3-HYDROXY-5-METHYLPROLINE, A NEW AMINO ACID IDENTIFIED AS A COMPONENT OF ACTINOMYCIN Z_1

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SUMMARY

3-Hydroxy-5-methylproline has been identified in hydrolysates of actinomycin Z_1 by ion-exchange and paper chromatography, paper electrophoresis, gas chromatography and mass spectrometry in comparison with the synthetic compound. The stereochemistry of this amino acid is under investigation. The amino acid composition of actinomycin Z_1 thus consists of threonine, hydroxythreonine, D-valine(2), 4-oxo-5-methylproline, 3-hydroxy-5-methylproline, sarcosine(2), N-methylalanine and N-methylvaline.

INTRODUCTION

The actinomycin Z complex (1) produced by Streptomyces fradiae consists of several compounds (designated Z_0 to Z_5) which differ markedly in amino acid content from other actinomycins described in the literature. The presence of N-methylalanine and the absence of proline is particularly unusual. In the case of actinomycin Z_5, for example, cis-5-methylproline has been identified (2,3) as an imino acid component while 4-oxo-5-methylproline (4,5) occurs in both actinomycins Z_1 and Z_5. Brockmann and Stähler (6) recently reported that reductive hydrolysis of actinomycin Z_1 with hydriodic acid produced 1.6 molar equivalents of 5-methylproline, and suggested that, in addition to 4-oxo-5-methylproline, one residue of 3-hydroxy-4-oxo-5-methylproline is present in the intact molecule. The latter proposal was based upon indirect evidence. For example, acetylation experiments revealed the presence of two hydroxyl groups in actinomycin Z_1,

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whereas elementary analysis of Z_1 indicated that a total of 19 oxygen atoms were present in the molecule. However, these arguments are invalidated by the recent discovery that one "threonine site" in actinomycin Z_1 is occupied by α-amino-β,γ-dihydroxybutyric acid (hydroxythreonine) (7), and not by N-methylthreonine as reported earlier (8). With a hydroxy-substituted methylproline in one peptide moiety, the presence of two hydroxyl groups and 19 oxygen atoms in actinomycin Z_1 would be explained. This communication presents direct evidence for the identity of 3-hydroxy-5-methylproline as a component of actinomycin Z_1.

MATERIALS AND METHODS

Actinomycin Z_1 was kindly provided by Dr. H. Brockmann, Göttingen University. Hydrolysis of the antibiotic in 6N HCl was carried out as reported in an earlier publication (9). High voltage electrophoresis (HVE) was employed using a Gilson Mfg. Co. instrument with 4% formate buffer for 3 hr at 200 mamps, 4000 volts (2,7). Data are expressed as electrophoretic mobility relative to sarcosine = 1.00. Paper chromatography as a second dimension (2) was performed using as solvent systems: PC_1 (butanol:acetic acid:water, 4:1:5) and PC_2 (methanol:water: pyridine, 20:5:1). Detection of amino acids was carried out with ninhydrin, isatin and Ehrlich's reagent. Analytical separation of amino acids was effected with a Beckman Spinco automatic amino acid analyzer (2,7), Model 120C, with 0.2M sodium citrate buffers, pH 3.05 and pH 4.25.

The synthesis of 3-hydroxy-5-methylproline (by a route analogous to that used for 3-hydroxyproline by Blake *et al.*)(10) and its separation into four diastereoisomeric racemates by ion-exchange column chromatography will be described elsewhere. The isomers, which were designated 1 to 4 according to their emergence from the column, were all obtained crystalline and characterized. The synthesis of 4-hydroxy-5-methylproline (isomeric mixture) was effected as described in the literature (5).

For gas-liquid chromatography a Shimadzu Model 4BM gas chromatograph, equipped with flame ionization detectors, was employed. The carrier gas was argon (40 ml/min) and the columns were glass, 2.5m x 3mm. Columns A and B were 3% OV17 and 3% OV225 (both on Gas Chrom Q, 100-120 mesh), respectively. Derivatization was effected as follows: an aliquot (0.1-0.5 nmol) of an amino acid or dried hydrolysate was treated with 4N methanolic HCl (0.5 ml) for 1 hr at 80°C (sealed tubes), then evaporated to dryness. The residue was treated with 10% trifluoroacetic anhydride in methylene chloride (0.05-0.2 ml) for 1 hr at 55°C. An aliquot (1 µl) of this solution was injected into the gas chromatograph.

For combined gas chromatography-mass spectrometry an LKB 9000 instrument was employed, with a 6 ft column of 1% OV17 (on

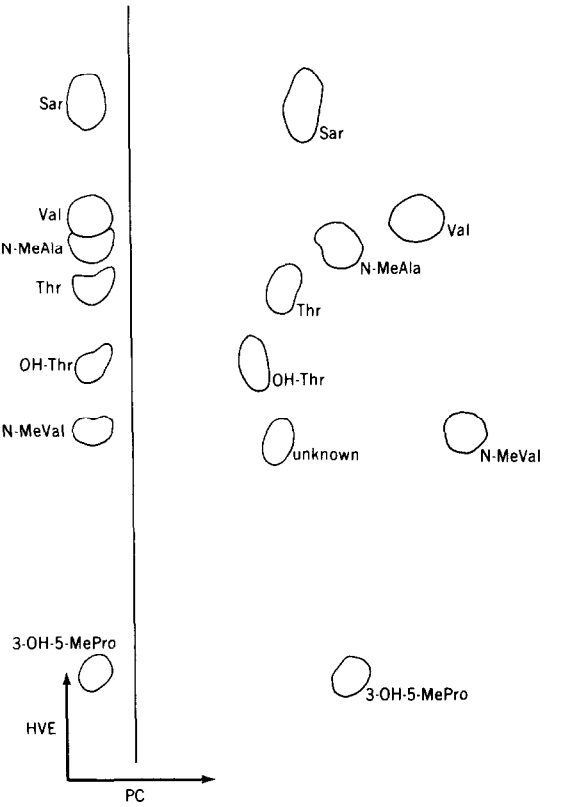

Figure 1. Separation of amino acids in hydrolysates of actinomycin Z_1 by the two-dimensional paper electrophoretic-chromatographic (PC_1) procedure. Sarcosine = Sar, Valine = Val, N-Methylalanine = N-MeAla, Threonine = Thr, Hydroxythreonine = OH-Thr, 3-Hydroxy-5-methylproline = 3-OH-5-MePro, N-Methylvaline = N-MeVal. The unknown ninhydrin-positive component may represent a degradation product derived from 4-oxo-5-methylproline which is reported to be destroyed during acid hydrolysis (4,5).

Gas Chrom Q) at 108°C. Electron impact mass spectra were obtained for each diastereoisomer of the derivatized 3-hydroxy-5-methylproline, for two diastereoisomers of 4-hydroxy-5-methylproline and for several gas chromatographic peaks from the actinomycin Z_1 hydrolysate.

RESULTS

Examination of hydrolysates of actinomycin Z_1 by high voltage electrophoresis and paper chromatography revealed that an imino acid (A, HVE = 0.48, PC_1 = 0.30, PC_2 = 0.51) was present

which gave a yellow color with ninhydrin but failed to react with isatin or Ehrlich's reagent (Fig. 1). It was also observed that the imino acid in Z_1 hydrolysates eluted early (89 min) on the Beckman Spinco amino acid analyzer (AAA). Reduction of actinomycin Z_1 with sodium borohydride yielded an additional imino acid component (B, HVE = 0.69, PC_1 = 0.30, AAA = 120 min) with no significant decrease in the amount of A. Chromatographic studies indicated that B was identical with one of the stereoisomers of 4-hydroxy-5-methylproline, and gave the same color reactions with ninhydrin (yellow), isatin (blue) and Ehrlich's reagent (red). By contrast, compound A possessed a lower electrophoretic mobility than any of the four stereoisomers of 4-hydroxy-5-methylproline. Since reduction of Z_1 generated only one new imino acid, these findings suggested that B results from reduction of 4-oxo-5-methylproline in Z_1. It was reasoned, therefore, that A cannot be 3-hydroxy-4-oxo-5-methylproline as reported by Brockmann and Stähler (6), although its failure to give a blue color with isatin (11) did suggest the presence of a 3-substituent in the prolyl ring. Comparison of the four diastereoisomers

Table 1. Electrophoretic and chromatographic comparisons of component A in actinomycin Z_1 hydrolysates with synthetic stereoisomers of 3-hydroxy-5-methylproline.

3-Hydroxy-5-Methylproline	HVE	PC_1	PC_2	AAA	GLC (Col. A)	GLC (Col. B)
	(sar = 1.00)	R_f	R_f	min	min	min
A (Z_1)	0.48	0.30	0.51	89	5.9	3.8
Isomer 1	0.48	0.30	0.50	89	5.9	3.8
" 2	0.58	0.29	0.60	103	6.9	4.4
" 3	0.78	0.30	0.59	144	11.4	6.8
" 4	0.71	0.31	0.60	144	8.8	5.0

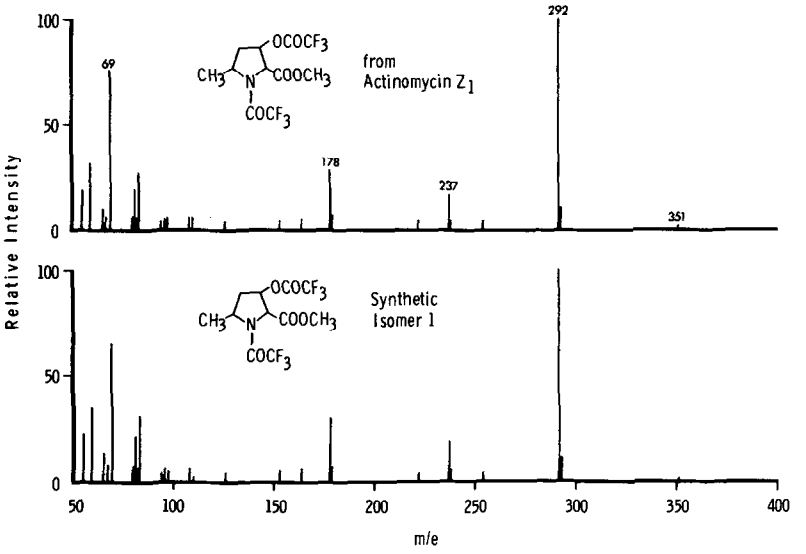

Figure 2. Mass spectra of derivatized 3-hydroxy-5-methyl-proline.

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of 3-hydroxy-5-methylproline with Z_1 hydrolysates (Table 1) indicated that synthetic isomer 1 corresponded with A.

Gas-liquid chromatography: The four diastereoisomers of 3-hydroxy-5-methylproline, when derivatized as the N, O-di-trifluoroacetyl methyl esters (see Materials and Methods), gave base-line separations on gas-liquid chromatography. On columns A at 115°C and B at 145°C isomer 1 corresponded in retention times with compound A (Table 1).

Combined gas chromatography-mass spectrometry: The electron impact mass spectra of isomer 1 of derivatized, synthetic 3-hydroxy-5-methylproline and of the corresponding peak in the actinomycin Z_1 hydrolysate are compared in Fig. 2 and are identical. The other three isomers (2, 3 and 4) gave slightly different spectra, the molecular ion (m/e = 351) being more pronounced. These spectra all showed prominent fragmentation peaks at m/e = (a) 292, (b) 237 and (c) 178, representing loss of (a) $COOCH_3$, (b) CF_3COOH and (c) both these fragments, respectively,

in addition to (d) 69 (CF_3^+). The mass spectra obtained from two diastereoisomers of 4-hydroxy-5-methylproline were markedly different in the relative intensities of the various fragmentation peaks.

DISCUSSION

The experimental evidence outlined here establishes 3-hydroxy-5-methylproline as a component of actinomycin Z_1 and thus completes the elucidation of this antibiotic's amino acid composition, the other components being threonine, hydroxythreonine, D-valine(2), 4-oxo-5-methylproline, sarcosine(2), N-methylalanine and N-methylvaline. Whereas both cis and trans-3-hydroxyprolines occur in the peptide antibiotic telomycin (12), its 5-methyl analogue has not been reported previously. Also, while 4-hydroxy, 4-oxo, and 4-oxo-5-methylproline all occur in various actinomycins (8,13), a proline oxygenated at the 3-position has not been observed hitherto. This observation raises interesting biosynthetic questions with respect to the temporal relationship of the hydroxylation and methylation reactions of a proline (prolyl) residue during antibiotic peptide synthesis. NMR spectroscopy permits tentative assignment of trans-3-hydroxy-cis-5-methyl stereochemistry (relative to the carboxyl group) to isomer 1 and hence to the natural amino acid. Studies designed to confirm this finding are in progress.

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