

Novel Actinomycins Formed by Biosynthetic Incorporation of *cis*- and *trans*-4-Methylproline

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Streptomyces parvulus (*Streptomyces parvullus*) normally produces actinomycin D; in the presence of *cis*-4-methylproline, this species synthesizes two additional actinomycins, designated K_{1c} and K_{2c}, in which one and two proline sites, respectively, are occupied by *cis*-4-methylproline. Analogously, actinomycins K_{1t} and K_{2t} are formed in the presence of *trans*-4-methylproline. Both mixtures were separated chromatographically, and the four novel actinomycins were obtained in crystalline form. Their biological activities were compared with that of actinomycin D in respect to inhibition of ribonucleic acid, deoxyribonucleic acid, and protein synthesis and antimicrobial potency. In all cases examined, the order of activity $D > K_{1t} > K_{1c} > K_{2t} > K_{2c}$ was observed, and the same sequence prevailed in a spectroscopic measure of their binding to deoxyribonucleic acid. In addition, proton nuclear magnetic resonance studies revealed that the replacement of proline by *cis*-4-methylproline alters the conformation of the antibiotic molecule.

In an earlier study of the effects of 3-, 4-, and 5-methylproline upon actinomycin biosynthesis by *Streptomyces antibioticus*, it was reported that new actinomycins were formed in the presence of 4-methylproline (isomeric mixture) (39, 40). Hydrolysis of the actinomycin mixture, which was too complex to separate, demonstrated that the 4-methylproline had been incorporated into the peptide moieties of the antibiotic. Similar observations were made with pipercolic acid and azetidine-2-carboxylic acid (17), and, more recently, various novel actinomycins containing the latter proline analogues have been isolated and characterized (10, 11, 18). The present study was designed to furnish individual actinomycins containing *cis*- and *trans*-4-methylproline for characterization and for comparisons of their biological activities with that of actinomycin D. For this purpose, *Streptomyces parvulus* (*Streptomyces parvullus*) was used with a modified culture medium (37). The advantages of using this species include higher yields of antibiotic and the fact that relatively few actinomycin analogues are synthesized since, under normal conditions, only one component (actinomycin D) is elaborated. *cis*- and *trans*-4-Methylproline were used in separate experiments; as each isomer was incorporated into one or both proline sites of the actinomycin molecule, a total of four new actinomycins (Fig. 1) was obtained.

cis- and *trans*-Methylproline have both been described as normal constituents of other

peptide antibiotics. Thus, *cis*-4-methyl-L-proline occurs in antibiotic ICI 13,959 (22), whereas *trans*-4-methyl-L-proline is a component of griselimycin (35) and the monamycins (13). In addition, the free *trans* isomer has been detected in apples (15). *cis*-3-Methylproline is a component of the peptide antibiotic bottromycin A (32), whereas *trans*-3-methylproline occurs in roseotoxin B (9). *cis*-5-Methylproline has been identified as a component of actinomycin Z₅ (7, 19), and 2,3-*trans*-2,5-*cis*-3-hydroxy-5-methylproline (20, 30) and 4-keto-5-methylproline (6) occur in actinomycin Z₁. A related example is 2,3-*trans*-3,4-*cis*-3-hydroxy-4-methylproline, which is a constituent of echinocandin B (3). The potent inhibitory effect of actinomycins upon deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) synthesis and their resulting antitumor activity have prompted the production and evaluation of numerous analogues (31). The present study with *cis*- and *trans*-4-methylproline is an example of the directed biosynthesis approach. Comparisons of the biological activities of these modified actinomycins with that of actinomycin D were made, and the Sobell model (16) for intercalation of the actinomycin chromophore between the DNA base pairs was considered in attempting to explain the structure-activity relationships observed in this series.

MATERIALS AND METHODS

Radioisotopes. L-[U-¹⁴C]valine (270 mCi/mmol),

[2-¹⁴C]juridine (60 mCi/mmol), [2-¹⁴C]thymidine (57 mCi/mmol), and [U-¹⁴C]adenosine-5'-triphosphate (555 mCi/mmol) were purchased from Amersham/Searle Corp., Des Plaines, Ill.

Actinomycins. Actinomycin D (IV) was provided by Merck Sharp & Dohme Research Laboratories, Rahway, N. J. Actinomycins K_{1c} , K_{11} , K_{2c} , and K_{2t} were purified from cultures of *S. parvulus* supplemented with *cis*- or *trans*-4-methylproline in the medium as described below. For biological studies, the antibiotics were dissolved in 50% ethanol (1 mg/ml). Dilutions were prepared in water or tris-(hydroxymethyl)aminomethane-NaCl buffer and pasteurized at 70°C for 20 min before use.

4-Methylproline isomers. Syntheses of *cis*-4-methyl-DL-proline-hydrochloride \cdot H₂O (95% *cis* + 5% *trans*) and of a mixture (1:1) of the *trans*-L and *cis*-D isomers will be described elsewhere. It was established that use of the latter material resulted in incorporation of only the *trans* isomer into actinomycins.

Chromatographic materials. Silicic acid (Sil B-200, 60 to 200 mesh) for column chromatography was purchased from the Sigma Chemical Co., St. Louis, Mo. Thin-layer chromatography sheets (Gelman ITLC) containing silicic acid (20 by 20 cm, 250 μ m thick) were obtained from the Gelman Instrument

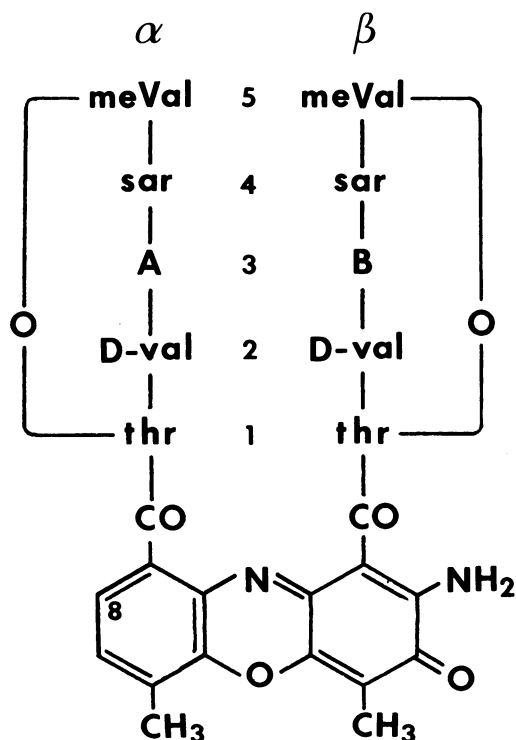


FIG. 1. Structures of actinomycins. Abbreviations: thr = L-threonine; val = D-valine; sar = sarcosine; meVal = N-methyl-L-valine. In actinomycin D, A = B = L-proline; in k_{1c} , A/B = L-proline/*cis*-4-methylproline; in K_{2c} , A = B = *cis*-4-methylproline; in K_{11} , A/B = L-proline/*trans*-4-methylproline; in K_{2t} , A = B = *trans*-4-methylproline.

Co., Ann Arbor, Mich. Acid-washed alumina was prepared from alumina (Brockmann activity no. 1, 80 to 200 mesh; Fisher Scientific Co., Pittsburgh, Pa.) by a published procedure (1).

Nucleic acids, nucleotides, and enzymes. *Micrococcus lysodeikticus* DNA and calf thymus DNA were obtained from Miles Laboratories, Inc., Elkhart, Ind. Crude *Bacillus subtilis* (Marburg strain) DNA was provided by O. Landmann, Department of Biology, Georgetown University, and purified by the Marmur procedure (26). Salmon sperm DNA (for DNA assays) and yeast RNA (for RNA assays) were obtained from the Sigma Chemical Co. *Escherichia coli* K-12 DNA-dependent RNA polymerase and guanosine-, uridine- and adenosine-5'-triphosphate were procured from Miles Laboratories. Cytidine-5'-triphosphate was from Boehringer Mannheim Biochemicals, Indianapolis, Ind., and bovine serum albumin (for protein assays) was purchased from Schwarz/Mann, Div. of Becton, Dickinson & Co., Orangeburg, N.Y.

Organisms and conditions of cultivation. *S. parvulus* (ATCC 12434) was employed for the synthesis of actinomycins. The organisms were maintained on slants of a glucose-yeast extract-malt extract agar medium (14), and were stored at 4°C. The procedure for preparation of vegetative mycelium (NZ-Amine liquid medium) as inoculum for actinomycin synthesis (glutamic acid-histidine-fructose-mineral salts medium) has been described previously (37). *Micrococcus luteus* (ATCC 15307), *Sarcina subflava*, *Staphylococcus aureus* (penicillin susceptible), and *B. subtilis* (Marburg strain, wild type) were employed for investigation of the biological properties of the actinomycins. The bacterial cultures were grown on nutrient agar slants at 37°C, except *M. luteus* which was incubated at 26°C.

Production of actinomycins. All experiments were carried out at 30°C on a gyratory shaking incubator (New Brunswick Scientific Co., New Brunswick, N.J.) at 250 rpm. For the production of actinomycins containing *cis*- or *trans*-4-methylproline, the proline analogue was added to the chemically defined medium (37) after 24 h of incubation (i.e., at the onset of actinomycin biosynthesis). The cultures were then reincubated for 5 days. The mycelium was separated from the culture medium by filtration and washed twice with an equal volume of water. After extraction of the filtered medium and washings with ethyl acetate, the extracts were evaporated under reduced pressure, and the residual actinomycin mixtures were separated into their components.

Separation of actinomycins containing *cis*-4-methylproline. Separation of actinomycins containing *cis*-4-methylproline was effected by descending preparative paper chromatography on paper sheets (Whatman 3MM, 18 by 20 inches [about 45.7 by 50.8 cm]) impregnated with 5% aqueous sodium *o*-cresotinate. The antibiotic mixture (15 mg) in acetone was applied as a streak to each paper, and the chromatograms were developed with amyl acetate for 10 to 12 h. Three zones were distinguished, corresponding to actinomycins D ($R_D = 1.00$), K_{1c} ($R_D = 2.13$), and K_{2c} ($R_D = 3.73$). The zones were cut out, chopped into small pieces, and blended with water and ethyl acetate in a Waring blender for 3 to 5 min.

The paper pulp was filtered off, and the ethyl acetate layer was washed with aqueous sodium bicarbonate (to remove *o*-cresotinate) and water and then evaporated to dryness. Actinomycin K_{1c} was rechromatographed by the same procedure to remove traces of K_{2c}.

Separation of actinomycins containing *trans*-4-methylproline. Separation of actinomycins containing *trans*-4-methylproline was accomplished by preparative thin-layer chromatography on Gelman ITLC silicic acid-impregnated glass-fiber sheets (20 by 20 cm), using ethyl acetate. Three zones were distinguished, which corresponded to actinomycins D ($R_D = 1.00$), K₁₁ ($R_D = 1.24$), and K₂₁ ($R_D = 1.42$). The zones were cut out and extracted with methanol-ethyl acetate (1:1), and the extracts were filtered and evaporated. Actinomycin K₁₁ was rechromatographed by the same procedure to remove traces of K₂₁.

Purification of separated actinomycins. Individual actinomycin preparations obtained as described above were chromatographed on columns of silicic acid with 10% methanol in ethyl acetate and finally purified on columns of acid-washed alumina (1), using chloroform. They were then recrystallized from chloroform-ethanol, with the exception of K₂₁, which crystallized from chloroform-benzene. Melting points were obtained using a Thomas micro hot-stage Kofler-type apparatus.

Circular paper chromatography. Homogeneity of the individual actinomycins described above was established by circular paper chromatography (11), using the solvent systems (i) di-*n*-butyl ether-*sym*-tetrachloroethane-10% aqueous sodium *o*-cresotinate (3:1:4 and 5:1:6), (ii) isopropyl ether-chloroform-10% sodium *o*-cresotinate (3:2:5), and (iii) amyl acetate-5% sodium *o*-cresotinate. In each case the paper was impregnated with the aqueous phase prior to chromatography.

Amino acid analysis. Each actinomycin (5 to 10 mg) was hydrolyzed in 5 ml of 6 N HCl for 3 h at 121°C. The hydrolysates were decolorized with Norit A charcoal (neutral), filtered, evaporated under reduced pressure, and redissolved twice in water and evaporated. The amino acid content of the residue was evaluated by using the following procedures.

(i) Quantitative amino acid analysis was carried out on a Beckman-Spinco automatic amino acid analyzer, model 120C, as described previously (38). The data were normalized to *D*-valine = 2.00 residues per actinomycin molecule.

(ii) Two-dimensional paper electrophoresis and paper chromatography (38) were effected on sheets of Whatman 3MM paper. The first dimension involved high-voltage (3,000 V) paper electrophoresis (Gilson Medical Electronics, Inc., Middleton, Wis.) with a 4% formate buffer at pH 1.9 for 3 h at 180 mA. For the second dimension, solvent system (i) 1-butanol-acetic acid-water (4:1:5, upper layer) or (ii) methanol-pyridine-water (20:1:5) were employed. Amino acids were visualized with 0.2% ninhydrin in acetone; cyclic imino acids were detected with 0.2% isatin in acetone.

(iii) In gas-liquid chromatography, the hydrolysates were evaporated in vacuo, and the residual amino acids were derivatized to their *N*-trifluoroacetyl methyl esters as described previously (29). Chromatography was effected on a gas chromatograph (Shimadzu, model 4BM) containing a column (6 feet [about 1.83 m] by 3 mm) of ethylene glycol adipate (0.5% on Chromosorb W, 60 to 80 mesh) at 100°C with argon (40 ml/min) as carrier gas.

NMR. Proton nuclear magnetic resonance (NMR) spectra were obtained in CDCl₃ solution (0.05 M) at 220 MHz using a Varian HR220 instrument, with tetramethylsilane as internal reference.

Biological activities. Determination of minimal inhibitory concentrations and of 50% inhibition of the exponential growth and biopolymer synthesis of *B. subtilis* (RNA, DNA, and protein) by the various actinomycins was accomplished by procedures that have been described in previous publications (12, 27). The inhibitory effect of different concentrations of the various actinomycins on the *E. coli* K-12 DNA-dependent RNA polymerase reaction was also studied (8).

Difference spectra. Difference spectra (12, 27) were obtained manually with a Gilford model 2000 spectrophotometer.

Actinomycin concentration. The actinomycin concentration in culture filtrates was carried out spectrophotometrically at 443 nm in ethyl acetate (21). In the case of pure actinomycins, determinations were effected in methanol at the same wavelength.

Radioactive measurements. Radioactive measurements on portions of solubilized protein, RNA, and DNA were made in 10 ml of Bray's liquid scintillation fluid (4). For the DNA-dependent RNA polymerase-catalyzed reaction, the acid-insoluble product was collected on a Whatman glass-fiber filter and counted in 10 ml of Omnifluor (New England Nuclear Corp., Boston, Mass.). Measurements were effected in a Mark I Nuclear-Chicago refrigerated liquid scintillation spectrometer.

RESULTS AND DISCUSSION

Synthesis of 4-methylproline-containing actinomycins. Preliminary experiments to determine the levels of *cis*- and *trans*-4-methylproline suitable for maximum production of modified actinomycins revealed that 250 and 200 µg/ml, respectively, represented the optimum concentrations. Furthermore, no significant inhibition of synthesis occurred with the *cis* isomer, whereas a moderate reduction (30 to 40%) was noted with the *trans* isomer relative to control cultures (550 to 600 µg of actinomycin D per ml). A total of 2.9 g of actinomycin mixture was obtained after supplying the *cis* isomer; correspondingly, 2.7 g of crude actinomycin mixture was obtained after supplementation with the *trans* isomer.

Separation and purification of modified ac-

tinomycins. The individual components of each of the above-mentioned actinomycin complexes were separated and purified by the chromatographic procedures described in Materials and Methods. Four novel actinomycins were obtained in crystalline form (red prisms) and designated K_{1c} and K_{2c} (from *cis*-4-methylproline) and K_{1t} and K_{2t} (from *trans*-4-methylproline). Their melting points were: actinomycin K_{1c} , 244 to 246°C; K_{2c} , 236 to 237°C; K_{1t} , 248 to 250°C; K_{2t} , 245 to 247°C.

Amino acid analysis. The quantitative data obtained with the amino acid analyzer are summarized in Table 1, and typical elution profiles are shown in Fig. 2. These results correlate with the outcome of the two-dimensional paper electrophoresis-paper chromatography separations; details of the mobilities and R_f values of these amino acids have been published (19, 29). Since these two methods make no distinction between *cis*- and *trans*-4-methylproline, gas-liquid chromatography (29) was used to identify the isomer present in each hydrolysate. The retention times (minutes) of standards were: proline, 12.3; *trans*-4-methylproline, 13.5; *cis*-4-methylproline, 15.0. In the hydrolysate chromatograms the following results were obtained: actinomycin K_{1c} contained proline, *cis*-4-methylproline, and a trace of *trans*-4-methylproline; K_{1t} contained proline, *trans*-4-methylproline, and a trace of *cis*-4-methylproline; K_{2c} contained *cis*-4-methylproline plus a trace of *trans*-4-methylproline; K_{2t} contained *trans*-4-methylproline and a trace of *cis*-4-methylproline. Some interconversion of *cis*- and *trans*-4-methylprolines occurs during hydrolysis of the actinomycins. These results form the basis for the designations given in Table 1.

NMR. The NMR data indicate that actinomycins K_{1c} and K_{1t} are both single entities, as distinct from mixtures of two possible isomers having the α - and β -peptide moieties inter-

changed. However, the question of which isomer is present in each case is not yet resolved. Spectra of K_{1t} and K_{2t} were remarkably similar to those of actinomycin D (2, 23, 24, 36), except for the presence of doublets at δ 1.17 and 1.12, respectively, which were assigned to the *trans*-4-methylprolyl methyl groups. On the other hand, spectra of K_{1c} and K_{2c} , especially the latter, displayed small but important differences in chemical shifts, revealing that conformational adjustments occur when proline is replaced by *cis*-4-methylproline, but not by *trans*-4-methylproline. The most striking example is that of the β -peptide threonine NH signal of K_{2c} , which occurs at δ 6.94 in $CDCl_3$. In other actinomycins, this resonance covers the range δ 7.50–7.86. The threonine NH coupling constants ($J_{NH,C\alpha H}$) are somewhat larger (7.0 and 7.5 Hz) than those of other actinomycins (typically, 6.2 and 6.8 Hz), reflecting slightly altered NH-C α H dihedral angles. The chromophoric 8-proton of K_{2c} is at higher field (δ 7.50) than in actinomycin D (δ 7.65), revealing a slight adjustment in the angle between the chromophoric plane and that of the actinocyl-threonine (α -peptide) amide bond. A fundamental difference in pyrrolidine ring conformation is evident from the 4-methylprolyl α -proton splittings. The latter, in the case of *trans*-4-methylproline (in K_{1t} and K_{2t}), resemble those of proline in actinomycin D (approximate doublet), whereas in the case of *cis*-4-methylproline (in K_{1c} and K_{2c}) a triplet pattern is observed. The latter formation (actually an overlapping doublet of doublets) results from two nearly equal $J_{\alpha,\beta}$ coupling constants, whereas the doublet situation arises when one $J_{\alpha,\beta}$ approximates to 0, due to a *trans*- α,β dihedral angle close to 90°. This conformational difference in the case of *cis*-4-methylproline in turn affects the peptide backbone conformation of K_{1c} and K_{2c} , with the result that, especially in K_{2c} , a noticeable de-

TABLE 1. Quantitative amino acid analyses of actinomycins

Actinomycin	Amino acid ^a						
	Valine	Sarcosine	Threonine ^b	N-Methylvaline	Proline	<i>cis</i> -4-Methylproline	<i>trans</i> -4-Methylproline
D	2.0	2.1	1.0	1.7	2.0	0.0	0.0
K_{1c}	2.0	2.0	0.9	1.8	0.9	1.0	0.0
K_{2c}	2.0	1.9	0.9	1.8	0.0	2.0	0.0
K_{1t}	2.0	1.8	0.9	1.6	0.7	0.0	1.0
K_{2t}	2.0	1.7	0.9	1.7	0.0	0.0	2.0

^a Residues per molecule normalized to valine = 2.0.

^b The low yields of threonine are due to destruction of the amino acid during acid hydrolysis of an actinomycin molecule.

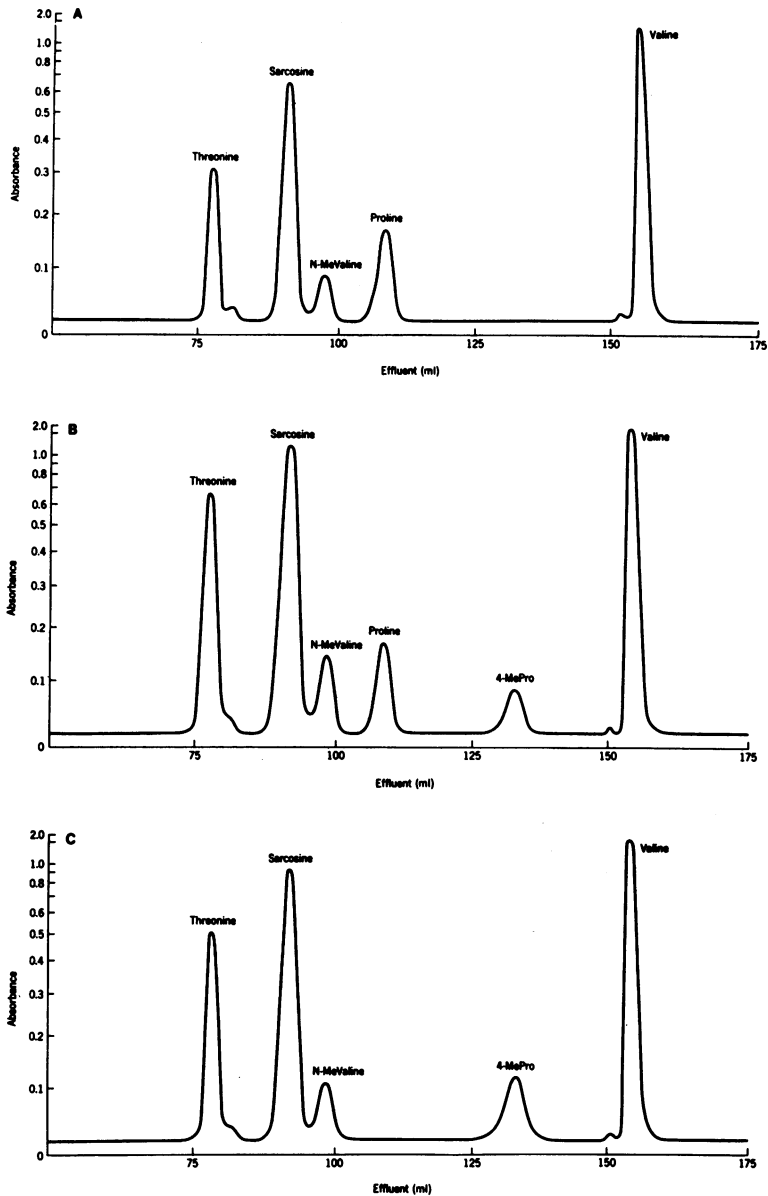


FIG. 2. Separation of amino acid hydrolysates from a crystalline actinomycin component: (A) actinomycin D; (B) actinomycin K_{1c} ; (C) actinomycin K_{2c} . The amino acids were eluted with 0.2 sodium citrate buffer at pH 3.05 and 4.25 from the column (0.9 by 69 cm). Flow rate of both the buffer and ninhydrin solutions were 34 ml/h. Absorbance was measured at 570 nm, except for the imino acids, which were measured at 440 nm (38). *N-MeValine* = *N-methyl-L-valine*.

parture from the uniform conformation observed for various other actinomycins (25, 28) ensues. This conclusion, which is also supported by ^{13}C NMR studies (W. A. Thomas and A. B. Mauger, unpublished data), will be described in detail elsewhere.

Biological activities. In qualitative aspects, the biological activities of these modified actinomycins *in vivo* and *in vitro* resemble that of actinomycin D. However, an exception is seen in the inhibition of DNA synthesis *in vivo* (Table 2), where markedly reduced activity was

exhibited by the modified actinomycins. In the other studies (difference spectroscopy with DNA, antibacterial activity, and inhibition of RNA and protein synthesis) a uniform trend was observed: $D > K_{11} > K_{1c} > K_{21} > K_{2c}$. The parallel behavior of these compounds under various experimental conditions is noteworthy. For example, the minimal inhibitory concentrations obtained with *B. subtilis* (Table 3) relate favorably to the 50% inhibition of the exponential growth of this organism and to the inhibition of its RNA and protein synthesis (Table 4). This uniform trend even extends to the difference spectra with DNA, strongly suggesting that differences in biological activity in this series of actinomycin analogues are directly related to the differences in their complexing with DNA (Fig. 3). Similar spectral data, although not presented here, were obtained with preparations of *B. subtilis* DNA. A comparable correlation between physicochemical and bio-

logical data was observed in the case of a series of actinomycins produced in the presence of pipecolic acid (12, 18). In contrast, there is a less direct comparison between these two criteria in the case of actinomycin Z components (27).

In comparison with actinomycin D, replacement of both proline residues by *cis*- or *trans*-4-methylproline resulted in greater loss of biological potency than replacement of one proline moiety. A similar effect was noted in the replacement by pipecolic acid (12, 18), but not by azetidine-2-carboxylic acid (10). It was also observed that substitution of proline by *cis*-4-methylproline reduced potency more than in the case of the *trans* isomer, i.e., $K_{11} > K_{1c}$ and $K_{21} > K_{2c}$. This effect may be related to the conclusions drawn from the NMR data, particularly, that the replacement of proline by *cis*-4-methylproline in actinomycin is accompanied by a conformational change that is not so apparent in the case of the *trans* isomer. It should also be noted, however, that the proline sites in actinomycin are in a critical location in respect to the interaction of the molecule with DNA, such that a substituent can exert a steric effect (16, 34). Thus, in the case of replacement of proline by *trans*-4-hydroxyproline (actinomycin $X_{\alpha\beta}$) or *cis*-4-(allo)-hydroxyproline ($X_{\alpha\beta}$) biological activities were considerably higher in the latter case (5, 33); the hydroxyl group of the imino acid (in the case of $X_{\alpha\beta}$) may sterically hinder complexing between the actinomycin molecule and DNA. In terms of the Sobell model (16, 34), an analogous difference (*cis* > *trans*) was expected in the case of the 4-methylproline substitution. The methyl groups of *trans*-4-methylproline in actinomycins K_{11} and K_{21} are located so that they contact the DNA

TABLE 2. Inhibition of DNA synthesis in *B. subtilis* by actinomycins

Actinomycin (5 μ g/ml)	DNA synthesis (% of control)	
	Expt 1 ^a	Expt 2 ^b
D	15	4
K_{11}	58	69
K_{1c}	77	85
K_{21}	65	74
K_{2c}	79	77

^a Ten milliliters of an exponentially growing culture of *B. subtilis* was added to a flask containing [2-¹⁴C]thymidine (1 μ Ci, 17.5 nmol) and actinomycin and incubated for 15 min at 37°C in a Dubnoff metabolic shaking incubator.

^b Ten milliliters of an exponentially growing culture of *B. subtilis* was preincubated with an actinomycin preparation for 10 min, and then [2-¹⁴C]thymidine (1 μ Ci, 17.5 nmol) was added. Incubation was then resumed as described in experiment 1.

TABLE 3. Antimicrobial activity of actinomycins

Species	Minimal inhibitory concentration (μ g/ml)				
	D	K_{11}	K_{1c}	K_{21}	K_{2c}
<i>M. luteus</i> ^a	0.03	0.06	0.08	0.11	0.16
<i>S. subflava</i> ^a	0.04	0.08	0.11	0.12	0.20
<i>S. subflava</i>	0.03	0.06	0.10	0.10	0.18
<i>B. subtilis</i>	0.09	0.25	0.45	0.60	0.90
<i>S. aureus</i>	0.2	0.6	1.2	3.5	4.0
Relative activity (%)	100	33-50	17-36	5-33	5-20

^a Incubation at 26°C for 40 h; all other incubations at 37°C for 20 h.

TABLE 4. Inhibition (50%) of bacterial growth and macromolecular synthesis by actinomycins

Expt	Actinomycin (μ g/ml)				
	D	K_{11}	K_{1c}	K_{21}	K_{2c}
Exponential growth ^a	0.045	0.13	0.24	0.32	0.47
RNA synthesis ^a	0.10	0.28	0.52	0.68	1.08
Protein synthesis ^a	0.21	0.56	1.09	1.28	2.2
DNA-dependent RNA polymerase-catalyzed reaction ^b	0.25	0.60	0.97	1.65	2.0

^a *B. subtilis*; procedures used are described in a previous publication (27).

^b *E. coli* DNA-dependent RNA polymerase; calf thymus DNA (6.7 μ g) was used as template. All other procedures used have been described (8, 27).

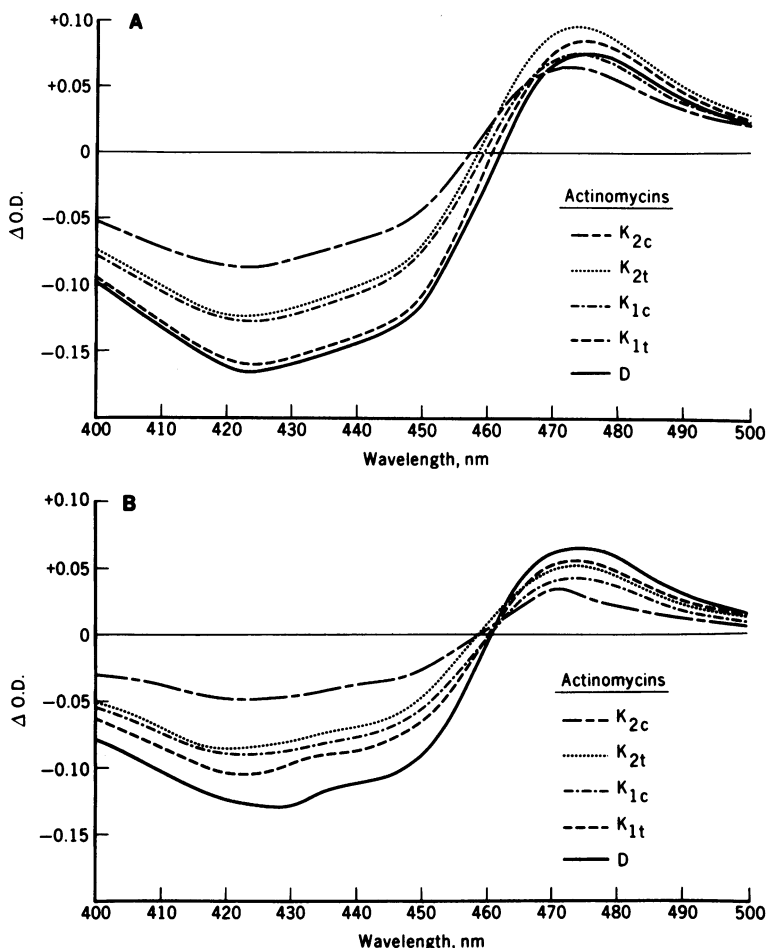


FIG. 3. Difference spectra obtained with: (A) *M. lysodeikticus* DNA (36.4 μg) and (B) calf thymus DNA (36.6 μg), using actinomycins D, K_{1t} , K_{2t} , K_{1c} , and K_{2c} in 0.001 M tris(hydroxymethyl)aminomethane-hydrochloride-0.005 M NaCl buffer, pH 7.4.

bases during intercalation, whereas the corresponding methyl groups in K_{1c} and K_{2c} extend away from the chromophore and, hence, the intercalation site. The fact that the *trans*-4-methylproline-substituted actinomycins were more active than the corresponding *cis* compounds could be explained by hydrophobic interactions as well as the conformational differences observed.

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