INFLUENCE OF METHYLPROLINE ISOMERS UPON ACTINOMYCIN BIOSYNTHESIS*

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The sole difference in the structure of the actinomycins produced by Streptomyces antibioticus resides in the "proline site" of the antibiotic peptides. For example, 4-hydroxy-L-proline and L-proline are present in actinomycin I, 4-oxo-L-proline and L-proline are found in actinomycin V, whereas actinomycin IV contains two residues of L-proline per molecule of antibiotic (Brockmann, 1960; Katz and Pugh, 1961). Previous investigations have shown that analogs of proline and also sarcosine can influence actinomycin formation by competing with, and replacing, endogenously synthesized proline in certain of the actinomycin components (Katz, 1960). As a consequence, the formation of minor components may be greatly enhanced or synthesis of new actinomycins takes place. In the light of these observations it was of interest to investigate the influence of 3-, 4- and 5-methyl-DL-proline upon actinomycin biosynthesis. The results of these studies are described in the present communication.

Methods and Materials: 3- and 4-Methyl-DL-proline (isomeric mixtures) were obtained by chemical synthesis (Cox, Johnson and Mauger, 1964). Separation of 3-methyl-DL-proline into cis and trans racemates was carried

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out as described elsewhere (Mauger, Irrevere and Witkop, 1966). The 5-methyl-DL-proline compound was supplied through the courtesy of Dr. H. Gershon.

S. antibioticus was grown in glutamic acid medium as previously described (Sivak, Meloni, Nobili and Katz, 1962). The concentration of actinomycin in the medium was determined by a spectrophotometric procedure (Katz and Weissbach, 1963). The actinomycin mixture formed was extracted from the medium into ethyl acetate (1:1) and concentrated to dryness under reduced pressure. The crude actinomycin complex was separated into individual components by circular paper chromatography with the solvent system: 10% aq. sodium o-cresotinate:dibutyl ether: sym. tetrachloroethane (3:2:1) (Katz, Prockop and Udenfriend, 1962). The actinomycin mixture formed after the addition of 4-methyl-DL-proline was purified further by silicic acid chromatography (Sivak, Meloni, Nobili and Katz, 1962). Vigorous hydrolysis of this mixture was carried out in 6N HCl at 121°C for 4.5 hr. in a sealed tube. After evaporation of the hydrolysate the amino acids were separated by electrophoresis (Gilson Medical Electronics) at 4600 volts on Whatman #3MM paper impregnated with 4% formate buffer (pH 1.9) and also by ascending paper chromatography on Whatman #1 paper in the following solvent systems: a) n-butanol:acetic acid:phenol:water (30:10:10g:50), b) 77% ethanol, c) n-butanol:water:formic acid (10:2:1), d) t-butanol:water: formic acid (5:1:1). The Rf values of 4-methylproline were found to be: a) 0.46 b) 0.63 c) 0.34 d) 0.58, respectively.

Radioisotope experiments were carried out with L-proline-U-C^{14}, L-threonine-U-C^{14}, L-valine-1-C^{14}, glycine-1-C^{14}, L-methionine-$C^{14}H_3$ and DL-tryptophan-C^{14}, labeled in the benzene ring. Radioactivity incorporated into actinomycin was measured by the procedure of Katz and Weissbach (1963) with a Nuclear Chicago liquid scintillation spectrometer. Samples were counted in a naphthalene-dioxane solution (Bray, 1960).

Results: When 3-, 4- or 5-methyl-DL-proline was supplied to S. antibioticus, actinomycin synthesis was inhibited; the order of effectiveness was found to be 3->4->5-methylproline. A comparison of the activity of pure cis- and trans-3-methyl-DL-proline revealed that the cis isomer is some 14-fold more active than the trans compound. The concentration of analog required to obtain a 50% inhibition is presented in Table I.

Table I

Concentration of Various Methylproline Analogs Required for 50 Percent Inhibition of Actinomycin Synthesis

Compound	Concentration for 50 Percent Inhibition M
cis-3-Methyl-DL-proline	3.3×10^{-7}
3-Methyl-DL-proline (cis + trans)	7.8×10^{-7}
trans-3-Methyl-DL-proline	4.5×10^{-6}
4-Methyl-DL-proline (cis + trans)	3.6×10^{-5}
5-Methyl-DL-proline (cis + trans)	5.2×10^{-4}

The methyl proline was added after 24 hr. incubation; actinomycin titer was determined spectrophotometrically at 48 hr.

The actinomycin mixture synthesized in the presence of each of the methylprolines was isolated and separated by circular paper chromatography as described in Methods and Materials. The composition of the actinomycin complex formed in each case is shown in Figure I. In the control, actinomycin I, IV and trace amounts of actinomycin V were synthesized. With 3-methyl-DL-proline, the mixture was similar to the control except that somewhat less actinomycin IV and a small amount of actinomycin III (Johnson and Mauger, 1959) were formed. By contrast, in the presence of 4-methyl-DL-proline, the amount of actinomycin IV was reduced from 80% to about 10 to 20% and three new actinomycins, representing more than 60% of the mixture, were synthesized. In the

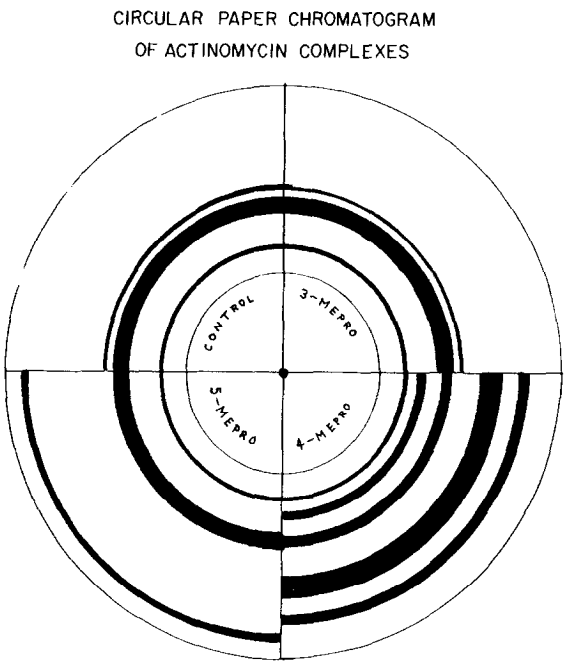

Fig. 1 - Schematic drawing of a circular paper chromatogram of the actinomycin complex formed in the presence of 3-, 4- and 5-methyl-DL-proline. The solvent system used consisted of 10% aq. sodium o-cresotinate: dibutyl ether: sym. tetrachloroethane (3:2:1). The actinomycin components observed from the origin in the control are actinomycins I, IV and V.

5-methyl-DL-proline-treated culture one new component (approximately 15%) was produced in addition to the mixture found in the control. After purification, the actinomycin mixture synthesized in the presence of 4-methyl-DL-proline was subjected to vigorous acid hydrolysis. In addition to sarcosine, valine, N-methylvaline, threonine and proline an imino acid, which possessed the identical electrophoretic mobility and similar Rf value by paper chromatography as authentic 4-methyl-proline, was present in the hydrolysate. It was concluded, therefore, that 4-methylproline had been incorporated into the actinomycins produced in the presence of this analog. The extent of incorporation of 4-methylproline into the actinomycins varied with the concentration of

the compound employed. For example, when 4-methylproline was supplied at a concentration of 7.8×10^{-5}M, the actinomycin mixture contained a proline: 4-methylproline ratio of 1:0.8; when employed at a concentration of 1.6×10^{-4}M, the ratio was 1:7. Further investigation of the actinomycins produced in the presence of 4-methylproline is in progress.

The inhibitory effect of 3-methyl-DL-proline (cis + trans) upon actinomycin formation was examined further. Conceivably, the analog might block antibiotic synthesis directly or it might exhibit an indirect action as a consequence of an inhibition of growth of S. antibioticus. Results of several experiments revealed that the inhibition is a direct one. In fact, the amount of mycelium formed in the presence of 3-methyl-DL-proline, in some cases, actually increased as the concentration of the inhibitor (over the range of 3.9×10^{-7} to 7.8×10^{-6}M) increased (Yoshida, Weissbach and Katz, 1966).

The effect of 3-methyl-DL-proline on the incorporation of C^{14}-labeled amino acids into actinomycin during a 60-minute incubation is shown in Table II. These results correlate well with the inhibition of actinomycin formation as measured by a spectrophotometric assay.

The analog inhibits actinomycin synthesis when supplied to S. antibioticus prior to or during actinomycin formation. However, when 3-methylproline is added after actinomycin formation has commenced an appreciable lag occurs before the maximum effect is obtained. Thus, with L-proline-C^{14} as precursor and 3-methyl-DL-proline (7.8×10^{-6}M), a 95% inhibition of antibiotic synthesis was achieved after 4 hr.; when the analog was used at 3.9×10^{-5}M, only 1 hr. was required to attain complete inhibition.

Experiments, to date, indicate that the inhibition of actinomycin synthesis by 3-methyl-DL-proline is related to an inhibition of the incorporation of L-proline into the antibiotic peptide. L-Proline and

analogs such as hydroxy-L-proline, DL-pipecolic and L-azetidine-2-carboxylic acids are capable of reversing the inhibition due to 3-methylproline.

Table II

Effect of 3-Methyl-DL-proline on the Incorporation of C^{14}-labeled Amino Acids into Actinomycin

Amino acid precursor	3-Methyl-DL-proline					
	0		7.8×10^{-7} M		7.8×10^{-6} M	
	Actinomycin					
	cpm/ml	cpm/ml	% Inhibition	cpm/ml	% Inhibition
DL-Tryptophan-C^{14}	2,910	1,340	54	348	88
L-Methionine-$C^{14}H_3$	5,320	1,990	63	257	95
L-Threonine-C^{14}	2,350	789	66	116	95
L-Valine-1-C^{14}	2,295	750	67	312	86
L-Proline-C^{14}	3,480	920	74	50	99
Glycine-1-C^{14}	790	208	74	16	98
Actinomycin titer, µg/ml	28	10	64	1	96

3-Methyl-DL-proline was added at 24 hours; ^{14}C-amino acid (0.9 to 1.1 X 10^4 cpm per mL) was supplied at 48 hours. Incubation with radioisotope was for 60 minutes; radioactivity incorporated into actinomycin was measured by the method of Katz and Weissbach (1963).

Discussion: The actinomycins constitute a model system for studying peptide synthesis. Thus, an investigation of the influence of proline analogs upon actinomycin formation may shed some light on the nature of this biosynthetic process and its relationship to protein synthesis. It is evident from previous findings as well as the results described here that small steric or structural differences, such as changes in the location of a substituent in a molecule, can profoundly affect the ability of a compound to influence actinomycin synthesis both qualitatively and quantitatively. For example, 3-methylproline is a highly active and selective inhibitor of antibiotic synthesis. While the site and nature of the inhibition remains unknown it is noteworthy that the compound is the most potent inhibitor of actinomycin synthesis discovered thus far. Although 4-methylproline and, to a lesser extent, 5-methylproline are somewhat inhibitory substances, they appear to be incorporated readily into the antibiotic molecule in place of proline. It would be of interest to determine the biological activity of the antibiotics produced in the presence of these analogs and to establish whether the methylprolines are incorporated into cellular proteins by S. antibioticus.

References

1. Brockmann, H. Ann. N.Y. Acad. Sciences 89, 323 (1960).
2. Katz, E. and Pugh, L.H. Appl. Microbiol. 9, 263 (1961).
3. Katz, E. Ann. N.Y. Acad. Sciences 89, 304 (1960).
4. Cox, D.A., Johnson, A.W. and Mauger, A.B. J. Chem. Soc. 5024 (1964).
5. Mauger, A.B., Irreverre, F. and Witkop, B. J. Amer. Chem. Soc. 88, 2019 (1966).
6. Sivak, A., Meloni, M., Nobili, F. and Katz, E. Biochim. Biophys. Acta 57, 283 (1962).
7. Katz, E. and Weissbach, H. J. Biol. Chem. 238, 666 (1963).
8. Katz, E., Prockop, D.J. and Udenfriend, S. J. Biol. Chem. 237, 1585 (1962).
9. Bray, G.A. Anal. Biochem. 1, 279 (1960).
10. Johnson, A.W. and Mauger, A.B. Biochem. J. 73, 533 (1959).
11. Yoshida, T., Weissbach, H. and Katz, E. Arch. Biochem. Biophys. 114, 252 (1966).