ADENYLYLATION OF TROSPECTOMYCIN BY CRUDE ENZYME PREPARATIONS FROM *ESCHERICHIA COLI*

Y. YAGI, L. BACZYNSKYJ, S. A. MIZSAK and A. D. ARGOUDELIS

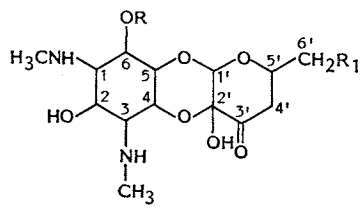
The Upjohn Company,
Kalamazoo, Michigan 49001, U.S.A.

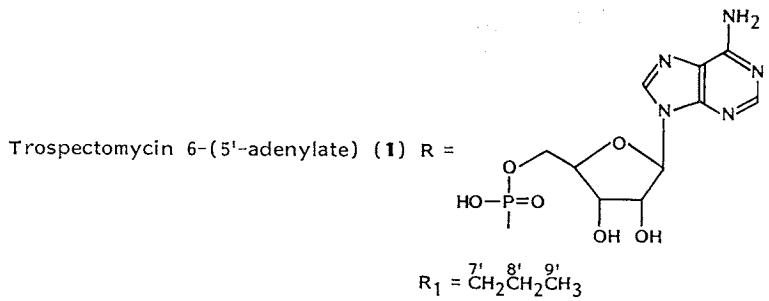
(Received for publication March 10, 1988)

Employing osmotically shocked lysate of a spectinomycin resistant strain of *Escherichia coli*, trospectomycin, a new alkylspectinomycin, was adenylylated in the presence of adenosine 5'-triphosphate and magnesium ion. A highly resistant strain of *E. coli* was obtained by transforming a laboratory strain with a newly constructed plasmid consisting of pBR322 and a determinant for spectinomycin resistance originally found on a low copy number plasmid in *E. coli* strain NR79. The biologically inactive adenylylated trospectomycin was found to be trospectomycin 6-(5'-adenylate).

Trospectomycin (U-63366; Fig. 1, **1a**) is a new alkylspectinomycin analog described by WHITE and his co-workers.[1] Recent *in vitro* studies indicate that trospectomycin is more effective than spectinomycin against various organisms[2] including *Haemophilus ducreyi*,[3] penicillin-resistant strains of *Neisseria gonorrohoeae*[2] and *Chlamydia trachomatis* (abstract: G. E. ZURENKO *et al.*, 2nd World Cong. on Sexually Transmitted Dis., Paris, 1986; abstract: E. NOVAK *et al.*, 15th Int. Cong. of Chemother., Istanbul, 1987). It has also been shown that pretreatment of a strain of *E. coli* with trospectomycin enhances ability of serum to kill the organism.[4] In the past, enzymatic inactivation has been reported on various antibiotics such as spectinomycin (Fig. 1, **1b**), lincomycin and clindamycin.[5~7] In case of spectinomycin, inactivation has been accomplished by an adenylylating enzyme

Fig. 1. Structures of trospectomycin 6-(5'-adenylate), trospectomycin and spectinomycin.





Trospectomycin 6-(5'-adenylate) (**1**) R =

$R_1 = {}^{7'}CH_2{}^{8'}CH_2{}^{9'}CH_3$

Trospectomycin (**1a**) R = H $R_1 = CH_2CH_2CH_3$

Spectinomycin (**1b**) R = H R_1 = H

Table 1. Strains.

	UC[a] I.D.	Additional strain designation	Plasmid[b]	Reference
Escherichia coli	UC 3833	NR79	pUC18	6
E. coli	UC 9224	MC 1061	None	12
E. coli	UC 9464	MC 1061 (pUC18)	pUC18	This study
E. coli	UC 12150	MC 1061 (pUC1153)	pUC1153	This study

[a] Upjohn Company culture collection.

[b] The designation "pUC" has been assigned to The Upjohn Company by Plasmid Reference Center (Stanford, CA, U.S.A.).

which acts on the hydroxyl group at the C-6 position of the actinamine moiety (Fig. 1, **1b**).[7] In this report we present data indicating that trospectomycin (**1a**) is adenylylated by a cell-free extract of an *E. coli* strain which harbors a recombinant plasmid to direct overproduction of an adenylylating enzyme, and that the product of adenylylation is trospectomycin 6-(5′-adenylate) (Fig. 1, **1**).

Materials and Methods

Bacterial Strain

E. coli strains used in this study are listed in Table 1. The plasmid pUC18 (Fig. 2), found in *E. coli* NR79, UC 3833, was transformed into *E. coli* MC 1061, UC 9224, to generate *E. coli* UC 9464.

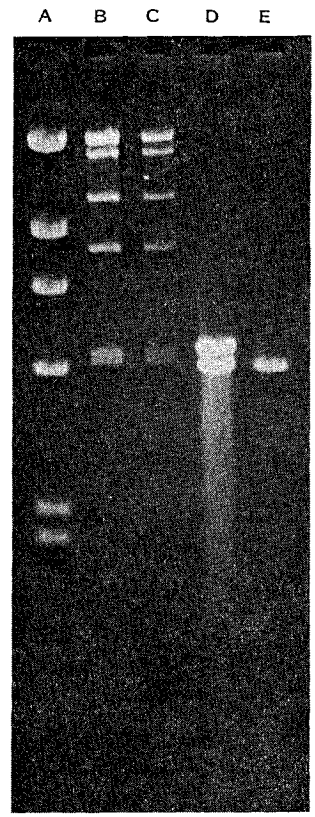
Sub-cloning of Spectinomycin-resistance Gene

Standard protocols were used for isolation of plasmids (pUC18, pBR322) from SDS lysates by ultracentrifugation and subsequent cloning of the spectinomycin-resistance gene of pUC18 into pBR322.[8] Selection for spectinomycin-resistant clones was carried out employing antibiotic medium No. 3 (Difco) with 100 μg of spectinomycin per ml. The enzymes necessary for molecular cloning were obtained from New England Biolabs (Beverly, MA, U.S.A.).

Determination of Antibiotic Resistance

The MIC was determined by the established tube dilution method employing the 0.5 McFarland turbidity standard.[9]

Fig. 2. Agarose gel (0.6%) electrophoresis of plasmid preparations.



A, phage λ digested by *Hin*d III; B, pUC18 from strain UC 3833; C, pUC18 from strain UC 9464; D, pUC1153 from strain UC 12150; E, pBR322, (B~E, digested by *Bam*H I).

Media

The modified medium B of NOSSAL and HEPPEL[10] was employed. For the MIC determination, Mueller-Hinton broth (Difco) was used.

Inactivation of Trospectomycin by Cell-free Extracts

The cells (*E. coli*, UC 12150) were grown to late logarithmic phase in 100 ml of the medium described above. After washing twice in 20 ml of TS buffer (0.03 M NaCl, 0.01 M Tris, pH 7.0) the cells were resuspended in 60 ml of STE buffer (20% sucrose, 1×10^{-3} M EDTA, 3.3×10^{-2} M Tris, pH 7.1) and stirred at room temperature for 40 minutes. This was then centrifuged ($13,000\times g$, 0°C, 10 minutes) to obtain a cell pellet, which was resuspended and stirred in 7.5 ml of ice cold distilled water for 25 minutes. Finally it was centrifuged ($13,000\times g$, 0°C, 20 minutes) to obtain clear supernatant (shockate), which represented a crude enzyme preparation. Protein in the shockate was quantitated by a Bio-Rad protein assay kit. For a larger scale inactivation of trospectomycin, a 4-liter culture was processed accordingly. Inactivation of trospectomycin was carried out (at 37°C, 11 hours) by mixing 125 μl of Tris-acetate buffer (0.5 M, pH 7.1), 75 μl of magnesium acetate (20 mM, pH 7.1), 50 μl of ATP (0.1 M), appropriate amount of the cell-free extract, trospectomycin (in water) and distilled water to make up a final volume of 500 μl.

Enzymatic Hydrolysis

Seven μg of inactivated trospectomycin was treated with 136 μg of venom phosphodiesterase (Worthington Biochemical) and 70 μl of buffer solution consisting of 0.05 M glycine - NaOH buffer (pH 9.0), 0.01 M $MgCl_2$ and 0.05 M KCl in a total volume of 160 μl. For a treatment with spleen phosphodiesterase (Worthington Biochemical) 300 μg of inactivated trospectomycin was mixed with 0.6 U of the enzyme and 100 μl of 0.25 M sodium succinate - HCl buffer (pH 6.5) in a total volume of 600 μl. In both cases the reaction mixtures were incubated at 37°C for 16 hours.

Spectroscopic Methods

^{1}H NMR spectra were recorded on a Varian XL-200 spectrometer operating at 100 MHz. Spectra were run in D_2O using sodium 2,2-dimethyl-2-silapentane-5-sulfonate (SDSS) as internal reference. ^{13}C NMR spectra were recorded on a Varian XL-200 spectrometer operating at 50.3 MHz. D_2O was used as the solvent. ^{1}H and ^{13}C NMR chemical shifts are reported as ppm relative to TMS. IR spectra were obtained in mineral oil suspension on a Digilab Model 14D Fourier Transform Spectrometer. Fast atom bombardment (FAB) spectra were obtained on a VG-ZAB-2F mass spectrometer.

Assay of Trospectomycin and Trospectomycin 6-(5′-Adenylate)

Since trospectomycin 6-(5′-adenylate) lacks *in vitro* antibacterial activity, its formation from trospectomycin can be followed by measuring the loss of its antibacterial activity. To determine the amount of trospectomycin in reaction mixtures or preparations obtained during purification a standard assay using either *E. coli* UC 51 or *Bacillus subtilis* UC 564 was employed. To assay for the presence of trospectomycin 6-(5′-adenylate), the phosphodiester bond was first hydrolyzed using snake venom phosphodiesterase by the procedure described above. The amount of trospectomycin in the hydrolysate was determined by a standard assay method.

TLC Analysis

The production and purification of trospectomycin 6-(5′-adenylate) were followed by TLC using Silica gel GF plates (Analtech) and water as the mobile phase. UV absorbing materials were detected by a short wavelength UV lamp. Bioinactive, UV-nonabsorbing materials were detected by permanganate-periodate spray reagent. Bioactive materials (trospectomycin) were detected by bioautography on agar seeded with *E. coli* UC 51. Trospectomycin 6-(5′-adenylate) exhibited an Rf value of 0.4 in this TLC system.

Isolation and Purification of Trospectomycin 6-(5′-Adenylate)

A reaction mixture containing approximately 500 mg of inactivated trospectomycin was adjusted to pH 7.5 and passed over a column containing 100 ml of Amberlite XAD-2 (Rohm and Haas Co., Philadelphia, PA). The spent was collected as one fraction. The column was washed with 300 ml of water and eluted with MeOH - water (70 : 30). The reaction mixture and the fractions obtained during the chromatography were analyzed for bioactivity before and after treatment with snake venom phosphodiesterase by UV determination at 260 nm as well as by TLC. Results indicated that the

starting material contained ATP, ADP, AMP and trospectomycin 6-(5′-adenylate); spent and aqueous wash contained mainly the different adenosine phosphates, while the methanolic eluates contained all of trospectomycin 6-(5′-adenylate) present in the reaction mixture. The methanolic eluates were combined and concentrated to an aqueous solution which was then freeze-dried to yield approximately 450 mg of a colorless material; UV λ_{max} nm (*a*), 260 (18.5). TLC on this preparation showed the presence of trospectomycin 6-(5′-adenylate) and trace of adenosine phosphates (ATP, ADP, AMP).

Isolation of Pure Trospectomycin 6-(5′-Adenylate)

A column was prepared from 200 ml of Amberlite XAD-2 in water. The starting material, 450 mg of the material isolated as described above, was dissolved in 20 ml of 0.1 M phosphate buffer (pH 7.0). This solution was passed over the column at a rate of 2 ml per minute. The column was washed with 600 ml of water, then with MeOH - water (10 : 90), 600 ml; MeOH - water (30 : 70), 600 ml; MeOH - water (50 : 50), 600 ml; finally MeOH - water (70 : 30), 600 ml. Fractions containing trospectomycin 6-(5′-adenylate) were combined, concentrated in an aqueous solution and freeze-dried to give 330 mg of pure trospectomycin 6-(5′-adenylate).

Anal Calcd for $C_{27}H_{42}N_7O_{13}P \cdot H_2O$ (MW 721): C 44.93, H 6.10, N 13.59, P 4.30.
Found C 45.07, H 5.62, N 13.18, P 3.69.

MW (FAB), 722 ($M+H^+ +H_2O$), 704 ($M+H^+$). Further characterization of trospectomycin 6-(5′-adenylate) is described in the following sections.

Results and Discussion

Subcloning a Spectinomycin-resistance Gene into pBR322

A spectinomycin adenylylating enzyme has previously been isolated from an *E. coli* strain NR73/W677 which is phenotypically similar to the original parent strain (NR79/W677) used in this study.[6] The initial examination revealed that the plasmid, pUC18, in NR79 exists as a low copy number molecule with molecular size of approximately 90 kb (Fig. 2). In order to facilitate large scale adenylylation of trospectomycin by a cell-free extract of a spectinomycin-resistant strain of *E. coli*, we attempted to subclone the spectinomycin-resistance gene into a high copy number plasmid pBR322. We hoped that either a high copy number of the gene or a more efficient promoter on pBR322 would result in better production of the adenylylating enzyme.

Fig. 3. Construction and a restriction map of pUC 1153.

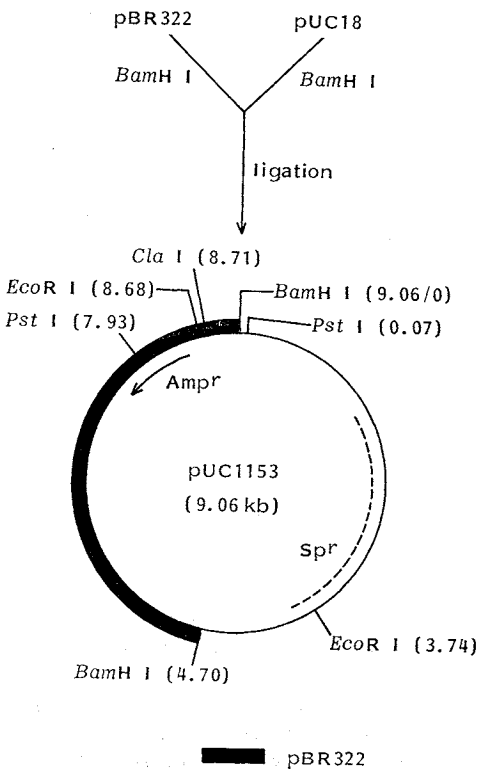


Table 2. MICs of drugs against *Escherichia coli* strains.

Strain	Spectinomycin (μg/ml)	Trospectomycin (μg/ml)
MC 1061, UC 9224	20	10
NR79, UC 3833	640	320
MC 1061 (pUC18), UC 9464	1,280	320
MC 1061 (pUC1153), UC 12150	>2,560	1,280

Table 3. Requirements for inactivation of trospectomycin.

Reaction conditions	Trospectomycin ($\mu g \times 10^{-1}$ ml)			
	0 hour	3 hours	6 hours	11 hours
Complete mixture	8.0	3.0	1.2	0
Without ATP	6.0	6.0	6.0	5.5
Without Mg^{2+}	6.0	6.0	5.5	5.5
Without shockate	6.0	6.0	6.0	5.0
With heated shockate (65°C, 10 minutes)	10.0	ND	ND	8.0

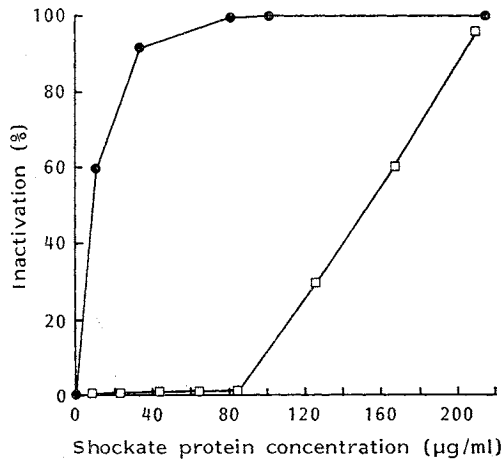
ND: Not determined.

As outlined in Fig. 3, the *Bam*H I digested preparations of pUC18 and pBR322 were ligated, and the mixture was transformed into *E. coli* MC 1061. Among the spectinomycin-resistant transformants (selected on a plate containing 100 μg of spectinomycin per ml), the isolate UC 12150 harbored a plasmid (designated pUC1153, Figs. 2 and 3) consisting of pBR322 and a 4.7-kb insert. When the MICs of trospectomycin were compared among the *E. coli* strains (Table 2), it was found that the newly constructed strain UC 12150 was much more resistant to the drug (MIC 1,280 μg/ml) than its isogenic parent UC 9464, which harbors the original low copy plasmid pUC18 (MIC 320 μg/ml). Similar differences in the spectinomycin MICs were observed among those strains (Table 2).

Trospectomycin Inactivating Activity in the Osmotic Shockate of UC 12150

Cell-free extracts were prepared from osmotically shocked cells of UC 9464 and the highly resistant strain UC 12150 in order to determine their enzymatic activities to inactivate trospectomycin (Fig. 4). For total inactivation of 50 μg of trospectomycin in 11 hours 20 μl (corresponding to osmotic shockate protein concentration of 108 μg/ml) of the high producer UC 12150 shockate was required in a total volume of 500 μl of the reaction mixture. On the other hand it took 50 μl (corresponding to 210 μg/ml of the shockate protein) of the osmotic shockate prepared from the isogenic parent strain UC 9464 in order to inactivate 96% of the same amount of trospectomycin. As indicated by the results in

Fig. 4. Inactivation of trospectomycin by cell-free extracts of *Escherichia coli* UC 12150 (●) and UC 9464 (□).



A total of 50 μg of trospectomycin was treated by various amounts of the shockate preparations as described in Materials and Methods. Inactivation was measured on *Bacillus subtilis* UC 564 as an assay organism.

Table 4. Reactivation of trospectomycin 6-(5′-adenylate).

Enzyme		*Bacillus subtilis* growth inhibition (mm zone)	
		0 hour	15 hours
Venom phosphodiesterase	+	0	26
	−	0	0
Spleen phosphodiesterase	+	0	0
	−	0	0

At the time indicated 75 μl of the reaction mixture was spotted on a 1.25-cm (diameter) filter disc, which was then placed on an agar-medium seeded with *B. subtilis* UC 564. A zone of inhibition was read after overnight incubation at 37°C.

Table 3, inactivation of trospectomycin requires ATP, $MgCl_2$, and cell-free extract of *E. coli* UC 12150. The inactivated trospectomycin was slowly reactivated by venom phosphodiesterase but not by spleen phosphodiesterase indicating linkage between 5'-P of ATP and trospectomycin (Table 4).

Production and Isolation of Trospectomycin 6-(5'-Adenylate)

A crude enzyme preparation was used to adenylylate 500 mg of trospectomycin as described in Materials and Methods. Since trospectomycin adenylate lacks *in vitro* antibacterial activity, its formation from trospectomycin was followed by measuring the loss of such antibacterial activity using *E. coli* UC 51 as the assay organism. Incubation of the reaction mixture for 15 hours (37°C) resulted in sufficient inactivation of trospectomycin and its transformation to a UV-absorbing material, which

Fig. 5. FAB-MS of trospectomycin 6-(5'-adenylate).

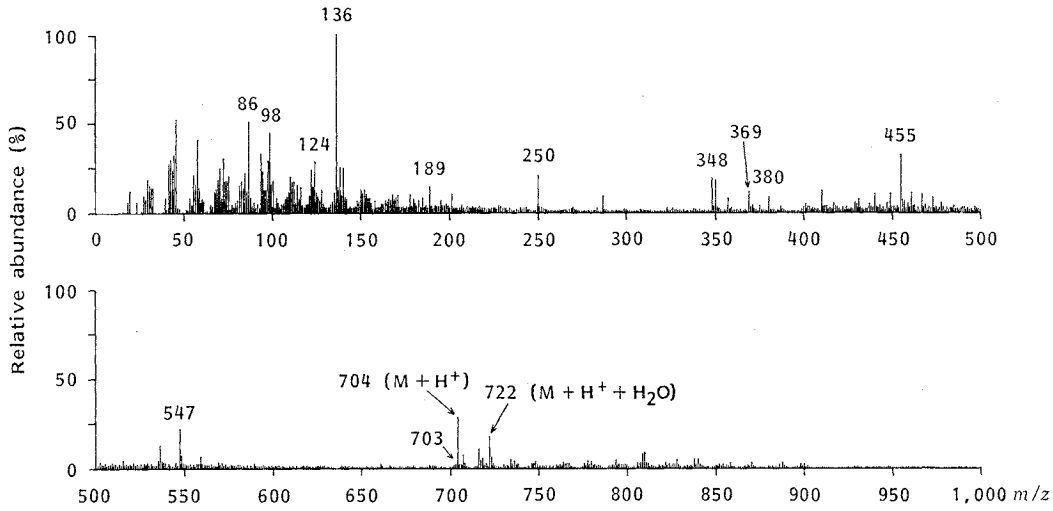
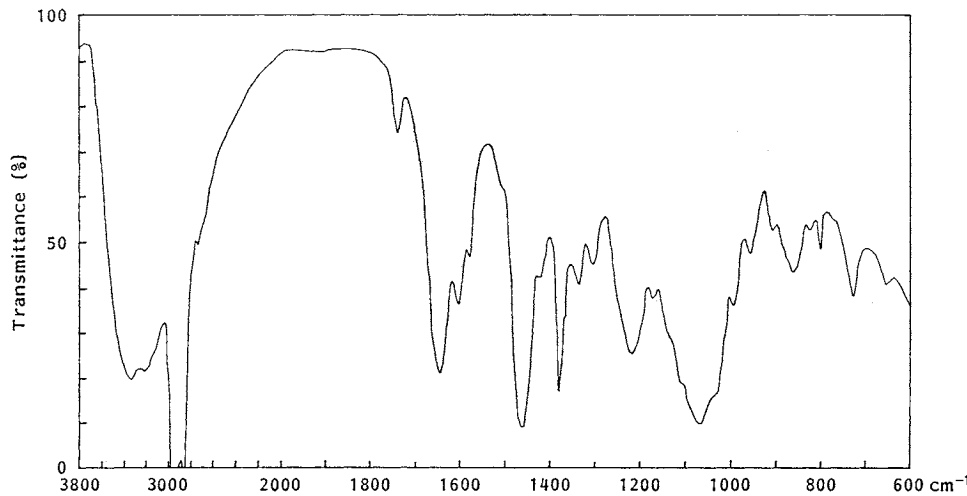


Fig. 6. IR spectrum of trospectomycin 6-(5'-adenylate).



yielded trospectomycin upon treatment with snake venom phosphodiesterase. Isolation and purification of trospectomycin adenylate were followed by TLC, UV absorption at 260 nm and *in vitro* assays before and after treatment with snake venom phosphodiesterase. Pure, "inactivated" trospectomycin was isolated from the reaction mixture by repeated chromatographies on Amberlite XAD-2 as described in detail in the Materials and Methods. Trospectomycin adenylate was eluted with methanol - water (30 : 70 to 50 : 50) and isolated as an amorphous colorless powder. TLC of this material showed the presence of only one compound.

Characterization and Structure of Trospectomycin Adenylate

Trospectomycin adenylate was isolated as an amorphous colorless material soluble in water and lower alcohols and practically insoluble in acetone as well as ethyl acetate. Analytical data combined with molecular weight determination by negative ion FAB-MS (Fig. 5 and Table 5) indicated the molecular formula of $C_{27}H_{42}NO_{18}P \cdot H_2O$; MW 703 and 721 (hydrated form). The IR spectrum (Fig. 6) showed weak carbonyl absorption at 1738 cm^{-1} indicating the presence of some trospectomycin adenylate in the nonhydrated form. The UV spectrum showed a maximum at 258 nm (a=17.27, E=12,150) supporting the presence of an adenosine moiety in the molecule of inactivated trospectomycin. The molecular formula (specifically the presence of one P atom per molecule) and the conversion of trospectomycin adenylate to trospectomycin by treatment with snake venom phosphodiesterase (but not with spleen phosphodiesterase) indicated that inactivated trospectomycin has a

Fig. 7. ^{1}H NMR (A) and ^{13}C NMR (B) spectra of trospectomycin 6-(5′-adenylate).

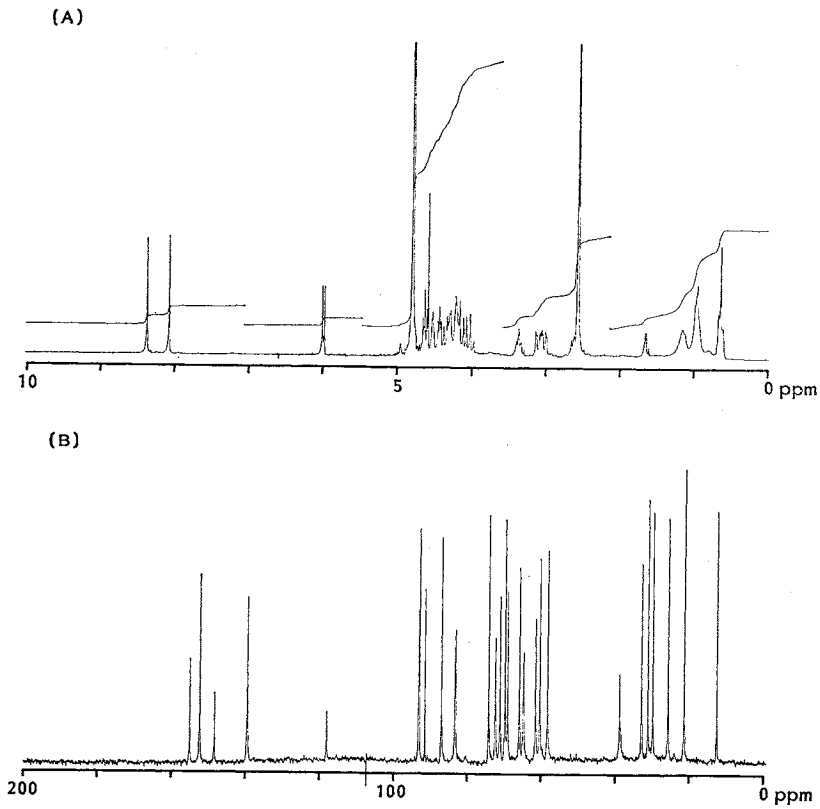
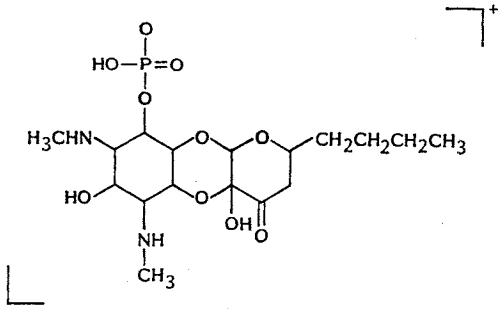


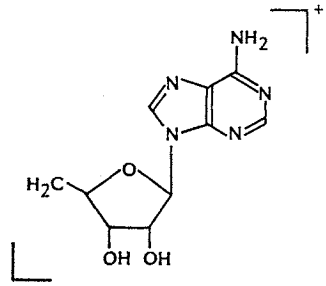
Fig. 8. Fragments observed in the high resolution-FAB-MS of trospectomycin 6-(5′-adenylate).



2

455 $C_{17}H_{30}N_2O_{10}P + H_2$

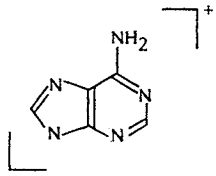
455.1794



3

250 $C_{10}H_{12}N_5O_3$

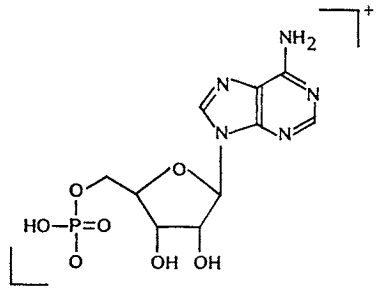
250.0940



4

136 $C_5H_4N_5 + H_2$

136.0623



5

348 $C_{10}H_{13}N_5O_7P + H_2$

348.0709

structure in which trospectomycin is linked to the phosphate group of adenosine-5′-phosphate. This is in agreement with the potentiometric titration (KOH, 80% aq ethanol) results which showed the presence of an acidic group in trospectomycin adenylate. The assignment of the phosphate diester linkage at the C-6 position of the actinamine moiety of trospectomycin (Fig. 1) is based on comparison of ^{13}C NMR spectra of trospectomycin and trospectomycin adenylate (Fig. 7 and Table 5). As shown in Table 5, there is an excellent agreement between the chemical shifts of 16 of the carbons of trospectomycin and the corresponding carbons of the adenylate. The chemical shift of the remaining carbon (C-6) of trospectomycin appears at δ 64.9 while C-6 of trospectomycin adenylate has a chemical shift of δ 72.9. This is due to the deshielding effect of the phosphate group attached at C-6 of trospectomycin adenylate.[11] Furthermore, the chemical shifts of the carbons of ribose and adenine moieties of trospectomycin adenylate are almost identical to those of adenosine 5′-phosphate (Table 5). It should be noted that the chemical shifts of C-5 of ribose in adenosine 5′-phosphate (δ 64.4) and trospectomycin 6-(5′-adenylate) (δ 65.3) are higher than the chemical shifts of C-5 of adenosine (δ 61.9). This is also due to the deshielding effect of the phosphate group at C-5 of ribose.

We, therefore, conclude that ^{13}C NMR also indicates a phosphodiester bond† between C-6 of trospectomycin and C-5 of the ribose moiety of adenosine as shown in Fig. 1, **1**. The ^{1}H NMR spectrum (Fig. 7) indicates the presence of 11 protons in the area of δ 0.6 to 1.7 assigned to the CCH_3 and the four CH_2 groups of trospectomycin 6-(5′-adenylate). Absorptions due to the two NCH_3 groups are present at *ca.* δ 2.58. Also complex absorptions due to the CHO and CHN of the remaining of the adenylate molecule are present at δ 3.0 to 4.8. The anomeric proton of adenosine appears at δ 6.02 (1H, doublet), and the two "aromatic" protons of adenine appear at *ca.* δ 8.15 and 8.43 as singlets. The fragmentation pattern obtained in the FAB spectrum of the adenylate (Fig. 8) is also in agreement with the conclusion that **1** (Fig. 1) is the structure of trospectomycin adenylate.

Table 5. Chemical shifts in the ^{13}C NMR spectra of trospectomycin[a], trospectomycin 6-(5′-adenylate)[b] and adenosine 5′-phosphate[c].

Chemical shift (ppm)			
Trospectomycin	Trospectomycin adenylate	Adenosine 5′-phosphate	Assignments[d]
29.8, 29.4	30.5, 31.7	—	NCH_3′s
60.6	62.0	—	C-1
58.6	60.9	—	C-2
57.6	58.8	—	C-3
64.6	66.4	—	C-4
68.7	69.6	—	C-5
64.9	72.9	—	C-6
92.6	93.6	—	C-1′
92.6	93.4	—	C-2′
91.0	91.8	—	C-3′
38.5	39.0	—	C-4′
70.9	71.6	—	C-5′
32.5	33.4	—	C-6′
25.4	26.3	—	C-7′
21.0	21.9	—	C-8′
12.3	13.1	—	C-9′
—	87.3	87.9	Ribose-1
—	70.3	71.4	Ribose-2
—	74.6	75.4	Ribose-3
—	83.7	85.1	Ribose-4
—	65.3	64.4	Ribose-5
—	152.6	153.3	Adenine-2
—	148.5	149.3	Adenine-4
—	118.3	118.9	Adenine-5
—	155.2	155.9	Adenine-6
—	139.7	140.8	Adenine-8

[a] ref 1.
[b] D_2O was used as the solvent with TMS as internal standard.
[c] ref 10.
[d] See Fig. 1.

Biological Properties of Trospectomycin 6-(5′-Adenylate)

Trospectomycin 6-(5′-adenylate) is bioinactive *in vitro*. *In vitro* treatment with venom phosphodiesterase slowly transforms the adenylate to trospectomycin which inhibits the growth of both Gram-positive (Table 4) and Gram-negative bacteria.[12] When, however, trospectomycin 6-(5′-adenylate) was administered subcutaneously at 30 mg/kg, it did not protect mice infected with *Streptococcus pyogenes* indicating that *in vivo* enzymatic transformation of the ribonucleotide prodrug **1** (Fig. 1) to its active form **1a** (Fig. 1) occurs slowly (if at all). (The CD_{50} of trospectomycin in the same system was *ca.* 3.0 mg/kg.)

Acknowledgments

The authors express their appreciation to Dr. D. R. WHITE for providing trospectomycin, Dr. C. W. FORD for the *in vivo* study, and Mr. K. J. GEIPEL for technical assistance.

References

1) WHITE, D. R.; C. J. MARING & G. A. CAIN: Synthesis and *in vitro* antibacterial properties of alkylspectinomycin analogs. J. Antibiotics 36: 339~342, 1983
2) ZURENKO, G. E.; B. H. YAGI, J. J. VAVRA & B. B. WENTWORTH: *In vitro* antibacterial activity of trospec-

† The ^{31}P-^{13}C coupling constants (Hz) observed were as follows: $J_{P\text{-}C\text{-}5}$ (ribose) 4.4; $J_{P\text{-}C\text{-}4}$ (ribose) 9.2; $J_{P\text{-}C\text{-}6}$ trospectomycin 6-(5′-adenylate) 6.1; $J_{P\text{-}C\text{-}1}$ trospectomycin 6-(5′-adenylate) 3.3.

tomycin (U-63366F), a novel spectinomycin analog. Antimicrob. Agents Chemother. 32: 216~223, 1988
3) SANSON-LEPORS, M.-J.; I. M. CASIN, M.-C. THEBAULT, G. ARLET & Y. PEROL: *In vitro* activities of U-63366, a spectinomycin analog; roxithromycin (RU 28965), a new macrolide antibiotic; and five quinolone derivatives against *Haemophilus ducreyi*. Antimicrob. Agents Chemother. 30: 512~513, 1986
4) CIALDELLA, J. I.; J. J. VAVRA & V. P. MARSHALL: Susceptibility of bacteria to serum lysis or phagocytosis following growth in subinhibitory levels of lincosaminide or spectinomycin related antibiotics. J. Antibiotics 39: 978~984, 1986
5) MARSHALL, V. P.; T. E. PATT & A. D. ARGOUDELIS: Enzymatic nucleotidylylation of lincosaminide antibiotics. J. Ind. Microbiol. 1: 17~21, 1986
6) BENVENISTE, R.; T. YAMADA & J. DAVIES: Enzymatic adenylation of streptomycin and spectinomycin by R-factor-resistant *Escherichia coli*. Infec. Immun. 1: 109~119, 1970
7) SHIMIZU, S.; M. INOUE, S. MITSUHASHI, H. NAGANAWA & S. KONDO: Enzymatic adenylylation of spectinomycin by *Acinetobacter calcoaceticus* subsp. *anitratus*. J. Antibiotics 34: 869~875, 1981
8) MANIATIS, T.; E. F. FRITSCH & J. SAMBROOK: Molecular Cloning (A Laboratory Manual). Cold Spring Harbor Laboratory, Cold Spring Harbar, New York, 1982
9) THORNSBERRY, C. (*Ed.*): Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically (Approved Standard M7-A). National Committee for Clinical Laboratory Standards, Villanova, 1985
10) NOSSAL, N. G. & L. A. HEPPEL: The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. J. Biol. Chem. 241: 3055~3062, 1966
11) STOTHERS, J. B. (*Ed.*): Carbon-13 NMR Spectroscopy. pp. 469, 471, Academic Press, New York, 1972
12) CASADABAN, M. J. & S. N. COHEN: Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. 138: 179~207, 1980