STRUCTURE OF LL-BM408, AN AMINOCYCLITOL ANTIBIOTIC

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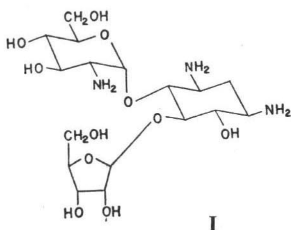
(Received for publication August 9, 1976)

An aminocyclitol antibiotic, LL-BM408, discovered in our screening program for new antibiotics, was produced by an undetermined species of *Streptomyces*, which also produced paromomycin I and paromomycin II. A purified sample of LL-BM408 was found to have activity against gram-negative bacterial infections in mice. LL-BM408 was identified as ribosyl paromamine (I) based on identification of acid hydrolysis fragments, nmr and mass spectral analyses. Although ribosyl paromamine was prepared by chemical degradation of antibiotic BU-1709E_2[2] and paromomycin,[3] this appears to be the first time that it has been detected as a direct fermentation product.

LL-BM408 was produced by the fermentation of the organism on a medium containing dextrose, glycerol, soy flour, sodium chloride and calcium carbonate. The course of antibiotic production was followed by inhibition of *Klebsiella pneumoniae* by the cup agar-diffusion method.



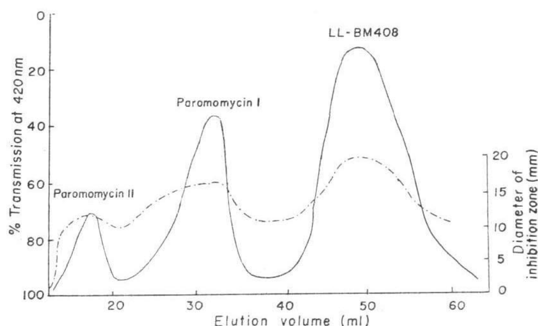
I

LL-BM408 was isolated by a sequence of steps involving ion-exchange chromatography on weak cationic exchange resins marketed as Amberlite IRC-50 (NH_4^+) and CG-50 (NH_4^+), and ion exclusion chromatography on a strong anion exchange resin marketed as Dowex 1-X2 (OH^-).

The mixture of antibiotics in the purified fractions prepared this way could be readily resolved on an analytical scale by ion exclusion chromatography (Fig. 1) as described by MAEHR and SCHAFFNER,[4] or by adsorption to Amberlite CG-50 (NH_4^+) resin and stepwise elution with 0.1 N NH_4OH by a procedure similar to that described by M. MURASE and co-workers.[5] The former procedure involved the use of a strongly basic anion exchange resin of low cross linkage, Dowex 1-X2 resin, in the hydroxyl form. In the application of this technique to the LL-BM408 complex, the column was eluted with water and the effluent was monitored simultaneously by an automated trinitrobenzenesulfonic acid procedure[6] and by *in vitro* bioactivity. LL-BM408 was obtained as a white powder, $[\alpha]_D^{25} +47° \pm 4°$ (*c* 0.256, H_2O).

Fig. 1. Analytical ion-exclusion chromatography on Dowex 1-X2 (OH^-) resin developed with water

—— Trinitrobenzenesulfonic acid color reaction
–·– Bioactivity, paper disc agar-diffusion assay *vs.* *Klebsiella pneumoniae*



Mass spectra of N-acetyl-O-trimethylsilyl derivatives and ion exclusion column retention times indicated two of the components were paromomycins I and II.

The fragmentation pattern of the N-acetyl-O-trimethylsilyl derivative of LL-BM408 is rationalized according to those commonly encountered for this type of derivative of aminocyclitol antibiotics.[7] The highest mass peak at *m/e* 1070 is attributed to the loss of a methyl radical. Peaks at *m/e* 838 and 767 are re-arrangement ions resulting from fragmentation and migration of TMSO groups. A strong *m/e* 838 ion is characteristic of antibiotics having paromamine linked to a sugar moiety. Fragmentations to give peaks at *m/e* 420 and 649 are indicative of a terminal aminohexose and a terminal pentose linked to deoxystreptamine.

Confirmation of these fragmentations was obtained by peak matching studies (Table 1) which showed the found masses are within 10 ppm of the calculated values. In addition, the low resolu-

Table 1. Relative abundance and composition of selected ions in the mass spectrum of the N-acetyl-O-trimethylsilyl derivative of LL-BM408

Relative abundance	Observed mass	Calculated mass	Composition
100	420.2047	420.2058	$C_{17}H_{38}NO_5Si_3$
22	649.3177	649.3192	$C_{27}H_{57}N_2O_8Si_4$
57	767.3602	767.3642	$C_{31}H_{67}N_2O_{10}Si_5$
72	838.3986	838.4013	$C_{34}H_{72}N_3O_{11}Si_5$
14	1070.4948	1070.4958	$C_{43}H_{92}N_3O_{14}Si_7$

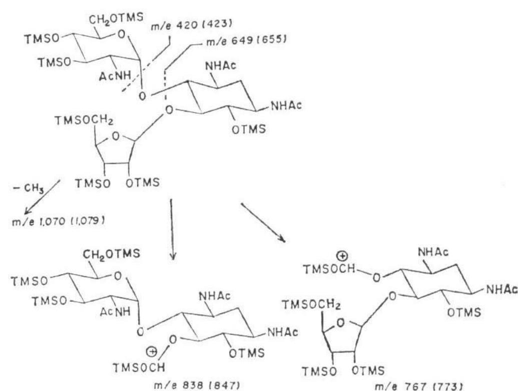
tion spectrum of an N-acetyl-d_3-O-trimethylsilyl derivative was studied and the peaks obtained are in agreement with the proposed number of nitrogens per fragment ion. Each acetylated nitrogen results in an increase of 3 mμ for a particular ion when the acetyl and acetyl-d_3 derivatives are compared. Consequently, the number of acetylated nitrogens per ion is readily determined.

From the mass spectral studies, the sequence of LL-BM408 was concluded to be an aminohexose linked to deoxystreptamine which in turn was linked to a pentose.

Acid hydrolysis studies provided further information about the structural components of LL-BM408. Mild conditions of 0.4 N HCl at 65°C for 1.5 hours yielded ribose, identified by paper chromatography on Whatman No. 1 paper developed with ethyl acetate, pyridine, water (360:100:115) for 20 hours at 25°C, allowing the solvent to drip off. Visualization with TOLLENS reagent gave the following R_{ribose} values: BM408

Fig. 2. Proposed fragmentation of N-acetyl-O-trimethylsilyl derivative of LL-BM408.

Number in parenthesis indicates mass of fragment ions from deuteroacetyl derivative.



hydrolysate, 1.00; glucose, 0.33; mannose, 0.50 and xylose, 0.73.

In addition, paromamine, deoxystreptamine and glucosamine were detected in a hydrolysate formed by heating LL-BM408 in 3 N HCl for 5 hours at 100°C. These fragments were identified by comparisons to authentic samples (a) by high-voltage paper electrophoresis on Whatman 3MM paper with a pH 3.8 buffer composed of pyridine, acetic acid, water: 1: 10: 289 at 2,500 V for 25 minutes and (b) by descending paper chromatography on Whatman No. 1 paper developed with 1-butanol-pyridine-water (6: 4: 3) for 20 hours at 25°C allowing the solvent to drip off. Zones of the three fragments were detected by ninhydrin.

Table 2. Antimicrobial spectra*

Test organism	MIC (mcg/ml)		
	LL-BM408	Ribostamycin	Paromomycin
Escherichia coli KC-74-1	32	2	4
Klebsiella pneumoniae W-75-1	64	2	4
Proteus mirabilis OSU-75-1	64	2	8
Salmonella enteritidis W-75-1	32	2	4
Shigella flexneri RB	16	1	2
Acinetobacter calcoaceticus Mayo 75-4	32	16	1
Pseudomonas aeruginosa 6236	>128	>128	64
Pseudomonas aeruginosa 12-4-4	>128	>128	16
Staphylococcus aureus OSU-75-2	8	0.25	0.25
Enterococcus KC-74-2	>128	32	32

* Agar dilution method on nutrient agar, pH 6.8

Glucosamine was also detected by a MORGAN-ELSON test. By electrophoresis the BM408 hydrolysis fragments migrated 23.6 cm (glucosamine), 32.4 cm (paromamine) and 34.1 cm (deoxystreptamine) toward the cathode. On paper chromatography, glucosamine and a hydrolysis fragment from LL-BM408 traveled the same distance from the origin (1.7 cm) and gave the same color reactions with the sprays.

The nmr spectrum of LL-BM408 is consistent with the proposed structure. It shows two anomeric protons at δ 5.45 and 5.63 with coupling constants of $\leq$4Hz, corresponding to those of the glucosamine and ribose moieties. The magnitudes of the coupling constants are in agreement with model systems having similar furanose configuration about the anomeric proton, but this assignment is not definitive.

LL-BM408 had $R_{paromamine}$ 0.83 in the system $CHCl_3$-MeOH-NH_4OH (1:3:2) on silica gel as would be predicted from the results reported.[3,8] This Rf is significantly different from that of the corresponding compound with the alternative location of ribose on deoxystreptamine. All of the data presented are consistent for LL-BM408 having structure **I**.

LL-BM408 protected mice (1/2 survivors at 64 mg/kg and 2/2 survivors at 128 mg/kg) infected with *Proteus mirabilis* when administered parenterally and inhibited *in vitro* a number of gram-negative bacteria (Table 2).

Acknowledgements

We wish to thank Mr. W. FULMOR and staff for spectral data, Dr. J. N. PORTER and staff for initial fermentation material, Dr. H. TRESNER and staff for isolation and taxonomic studies of the organism, Mrs. N. A. KUCK, for *in vitro* antimicrobial determinations and Mr. G. REDIN for the *in vivo* antibacterial assays.

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