THE PRESENCE OF α-AMINO-β,γ-DIHYDROXYBUTYRIC ACID IN HYDROLYSATES OF ACTINOMYCIN Z_1

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Actinomycin Z_1, synthesized by *Streptomyces fradiae* contains only one residue of the amino acid, threonine. Hydrolysates of actinomycin Z_1 were investigated using paper, gas and ion-exchange chromatographic procedures. Identification of an unknown amino acid in actinomycin Z_1 (and other actinomycins of the Z series) as α-amino-β,γ-dihydroxybutyric acid (hydroxythreonine) was confirmed by mass spectrometry.

The actinomycin Z complex, produced by a strain of *Streptomyces fradiae*, was first described by BOSSI *et al*[1]. Total hydrolysates of the actinomycin Z components were shown to contain threonine, valine, sarcosine, N-methylvaline and N-methylalanine, but no proline. Subsequently, BROCKMANN and MANEGOLD confirmed the presence in actinomycin Z_1 of these amino acids in addition to three previously unidentified amino acids[2]. Two of these were tentatively identified as L-4-oxo-5-methylproline[2,3] and its 3-hydroxy derivative[4], L-3-hydroxy-4-oxo-5-methylproline, while the third was said to be an hydroxy amino acid which differed from serine and threonine. While several publications[5~7] have referred to N-methylthreonine as an amino acid component of actinomycin Z_1 and have quoted a private communication from BROCKMANN, no experimental evidence for this finding has appeared in the literature.

We compared hydrolysates of actinomycins Z_1, Z_5 and the complete Z complex with authentic N-methylthreonine in several chromatographic systems, but none of these experimental procedures revealed the presence of any N-methylthreonine in hydrolysates. It was concluded that N-methylthreonine, which is a component of the peptide antibiotic stendomycin[8], is not present in the actinomycins of the Z complex. We now report that the unknown hydroxy amino acid in actinomycin Z_1 is α-amino-β, γ-dihydroxybutyric acid (hydroxythreonine). This observation was prompted by the work reported by WESTLEY *et al.*[9], who found that an unidentified *Streptomycete* produced the antimetabolite L-*threo*-α-amino-β,γ-dihydroxybutyric acid and that this amino acid is also a component of an actinomycin preparation (Ro 2-6329) elaborated by another organism[10]. Chromatographic studies in our laboratories have recently demonstrated that the latter actinomycin preparation may have components in common with the Z complex.

Materials and Methods

Compounds. Actinomycins Z_1, Z_5 and the Z complex were kindly provided by Prof. H.

BROCKMANN, Göttingen University, West Germany. Actinomycin Ro 2-6329 and L-threo-α-amino-β,γ-dihydroxybutyric acid (hydroxythreonine) were obtained through the courtesy of Drs. J. BERGER and J. WESTLEY, Hoffmann-La Roche Inc., Nutley, N.J. N-Methyl-DL-threonine was a gift from Drs. M. BODANSKY, Case Western Reserve University, Cleveland, Ohio, and A. FELIX, Hoffmann-La Roche Inc. All other compounds employed were purchased from commercial sources.

Paper electrophoretic and chromatographic procedures. Actinomycins were hydrolyzed in 6 N HCl as described previously[11]. Amino acids in hydrolysates were separated by high voltage electrophoresis in one dimension and ascending chromatography in the second dimension, using Whatman 3 MM paper[11]. After drying, amino acids were visualized with 0.2% ninhydrin/acetone.

Amino acid analyses. Analytic 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[11]. The flow rate of the buffer and ninhydrin solution were both 34 ml/hour.

Gas chromatography. For gas-liquid chromatography (GLC) a Shimadzu Model 4BM chromatograph equipped with flame ionization detectors was employed. The carrier gas was argon (40 ml/min.) and the columns were glass, 6 ft. × 3 mm I.D. Column A was packed with 3% OV17 on Gas Chrom Q (100~120 mesh) and column B with 3% OV225 on Gas Chrom Q (100~120 mesh). An aliquot (0.1 μM) of actinomycin Z_1 hydrolysate was dried in high vacuum and the residue treated in a sealed tube with 25% bis-(trimethylsilyl)-trifluoroacetamide in acetonitrile (25 μl) at 80°C for 45 minutes. Aliquots (2 μl) of the resulting solution were injected directly into the gas chromatograph. Authentic L-*threo*-α-amino-β,γ-dihydroxybutyric acid and other amino acids present in actinomycin were derivatized in the same way.

Gas chromatography-mass spectrometry. An LKB9000 combined gas chromatograph-mass spectrometer equipped with a 6-ft. column of 1% OV17 on Gas Chrom Q was used. Electron impact mass spectra of various chromatographic peaks were obtained.

Results and Discussion

Paper electrophoresis, paper chromatography and the use of the amino acid analyzer have confirmed the presence in actinomycin Z_1, Z_5 and the Z complex of sarcosine, valine, N-methylalanine, threonine and N-methylvaline (see Table 1). The presence of *cis*-5-methylproline in actinomycin Z_5 was reported earlier[11]. In addition, the hydrolysate of actinomycin Z_1 contains three unknown amino acids, one of which gives a yellow ninhydrin spot on paper chromatograms, while the other two give purple spots. One of the latter amino acids displayed an electrophoretic mobility intermediate between those of threonine and N-methylthreonine, and a lower R_f on paper chromatography than either of these hydroxy amino acids. This result suggested that an additional hydroxy group might be present in the molecule. On the amino acid analyzer the unknown had a retention time intermediate between those of threonine and N-methylthreonine. In all these systems the unknown behaved in an identical manner with hydroxythreonine and cochromatographed with an authentic sample of the L-*threo* isomer of this amino acid. Similar results were observed with an hydrolysate of actinomycin Z_5 and the Z complex.

The conclusion that actinomycin Z_1 contains hydroxythreonine was further supported by gas chromatography after trimethylsilylation of the hydrolysate amino acids[9]. On column A (see Materials and Methods) at 140°C threonine and hydroxythreonine had retention times of 2.5 and 6.4 minutes, respectively; on column B at 135°C the corresponding retention times

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_1	Z_5	std.	Z_1	Z_5	std.	Z_1	Z_5	std.
Sarcosine	1.00	1.00	1.00	0.28	0.26	0.27	158	160	158
Valine	0.89	0.89	0.89	0.47	0.47	0.49	315	313	311
N-Methylalanine	0.86	0.86	0.86	0.35	0.34	0.38	158	160	158
Threonine	0.82	0.82	0.82	0.26	0.25	0.27	133	135	133
Hydroxythreonine	0.75	0.75	0.75	0.20	0.18	0.20	105	107	105
Cis-5-Methylproline	—	0.72	0.72	—	0.45	0.44	—	177	176
N-Methylvaline	0.69	0.68	0.69	0.56	0.54	0.56	165	171	168
Unknown	0.69	0.68	—	0.24	0.21	—	109	111	—
N-Methylthreonine	—	—	0.54	—	—	0.32	—	—	88
Imino acid*	0.48	—	—	0.35	—	—	89	—	—

* Identified by BROCKMANN and STAHLER[4] as 3-hydroxy-4-oxo-5-methylproline.

were 2.0 and 4.4 minutes. On both columns the derivatized actinomycin Z_1 hydrolysate gave corresponding peaks. The identity of the hydroxythreonine peak was confirmed by combined gas chromatography-mass spectrometry (see Materials and Methods). Mass spectra from the authentic and actinomycin Z_1-derived hydroxythreonine peaks were identical (see Table 2). The principal fragmentations observed have been discussed by WESTLEY *et al.*[6].

The results obtained by us have recently been confirmed by a modified fluorescamine analysis[12].

Table 2. Mass spectra of trimethylsilylated hydroxythreonine.

m/e	Relative abundance	
	Authentic hydroxythreonine	Z_1 hydroxy threonine
408	0.07	0.06
380	0.06	0.06
306	1.00	1.00
291	0.41	0.30
219	0.87	0.90
218	0.91	0.90
205	0.32	0.34
147	0.70	0.88
117	0.33	0.41
102	0.52	0.56

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