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LL-A491, A Monazomycin-like Antibiotic

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A new antibiotic, designated LL-A491, was isolated by butanol extraction and crystallization from beers of an unidentified streptomycete, Lederle culture A491. LL-A491 was primarily active against gram-positive bacteria and was related to monazomycin. A tentative molecular formula of $C_{72\pm2}H_{144\pm8}O_{25\pm1}N$ for the antibiotic, based on analyses of the crystalline hydrochloride, picrate, and picrolonate salts, is considerably larger than that proposed for monazomycin, from which LL-A491 was not differentiated by paper chromatography. Analysis of the amorphous polyacetate ester suggested 15 to 16 acetylable groups. Upon hydrolysis, LL-A491 liberated ammonia and a reducing sugar which appeared to be mannose. LL-A491 was dextrorotatory, $[\alpha]_D^{25}$ +14°, and exhibited only end absorption in the ultraviolet region.

In the course of our antibiotic screening program, an unidentified streptomycete, isolated from a soil sample, was found to produce a new antibiotic. This antibiotic, designated as LL-A491, is primarily active against gram-positive bacteria. This paper describes a fermentation process for the production of the antibiotic, the isolation of crystalline LL-A491, its chemical and biological characteristics, and a comparison of some of its properties with those of monazomycin (1).

MATERIALS AND METHODS

Production. A subculture of Lederle strain A491, carried at 28 C on a yeast-malt-agar slant, was transferred directly to an inoculum flask containing 100 ml of sterile medium of the following composition: 1% soybean oil meal, 2.0% glucose, 0.5% corn steep liquor, and 0.3% calcium carbonate. The inoculated flask was incubated at 28 C for 72 hr on a rotary shaker, transferred to a bottle containing 1 liter of sterile medium, and incubated with shaking for an additional 48 hr. This two-stage vegetative inoculum was introduced into a 40-liter fermentor containing 30 liters of a medium composed of 0.5% N-Z Amine type A (Sheffield Chemical Division, National Dairy Products Corp., Norwich, N.Y.), and 2.0% glucose, with the *p*H adjusted to 6.0 to 6.3 before sterilization. The fermentation was supplied with sterile air at the rate of 0.7 liter per liter of medium per min and was agitated at 800 rev/min. During the course of the fermentation, samples of the mash filtrate were tested for potency by a *Bacillus subtilis* agar diffusion assay. After 4 to 5 days of incubation at 28 C, the antibiotic activity had reached a maximum, and the mash was harvested.

Isolation. The fermentation broth was adjusted to *p*H 9.0 and was filtered with the aid of diatomaceous earth. The filter pad was suspended in aqueous acid (*p*H 3.0), and, after filtration, the activity was extracted from the filtrate with one-fourth volume of *n*-butanol. The butanol extract was concentrated to a small volume under reduced pressure, and 1.5 to 2.5 g of crude LL-A491 hydrochloride was precipitated by addition of acetone. The overall recovery of activity at this stage was 40%. The precipitate was dissolved in methanol and reprecipitated with acetone. The product became crystalline when the suspension was agitated intermittently several hours at room temperature.

RESULTS AND DISCUSSION

LL-A491 was difficult to characterize because of its unfavorable physical properties. LL-A491 hydrochloride had no readily reproducible melting point, but became fluid between 125 to 135 C, depending upon the temperature of immersion and the rate of heating and stirring. The antibiotic was dextrorotatory, $[\alpha]_D^{25}$ +14° (*c*, 0.984, methanol), and exhibited only end absorption in the ultraviolet region at concentrations ranging from 10 to 1,000 μg/ml in methanol or ethyl alcohol. At room temperature, the free base was readily soluble in *n*-butanol, 3-pentanol, dioxane, and ethyl cellosolve, very soluble in methanol, ethyl alcohol, acetic acid, and pyridine, and slightly soluble in water, acetone, ethyl acetate, benzene, ether, and chloroform. The hydrochloride did not reduce Fehling's solution, and was negative to anthrone reagent and 2,4-dinitrophenylhydrazine. The antibiotic hydrochloride decolorized aqueous permanganate solution, and, at *p*H 4.5, consumed 1 mole of sodium metaperiodate in 1 hr. On prolonged standing, a 2nd mole was slowly consumed.

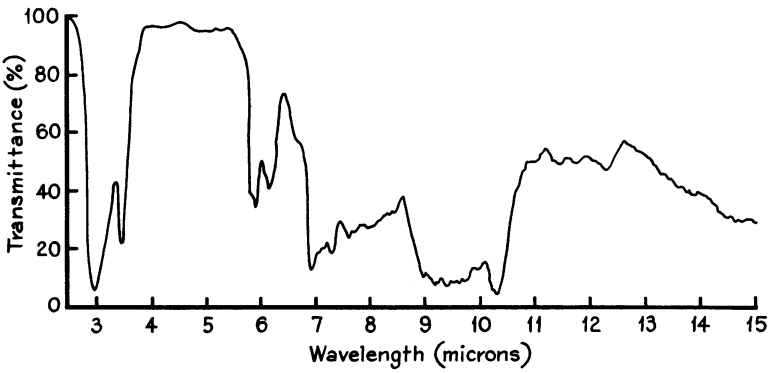

FIG. 1. *Infrared spectrum (KBr disc) of LL-A491 hydrochloride.*

Ammonia was liberated upon treatment with strong base. The ammonia was trapped in dilute acid and identified paper chromatographically by use of Whatman no. 1 filter paper strips and a system composed of *n*-butanol saturated with 0.1 N HCl. Control strips of the hydrochlorides of ammonia, ethylamine, methylamine, and dimethylamine were run along side and in admixture. The spots were visualized by use of Folin's reagent as modified by Blau (2). Upon hydrolysis with 1 N hydrochloric acid, a reducing sugar was produced which had the same paper chromatographic mobilities as mannose in three diverse solvent systems: system A, ethyl acetate-water-pyridine (2:2:1); system B, ethyl acetate-water-acetic acid (3:3:1); and system C, *s*-Collidine saturated with water. No R_F values were obtained, as the solvent systems were allowed to drip off the end of the strips to insure optimal resolution. The hydrolysate was run in admixture with glucose, glucosamine, and mannose, and the spots were visualized by spraying with aniline phthalate reagent.

The infrared absorption spectrum in a pressed KBr disc is shown in Fig. 1. A nuclear magnetic resonance spectrum in water solution was unrevealing in that the numerous protons were clumped together in a featureless mass. There was insufficient pure monazomycin available for a comparison. An apparent molecular weight of 744 was determined by isothermal distillation in methanol. Allowing for two nonvolatile ions per molecule of the hydrochloride salt, the true molecular weight would be 1,488 by this method. Despite the excellent agreement of this value with those obtained by spectrophotometric measurements (vide infra), its validity is open to question. Because LL-A491 hydrochloride is probably not completely ionized under these conditions, three nonvolatile particles are anticipated—the chloride ion and the amine salt ion in equivalent amounts and the nonionized hydrochloride particle in an undetermined amount. Furthermore, there appear to be 2 moles of water of crystallization in the salt based upon the microanalytical figures, and these would be expected to modify the vapor pressure difference observed during the determination in a way which is very hard to correct by calculation. The close agreement of this value, then, is probably largely fortuitous. An attempt to determine the molecular weight of LL-A491 by mass spectrometry failed because of the poor volatility of the antibiotic. On the basis of numerous microanalyses, a tentative molecular formula of $C_{72\pm2}H_{144\pm8}O_{25\pm1}NCl$ can be proposed. The samples were dried for 4 hr at 100 C under 0.01 mm of pressure before analysis.

Calculated for $C_{72}H_{144}O_{25}NCl$ (1,459): C, 59.29; H, 9.88; N, 0.96; Cl, 2.43; O, 27.43; $2H_2O$, 2.46; 1 *O*-acetyl, 2.96; 10 *C*-methyl, 10.28; and NH_2-N, 0.96. Found: C, 59.08; H, 9.71; N, 1.32; Cl, 3.17; O, 27.33; H_2O, 1.94; *O*-acetyl, 1.73; *C*-methyl, 10.22; and NH_2-N, 0.85.

Addition of a saturated aqueous solution of picric acid to an aqueous solution of LL-A491 hydrochloride produced a crystalline precipitate of the picrate salt. The picrate had no definite melting point. Determination of the molecular weight from the optical density of the picrate salt (3) at 360 mμ gave a value of 1,688 (equivalent to 1,495 for the hydrochloride).

Calculated for $C_{78}H_{146}O_{32}N_4$ (1,652): C, 56.72; H, 8.85; N, 3.39; and $2H_2O$, 2.18. Found: C 56.92; H, 9.00; N, 3.48; and H_2O, 2.28.

The addition of a saturated aqueous solution of picrolonic acid to an aqueous solution of LL-A491 hydrochloride produced a microcrystalline precipitate of the picrolonate salt. The picrolonate had no definite melting point. Determination of the molecular weight spectrophotometrically in the same manner as for the picrate salt led to a

TABLE 1. *Antibacterial spectrum[a] of LL-A491*

Test organism	Minimal inhibitory concn[b]
Staphylococcus aureus ATCC 6538P	6.2
S. aureus Lederle 69	6.2
S. aureus Smith ATCC 13709	6.2
S. aureus Rose ATCC 14154	6.2
Streptococcus faecalis ATCC 8043	6.2
S. pyogenes C203	6.2
Streptococcus sp. Lederle 80	6.2
Streptococcus sp. Lederle 11	6.2
Bacillus subtilis ATCC 6633	6.2
B. cereus ATCC 10702	3.1
Mycobacterium smegmatis 607	3.1
Enterobacter cloacae 529	125
Salmonella typhosa ATCC 6539	>250
Proteus vulgaris ATCC 9484	>250
Escherichia coli ATCC 9637	>250
Klebsiella pneumoniae ATCC 10031	>250
Salmonella gallinarum Lederle 604	>250
Pseudomonas aeruginosa ATCC 10145	>250

[a] Determined by agar dilution technique.
[b] Expressed in micrograms per milliliter, two-fold dilutions up to 250 μg/ml.

TABLE 2. *Paper chromatographic comparison of monazomycin and LL-A491*

System	R_F	
	Monazomycin	LL-A491
Isoamyl alcohol-0.2 M morpholine-0.2 M acetic-acid (10:7:3)	0.72	0.72
Hexanol saturated with water	0.08	0.07
Diethylketone-pyridine-water-acetic acid (20:14:5:4)	0.48	0.45
5% Ammonium chloride solution	0.78	0.76
n-Butanol-isoamylalcohol-dichloroacetic acid-water (100:50:1:75)	0.82	0.84
n-Butanol-acetic-acid-water (4:2:1)	0.72	0.74
n-Butanol saturated with water	0.17	0.15

value of 1,665 (which is equivalent to 1,438 for the hydrochloride).

Calculated for $C_{82}H_{151}O_{30}N_5$ (1,687): C, 58.41; H, 8.97; and N, 4.15. Found: C, 58.31; H, 8.91; and N, 4.12.

An amorphous polyacetate ester was formed upon treatment of LL-A491 with acetic anhydride and pyridine at room temperature overnight. Removal of the solvents under reduced pressure produced a thick oil, which was dissolved in chloroform and was chromatographed on silica gel. The ester was eluted by 5% methanol, the solvents were removed under reduced pressure, and the residue was dissolved in acetone and precipitated as an oil by the addition of water. The oil was dissolved in ether and the solution was washed successively with 5% sodium bicarbonate and 5% hydrochloric acid solutions. After drying over sodium sulfate, filtering, and evaporating, a white foam resulted. Microanalysis suggested 15 to 16 acetylated functions.

Calculated for $C_{102}H_{170}O_{38}N$ (2,018): C, 60.70; H, 8.47; N, 0.69; and 15 acetyl, 31.95. Calculation for $C_{104}H_{172}O_{39}N$ (2,059): C, 60.61; H, 8.40; N, 0.68; and 16 acetyl, 33.41. Found: C, 60.33; H, 7.82; N, 0.66; and acetyl, 32.15.

The antibacterial spectrum (Table 1) of the hydrochloride salt was determined by the agar-dilution procedure on Trypticase Soy Agar (BBL). LL-A491 is primarily active against gram-positive bacteria.

After the completion of this phase of our work, a limited sample of monazomycin (1) was received through the courtesy of Hamao Umezawa. The paper chromatographic behavior of monazomycin closely paralleled that of LL-A491 (Table 2). In fact, the two substances could not be resolved by mixed-spot paper chromatography in seven systems selected as having the greatest potential for separating the two samples. The micro-infrared spectra of the two substances are reproduced in Fig. 2. The spectra were obtained by

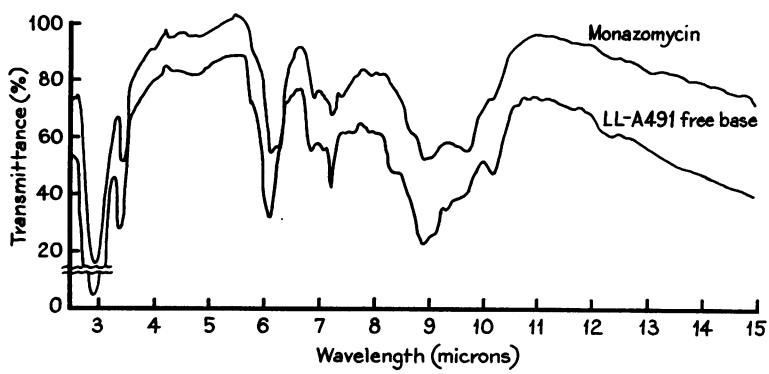

FIG. 2. *Infrared comparison of monazomycin and LL-A491 lyophilizates.*

TABLE 3. *Comparison of reported properties of monazomycin and LL-A491*

Property	Monazomycin	LL-A491
Producing organism	*Streptomyces mashuensis*	Unidentified *Streptomyces* sp.
Bioactivity against	Gram-positive organisms	Gram-positive organisms
Melting point	126–128 C	Indefinite
Rotation	$[\alpha]_D^{16}$ +8.0 (*c*, 1, methanol)	$[\alpha]_D^{25}$ +14° (*c*, 0.984, methanol)
Ultraviolet	End absorption	End absorption
Tentative molecular formula	$C_{62}H_{119}NO_{20}$	$C_{72}H_{143}NO_{25}$
Molecular weight	1,051–1,200	~1,470

evaporating aqueous solutions of monazomycin and LL-A491, containing 50 μg of antibiotic, in the presence of spectral grade potassium bromide, and preparing micropellets. The two spectra are similar but not identical. The difference may reflect their somewhat different histories. The close similarity of infrared spectra, paper chromatography characteristics, and general properties (Table 3) suggest that the two antibiotics are closely related, if not identical. Further comparison must await the availability of additional supplies of monazomycin.

It is frequently difficult to reach a completely definitive position on the differentiation of high molecular weight products when physicochemical and analytical characteristics are unfavorable and when quantities for comparative studies are extremely limited. The comparison of LL-A491 with monazomycin would be facilitated if the products could be degraded to specific fragments. A minor difference in stereochemistry, oxidation level, or chain length may at times be detected by subtle differences only after much study. The literature contains numerous examples of such difficulties, with the fungichromin-lagosin comparison as an extreme case. Although identical gross structures were established for both fungichromin and lagosin, uncertainty persists about their differentiation even after exchange of samples (4).

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