A NEW BROAD SPECTRUM AMINOGLYCOSIDE ANTIBIOTIC, G-52, PRODUCED BY *MICROMONOSPORA ZIONENSIS*

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G-52 is a new broad spectrum aminoglycoside produced by a species of the genus *Micromonospora, Micromonospora zionensis*. It has been differentiated from other known related antibiotics by a variety of chemical and biological methods. Its *in vitro* and *in vivo* spectrum of activity appears to be quite similar to that of verdamicin and gentamicin but is differentiated from them by its increased activity against 6′-N-acetylating strains.

Micromonospora inyoensis produces a broad spectrum aminoglycoside antibiotic named sisomicin which was found to be the first to contain a double bond in one of the sugar moieties.[1,2] Our laboratories later discovered a species of *Micromonospora, M. grisea,* which was found to produce sisomicin and a new antibiotic verdamicin, 6′-C-methylsisomicin.[3,4] Another species of *Micromonospora, M. zionensis* has now been discovered which produces an antibiotic complex consisting of sisomicin and a new broad spectrum antibiotic named G-52. This report presents initial data concerning the morphology of the producing organism as well as chemical and biological characteristics of the novel antibiotic produced. Its chemical structure is elucidated in the succeeding paper.[5]

Materials and Methods

Organism and Culture Conditions

The organism producing the antibiotic is a species of the genus *Micromonospora* named *Micromonospora zionensis*. It was isolated from a soil sample in Zion National Park, Utah, U.S. A culture of *M. zionensis* has been deposited in the collection of the U.S. Department of Agriculture, Northern Utilization Research and Development Division, Peoria, Illinois where it has been designated as NRRL 5466.

For laboratory production of G-52 a loopful of *M. zionensis* from an agar slant or a 0.5 ml of a frozen whole broth preparation was used to inoculate 100 ml of a medium consisting of (g/liter); yeast extract, 5 g; corn steep liquor, 5 g; calcium carbonate, 5 g; N-Z amine (Hunko Chemical Co.), 5 g; and sucrose, 25 g contained in a 300 ml Erlenmeyer flask and incubated for 2～4 days at 35°C on a rotary shaker. Five ml of inoculum were transferred to a 500-ml Erlenmeyer flask containing 100 ml of a fermentation medium consisting of (g/liter); dextrose, 5 g; calcium carbonate, 7 g; soybean meal, 30 g; dextrin, 50 g; and cobalt chloride, (10^{-6} moles/liter). The fermentations were carried out at 26～35°C for 4～7 days with continual agitation on a rotary shaker at 200～350 rpm.

Microbiological Assay

Antibiotic potencies were determined by means of a disc plate assay similar to that described for gentamicin with *Staphylococcus aureus* ATCC 6538P as the test organism.[6] The G-52 standard was established after separation of the components. A unit of antibiotic G-52 activity is the amount of material which produces a zonal response of 17.7 ± 1.0 mm under the conditions of the assay and has been defined as 1 mcg.

Antibiotic Isolation

The G-52 complex was isolated from the fermentation broth by an ion-exchange procedure.

The pH of the whole broth was adjusted to 2.0 with sulfuric acid, filtered, neutralized, and adsorbed to an IRC 50 ion-exchange resin column. The antibiotic mixture was eluted from the resin with 2 N ammonium hydroxide. Separation and isolation of the antibiotic moieties was achieved by silica gel chromatography using the lower phase of a solvent mixture consisting of chloroform, isopropanol and concentrated ammonium hydroxide (2: 1: 1, v/v).

Chromatographic Methods

G-52 was compared with a variety of gentamicin-related antibiotics by paper chromatography and subsequent bioautography in a series of solvent systems. The following antibiotics as their sulfates were used: G-52-components 1 and 2, verdamicin, sisomicin, gentamicins C_1, C_2 and C_1a.

Hydrolysis of Antibiotics

All antibiotics were hydrolyzed for comparative purposes as the free bases and sulfate derivatives (10 mg/ml) in 6 N hydrochloric acid in sealed tubes at a 100°C for 2 hours.

In vitro Studies

For determination of *in vitro* sensitivity, all test organisms were incubated in pH 7.4 MUELLER-HINTON broth at 37°C for 18~24 hours except where indicated. The volume in each tube was 3 ml and the inoculum was 0.05 ml of a 1: 1,000 dilution of an 18-hour broth culture.

In vivo Studies

Animal studies were carried out in CF-1 male albino mice weighing approximately 20 g each. The G-52 sulfate derivative was used for these studies. Drug solutions were prepared in sterile distilled water and all doses expressed in terms of base activity. In therapeutic tests, animals were treated once, 1 hour after intraperitoneal infection with approximately 10^7 organisms per mouse. Control infected mice died in 18~24 hours; survivors in treated groups were determined 48 hours after infection. Generally, groups of 7 mice each at 5 dose levels in addition to 10 controls were used for each test. PD_{50} and LD_{50} values were determined by probit procedures.

Results and Discussion

Macroscopic examination of the culture on NZ amine-dextrose agar indicates that colonial growth was fair, flat, furrowed; no aerial mycelia was seen and no diffusible pigment was produced. On a yeast extract-sucrose medium after a 60-day incubation period spores were abundant; microscopic examination of the spores showed them to be globose to spherical, 1.0~1.5 μm in diameter; produced on short sporophores. *M. zionensis* was distinguished from other, related *Micromonospora* species by color difference on a variety of media, various chemical tests and particularly by the fact that it grows well on rhamnose as a carbohydrate source as compared to poor growth by related species. Detailed taxonomic studies will be published elsewhere.

The antibiotic titer reached a maximum after 4~5 days of fermentation at 35°C under the conditions described. Isolation of the antibiotic complex from the fermentation broth *via* resin extraction afforded an antibiotic mixture consisting of 2 major (components 1 and 2) and several minor components. G-52-components 1 and 2 were compared with a variety of antibiotics by paper chromatography and subsequent bioautography as shown in Table 1. The Rf values of the two major components were distinguishable from those of all other related antibiotics with the exception of G-52-component 2 which was similar to sisomicin. The hydrolytic patterns indicated that 2-deoxystreptamine is common to the G-52 components and also clearly differentiated G-52-component 1 from other known compounds. The hydrolytic pattern of G-52-component 2 however, was similar to that of sisomicin. Further chromatographic and chemical comparisons as well as nmr, mass and i.r. spectra, *in vitro* and *in vivo* studies conclusively demonstrated that G-52-component 2 was indeed identical to sisomicin which has previously been described.[1]

Table 1. Comparative Rf values of antibiotic G-52 and other aminoglycoside antibiotics

Paper-chromatographic system	Rf's of Antibiotics									
	G-52 Components		Verdamicin	Sisomicin	Gentamicin components			Neomycin	Kanamycin	Paromomycin
	1	2			C_1	C_2	C_1a			
80% Methanol-3% sodium chloride, 1: 1 (w/v), descending*	0.55	0.45	0.55	0.45	0.57	0.56	0.48	0.0, 0.17	0.0, 0.28	0.0, 0.28
Propanol - pyridine - acetic acid - water, 6: 4: 1: 3 (v/v), ascending	0.29	0.22	0.30	0.22	0.34	0.30	0.22	0.05	0.08	0.07
80% Phenol (v/v), ascending	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.0, 0.12	0.0, 0.17	0.0, 0.2
Benzene - methanol, 9: 1 (v/v), descending	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
n-Butanol - water - acetic acid, 4: 5: 1, upper phase used, ascending	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Rt's of Antibiotics, ** t=4 hours										
Chloroform - methanol - 17% ammonium hydroxide, 2: 1: 1	0.48	0.21	0.40	0.21	0.67	0.40	0.21			
Rt's of Antibiotics, t=16 hours										
2-Butanone - *tert*-butanol - methanol - conc. ammonium hydroxide, 16: 3: 1: 6	0.73	0.59	0.73	0.59	0.88	0.75	0.65			

* Paper buffered with 0.95 molar Na_2SO_4+0.05 molar $NaHSO_4$.

** $Rt = \frac{\text{distance of zone from origin}}{\text{distance from origin to end of paper}}$ at time t

Table 2. Chemical and physical properties of antibiotic G-52

	Sulfate	Base
Rotation $[\alpha]_D^{26}$*	89.3°	157.9°
Equivalent weight		106.8
pKa		8.2
Microanalysis**		
C	28.75	47.94
H	6.23	8.19
N	8.30	13.43
O by difference		30.45
SO_4	30.43	
For $C_{20}H_{39}N_5O_7 \cdot H_2CO_3$		
C		48.17
H		7.89
N		13.38

* (c 0.3, H_2O)
** Average of 4.

Table 3. *In vitro* activity of G-52

Organism	No. Strains	Range of MIC's* (mcg/ml)
Bacillus subtilis	1	0.01
Staphylococcus aureus	6	0.05~3.0
Streptococcus pyogenes	6	3.0 ~17.5
Escherichia coli	6	0.3 ~3.0
Klebsiella pneumoniae	5	0.8 ~7.5
Proteus sp.	2	0.7 ~7.5
Pseudomonas aeruginosa	9	0.3 ~7.5
Salmonella sp.	1	0.8

* MUELLER-HINTON broth, pH 7.4.

Antibiotic G-52-component 1, now referred to as G-52 is stable to boiling for at least 30 minutes in the pH range of 2~10. G-52 exhibits no absorption in the ultraviolet range (200~400 nm) and i.r. and nmr data indicate the presence of a double bond showing its relationship to verdamicin and sisomicin. It forms salts with mineral acids to yield the respective hydrochloride, sulfate, phosphate *etc.* The base is soluble in water. Some chemical and physical characteristics of G-52 are listed in Table 2.

A summary of the *in vitro* activity of G-52 using conventional procedures in MUELLER-HINTON broth is given in Table 3. The antibiotic was found to have activity against a number of strains of gram-positive and gram-negative organisms. The activities obtained are similar to those seen with verdamicin and sisomicin.

Table 4 shows the results of agar dilution tests with organisms producing kanamycin acetyl-transferase (AAC-6′). The lack of activity of kanamycin, tobramycin and amikacin is due to this enzyme which acetylates the 6-amino group of these antibiotics. Gentamicin is active against these organisms based on the fact that two of the three gentamicin components, C_1 and C_2, are not acetylated by this organism although gentamicin C_1a is inactivated.[7] Note the 48-hour values of G-52 against both *Escherichia coli* strains compared to other related aminoglycosides, in particular, those containing

Table 4. Activity *vs.* 6′-N acetylating strains

Antibiotic	MIC (mcg/ml)					
	E. coli R5/677		*E. coli* HL97/677		*Pseudomonas* GN 315	
	24 hrs.	48 hrs.	24 hrs.	48 hrs.	24 hrs.	48 hrs.
G-52	0.3	0.3	0.8	3.0	0.3	0.3
Gentamicin	0.5	1.0	4.0	4.0	2.0	4.0
Kanamycin	>32	>32	>32	>32	>32	>32
Tobramycin	32	>32	>32	>32	32	>32
Amikacin	32	>32	>32	>32	16	32
Sisomicin	4	8	32	>32	8	16
Verdamicin	0.5	1.0	2.0	4.0	1.0	2.0

a double bond in the same carbohydrate moiety, sisomicin and verdamicin. In both instances, G-52 is more active than the latter two antibiotics. An even greater difference exists in the activity against the *Pseudomonas* strains examined with G-52 showing at least a 6-fold increase in activity compared to verdamicin and even greater activity compared to sisomicin and gentamicin. This suggests that G-52 is unaffected by the acetylating enzyme due to possible steric hindrance at the 6-amino site. The structure/activity relationship of G-52 is discussed in the succeeding paper.[5]

Table 5. *In vivo* activity of G-52

Infectious Organisms	PD_{50} (mg/kg)	
	G-52	Gentamicin
Staphylococcus aureus Gray	2.5	1.9
Escherichia coli Sc.	2.5	1.5
Pseudomonas aeruginosa Sc.	1.5	0.8

Acute toxicity of G-52

Route	Mean lethal dose (mg/kg)	
	G-52	Gentamicin
I.V.	50	75
S.C.	400	485
I.P.	200	430

The comparative *in vivo* activity and acute toxicity between G-52 and gentamicin is shown in Table 5. Antibiotic G-52 shows broad spectrum protection in mice, but is slightly less active than gentamicin. The acute toxicity expressed as LD_{50} is comparable to gentamicin *via* the intravenous and subcutaneous routes but it is more toxic *via* the intraperitoneal route. Based on the data described further studies are in progress with this novel antibiotic.

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