin Z_5 contains 5-methylproline was further supported by gas chromatography after derivatization of the hydrolysate amino acids as N-trifluoroacetyl methyl esters. On column A at 120°, both synthetic 5-methylproline and an amino acid from actinomycin Z_5 had the same retention time

Table 2. Mass spectra of N-TFA-cis-5-methylproline methyl ester from actinomycin Z_5 (Z_5) and standards (std.)

M/e	Rel. abundance (Z_5)	Rel. abundance (std.)
239	0.031	0.029
180	1.00	1.00
164	0.091	0.097
142	0.028	0.028
126	0.045	0.049
83	0.027	0.032
69	0.14	0.16
67	0.57	0.72

(10.9 min) and this comparison was confirmed by co-chromatography. With the combined gas chromatograph-mass spectrometer, mass spectra from the two corresponding peaks were obtained and observed to be identical. Relative abundances of the 8 most prominent peaks are given in Table 2; peaks at m/e 239 and 180 were identified as M and M-CO_2CH_3, respectively.

In order to establish the stereochemistry of the 5-methylproline in actinomycin Z_5, it was necessary to know the configuration of the synthetic sample supplied by Dr. H. Gershon. The latter material gave a single peak both on the amino acid analyzer and upon gas chromatography of the N-acetyl (but not N-trifluoroacetyl) methyl ester. Epimerization generated a second peak in both systems, the 5-methylproline from actinomycin Z_5 corresponding with the original diastereoisomer. Since the latter was synthesized (11) via a catalytic hydrogenation of 2-methyl-Δ^1-pyrroline-5-carboxylic acid (and an analogous synthesis (12) has

been reported in which the product was designated cis) we conclude that this isomer possesses the cis configuration, and that generated by epimerization, the trans. This assignment was confirmed by direct correlation of the original synthetic 5-methylproline with cis 2,5-dimethylpyrrolidine (details to be published elsewhere). On column A at 150°, gas chromatography of the derivatized (N-acetyl) actinomycin Z_5 hydrolysate gave a peak with a retention time (14.1 min) corresponding with that of cis-5-methylproline, and a much smaller (ca. 5%) peak at 13.2 min corresponding with the trans isomer. A similar result was obtained on column B at 150° (retention times: cis, 8.4 min; trans, 7.8 min). The gas chromatographic data, like that from the amino acid analyzer, also served to exclude the possibility of 3- or 4-methylproline.

In view of the finding that actinomycin Z_5 contains cis-5-methylproline, it was of interest to review the effect of adding this amino acid to the culture medium of a different actinomycin-producing organism which normally does not appear to produce any 5-methylproline-containing actinomycins. In earlier experiments (13) we investigated the effects of 3-, 4- and 5-methylprolines upon actinomycin production by S. antibioticus. 4-Methylproline was readily incorporated into the proline sites of the molecule, while 3-methylproline was not. Small amounts of a new component appeared to be produced in the presence of 5-methylproline (10^{-3} molar). Recently it was confirmed by the techniques described above that 5-methylproline is incorporated, to a small extent, into the peptide moiety of actinomycin.

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