

# Analytical Method for Streptothricin-Type Antibiotics: Structure of Antibiotic LL-BL136<sup>1</sup>

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An analytical procedure was devised which can distinguish members of the streptothricin family of antibiotics. It is based upon an analysis of the hydrolysis products of the antibiotics using a Technicon amino acid autoanalyzer under special conditions. The various fragments including the different streptolidine-amino sugar compounds were well resolved. A basic water-soluble antibiotic discovered in our laboratories and named LL-BL136 was compared to other members of this group by this technique. It was not differentiated from the antibiotic SF-701 reported by Tsuruoka. The autoanalyzer results along with other physicochemical data permitted a structure proposal for this antibiotic, which is the *N*-methyl-desformimino derivative of antibiotic LL-AC541.

Identification of various streptothricin-type antibiotics with the aid of paper chromatography of hydrolysis products has been frequently reported. An extension of this method is the use of an amino acid autoanalyzer to obtain more quantitative and precise data. Concurrent with our initial studies in this area, Egorov and co-workers (2) published a procedure very similar to the method we were developing. The procedure that we describe below extends this general method to significantly different streptothricin-type antibiotics and also shows that the streptolidine-sugar compounds from mild acid degradations can be readily differentiated by this technique.

In the course of these studies, a basic water-soluble antibiotic, LL-BL136, discovered in our laboratories was compared to other streptothricin-type antibiotics by this technique. It was not differentiated from antibiotic SF-701 (6). The autoanalyzer results along with other physicochemical data permitted a structure proposal for this antibiotic.

## MATERIALS AND METHODS

**General.** All reference compounds used for nuclear magnetic resonance (nmr) studies or for autoanalyzer retention time and color yields were analytical reagent grade or samples with purity and analytical properties previously reported (1). The nmr spectra of LL-BL136

were obtained from summarized scans (100 ×) on a Varian A60D spectrometer equipped with a Varian CAT C1024. Hydrolysates were prepared in sealed vials with 3 *N* HCl at 100 to 110 C for 5 hr or with 6 *N* HCl at 110 to 120 C for 16 hr. The resulting solutions were evaporated under reduced pressure to residues which were dissolved in water to give ~1% solutions used for autoanalyzer and paper chromatography studies.

**Autoanalyzer determinations.** The compositions of the hydrolysates were determined with a Technicon amino acid autoanalyzer under the conditions described below. The columns were eluted continuously with a pH 5.0 buffer composed of 14.71 g (0.05 mole) of sodium citrate dihydrate, 900 ml of water, 25 ml (0.05 mmole) of 0.002 *N* sodium hydroxide, 35.07 g (0.60 mole) of sodium chloride, and 10 ml of Bridge detergent [polyoxyethylene (23) lauryl ether (10 g) dissolved in 200 ml of water] adjusted to pH 5.0 with 6 *N* HCl and diluted to 1 liter with water.

**Condition A.** A column (0.6 by 130 cm) of Chromobeads type A (sulfonated polystyrene resin from Technicon Chromatography Corp., Chauncey, N.Y.; type indicates degree of cross-linking) was maintained at 60 C by a water jacket and eluted with pH 5.0 buffer. The column effluent was monitored by a standard automated ninhydrin-hydrindantin procedure (4).

**Condition B.** Condition B was the same as condition A, except that the column was 0.6 by 75 cm of Chromobeads type C2, and the column effluent was monitored by an automated ninhydrin-hydrazine procedure (5). The prepared reagent for this modified detection system is more stable than that used in condition A.

**Antibiotic LL-BL136.** The following procedure was used to obtain and identify antibiotic LL-BL136.

**Fermentation.** Antibiotic LL-BL136 is produced by an unidentified *Streptomyces* species designated as culture BL136 in our laboratories. An inoculum of

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culture BL136 was prepared by growing the organism at 28 C in aerated 20-liter bottles for 48 hr in a medium of the following composition (g/liter): soybean meal, 10; glucose, 20; corn steep liquor, 5; and calcium carbonate, 3. Twelve liters of inoculum was used to seed 300 liters of medium containing the following ingredients (g/liter): cane molasses, 20; glucose, 10; Difco peptone, 5; NaOH to adjust pH to 7.2. The fermentation was carried out for 131 hr with an aeration rate of 0.8 liter of air per liter of mash per min and an agitator speed of 300 rev/min. The course of antibiotic production in the fermentation was followed by inhibition of *Klebsiella pneumoniae* by the agar-diffusion method. The assay organism was grown on Mycin Assay Agar (nutrient agar, pH 7.9, Difco).

**Isolation.** The 300 liters of harvested mash was filtered, the filtrate was adjusted to pH 8.0 with dilute sodium hydroxide, and the resulting solution was passed through a column (800 ml) of weakly acidic cation exchange resin (Amberlite CG50) which had been adjusted to pH 8.5 with ammonium hydroxide. The resin was washed with 4 liters of water, and the antibiotic was eluted with 0.5 N ammonium hydroxide. The first four liters of column effluent (pH 11.0) were combined, evaporated under reduced pressure to remove ammonia, adjusted to pH 6.7 with dilute hydrochloric acid, and freeze-dried to obtain 3.1 g of tan powder.

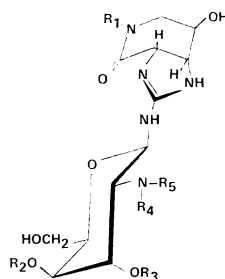
A 400-mg amount of the tan powder was dissolved in a small volume of water and adsorbed onto a column (2 by 32 cm) of charcoal (neutral Norit)-cellulose powder (1:3). The column was eluted with 425 ml of water followed by a linear gradient between water and 14% aqueous acetone. The antibiotic was detected in 21 to 220 ml of column effluent from the water-acetone gradient. The active fractions were determined by the agar diffusion paper disc assay against *K. pneumoniae*. The fractions containing the antibiotic were combined, evaporated to remove acetone, and freeze-dried to obtain 29 mg of white powder.

**Paper chromatography.** Antibiotics SF-701 and LL-BL136, both as hydrochloride salts, were not differentiated by paper chromatography in the following systems: wet 1-butanol containing 2% *p*-toluenesulfonic acid,  $R_F$  0.21; pyridine-*S*-collidine-tetramethylammonium hydroxide-water (50:25:1:125),  $R_F$  0.70; 90% phenol-*m*-cresol-acetic acid-pyridine-water (100:25:4:4:75),  $R_F$  0.55 and 0.68; 1-butanol-methanol-water-*p*-toluenesulfonic acid (40:10:20:1),  $R_F$  0.63.

The antibiotic hydrolysates were chromatographed with the system *t*-butyl alcohol-acetic acid-water (2:1:1), and the zones were detected with ninhydrin. Reference compounds gave the following  $R_F$  values in this system: *N*-guan-streptolidyl *N'*-methylgulosaminide, 0.14; streptolidine, 0.24; glycine, 0.39; methylamine hydrochloride, 0.34 and 0.43 (presumed effect of salt forms); sarcosine, 0.48.

## RESULTS AND DISCUSSION

The relationships of structures for some previously reported streptothricin-type antibiotics are summarized in Fig. 1, structures Ia-If (1, 7). These antibiotics differ in the amino acid side



Compound	Substituents				
	R <sub>1</sub>	R <sub>2</sub> , R <sub>3</sub>	R <sub>4</sub>	R <sub>5</sub>	
Ia Streptothricin	H	$\begin{matrix} \text{O} \\   \\ \text{H, CNH}_2 \end{matrix}$	H	$\begin{matrix} \text{O} \\   \\ \text{NH}_2 \\   \\ \text{CCH}_2 \text{ CH CH}_2 \text{ CH}_2 \text{ CH}_2 \text{ NH}_2 \end{matrix}$	
Ib Streptolin	H	$\begin{matrix} \text{O} \\   \\ \text{H, CNH}_2 \end{matrix}$	H	$\begin{matrix} \text{O} \\   \\ \text{NH}_2 \\   \\ (\text{CCH}_2 \text{ C CH}_2 \text{ CH}_2 \text{ CH}_2 \text{ NH}_2) \text{ H} \end{matrix}$	
Ic LL-AC541	H	$\begin{matrix} \text{O} \\   \\ \text{H, CNH}_2 \end{matrix}$	CH <sub>3</sub>	$\begin{matrix} \text{O} \\   \\ \text{NH} \\   \\ \text{C CH}_2 \text{ NH CH} \end{matrix}$	
Id Desformimino LL-AC541	H	$\begin{matrix} \text{O} \\   \\ \text{H, CNH}_2 \end{matrix}$	CH <sub>3</sub>	$\begin{matrix} \text{O} \\   \\ \text{C CH}_2 \text{ NH}_2 \end{matrix}$	
Ie LL-AB664	CH <sub>3</sub>	$\begin{matrix} \text{O} \\   \\ \text{H, CNH}_2 \end{matrix}$	CH <sub>3</sub>	$\begin{matrix} \text{O} \\   \\ \text{NH} \\   \\ \text{C CH}_2 \text{ NH CH} \end{matrix}$	
If Desformimino LL-AB664	CH <sub>3</sub>	$\begin{matrix} \text{O} \\   \\ \text{H, CNH}_2 \end{matrix}$	CH <sub>3</sub>	$\begin{matrix} \text{O} \\   \\ \text{C CH}_2 \text{ NH}_2 \end{matrix}$	
Ig LL-BL136	H	$\begin{matrix} \text{O} \\   \\ \text{H, CNH}_2 \end{matrix}$	CH <sub>3</sub>	$\begin{matrix} \text{O} \\   \\ \text{C CH}_2 \text{ NH CH}_3 \end{matrix}$	

FIG. 1. Structures of streptothricin and some related antibiotics.

chain and in methyl group substitution on the amino sugar and streptolidine moieties. Previously, substitution of the carbamate group on the hydroxymethyl of the sugar was considered a possibility for streptolin and streptothricin (7). Substitution in this position has now been excluded for streptothricin (Ia) by nmr studies similar to those for LL-AC541 and LL-AB664 (1), since the chemical shift of the hydroxymethyl protons was almost the same in the antibiotic ( $\delta$  4.23 relative to external tetramethylsilane reference) and in *N*-guan-streptolidyl gulosaminide ( $\delta$  4.27). The same substitution pattern is most likely valid for streptolin (Ib).

Hydrolysis products for a typical member of this antibiotic family are summarized in Fig. 2. (1). The equation for the vigorous condition shows only the products which were readily identified. Also obtained was some dark polymeric material probably resulting from degradation of the sugar.

In the classification of an antibiotic as streptothricin-like, paper chromatography is conventionally used to identify hydrolysis fragments. A method for the quantitative analysis of

TABLE 1. Autoanalyzer retention times for antibiotic hydrolysis fragments and related compounds

Compound	Condition A (14-hr run)		Condition B (5-hr run)	
	Retention time <sup>a</sup> (min)	Color yield <sup>b</sup> (area/mole)	Retention time (min)	Color yield (area/mole)
Glycine	73	35.3	35	19.1
Glucosamine	159 <sup>c</sup>	33.3	61	15.7
Galactosamine	164 <sup>c</sup>	32.6		
Gulosamine	173 <sup>c</sup>		69	14.3
Lysine	204	34.4		
Streptolidine	235	29.6	93	8.3
$\beta$ -Lysine	258		99	15.4
Ammonia	307	12.7	113	6.4
Methylamine	344	10.0		
Arginine	437	31.6		
<i>N-guan</i> -streptolidyl gulosaminide	605		236	9.8
<i>N-guan</i> -streptolidyl <i>N'</i> -methylgulosaminide	767	31.9	302	9.9

<sup>a</sup> Retention time averages for a particular compound were usually  $\pm 2$  min from given value.

<sup>b</sup> Area in arbitrary units.

<sup>c</sup> Separation of sugars confirmed by a mixed run.

tolidine, and the same streptolidine-sugar compound (Table 3). LL-AC541 is included for purposes of comparison. Both antibiotics had approximately a 1:1 ratio of total streptolidine to ammonia. The retention time of the streptolidine-sugar compound suggested that it was *N-guan*-streptolidyl *N'*-methylgulosaminide which is also a hydrolysis product of antibiotic LL-AC541 (1). This assignment was further supported by the fact that, under the more vigorous hydrolysis conditions, both LL-BL136 and SF-701 yielded methylamine due to degradation of the streptolidine-sugar compound. This feature is characteristic of streptothricin-type antibiotics containing *N*-methylamino sugars (1).

Tsuruoka reported the presence of sarcosine in the hydrolysate of antibiotic SF-701 (6). Paper chromatographic comparisons of the SF-701 and LL-BL136 hydrolysates and sarcosine also indicated the presence of a sarcosine moiety in LL-BL136. Although sarcosine was readily detected on papergrams with ninhydrin, it was not sensitive to the autoanalyzer system.

The evidence cited above and paper chromatography comparisons of the intact antibiotics indicated that SF-701 and LL-BL136 were identical and closely related to LL-AC541. The nmr spectrum of LL-BL136 had characteristic absorptions which were consistent with the struc-

TABLE 2. Autoanalyzer comparisons of ninhydrin-positive fragments from hydrolysates of various streptothricin-type antibiotics<sup>a</sup>

Antibiotic fragment	Observed mole ratios of fragments			
	Streptothricin	LL-AC541	Desformimino LL-AC541	LL-AB664 <sup>b</sup>
Glycine		1.0	1.0	1.0
Gulosamine	0.2			
Streptolidine	0.6	0.2	0.1	
$\beta$ -Lysine	1.0			
Ammonia	1.1	2.3	1.3	2.4
<i>N-guan</i> -streptolidyl gulosaminide	0.4			
<i>N-guan</i> -streptolidyl <i>N'</i> -methylgulosaminide		0.8	0.6	

<sup>a</sup> Values observed from hydrolyses with 3 N HCl at 100 to 110 C.

<sup>b</sup> The *N*-methylstreptolidine and streptolidine-sugar compound from LL-AB664 (1e) were essentially ninhydrin-negative.

TABLE 3. Hydrolysis fragments of LL-BL136, SF-701, and LL-AC541

Fragments	Retention time (min) <sup>a</sup>	Mole ratios		
		LL-BL136	SF-701	LL-AC541
Glycine	35			1.0
Streptolidine	93	0.3	0.2	0.2
Ammonia	113	1.0	1.1	2.3
<i>N-guan</i> -streptolidyl <i>N'</i> -methylgulosaminide	302	0.7	0.8	0.8

<sup>a</sup> Condition B (5-hr run).

tural assignments given above. It showed readily discernable signals attributed to the two *N*-methyl groups of the sarcosine and the sugar, the hydroxymethyl and anomeric protons of the sugar, and the methylene of the sarcosine. A comparison of LL-BL136, LL-AC541, and desformimino LL-AC541 spectra is given in Table 4.

The LL-BL136 does not have an nmr signal for a formimino group; therefore, the mole of ammonia detected in the hydrolysate probably is derived from a carbamate group. This group commonly occurs in other antibiotics of this family (1, 7).

From the amino acid autoanalyzer studies and nmr spectra, it was concluded that the mole ratio of primary fragments obtained from LL-BL136 was ammonia, sarcosine, *N-guan*-strep-

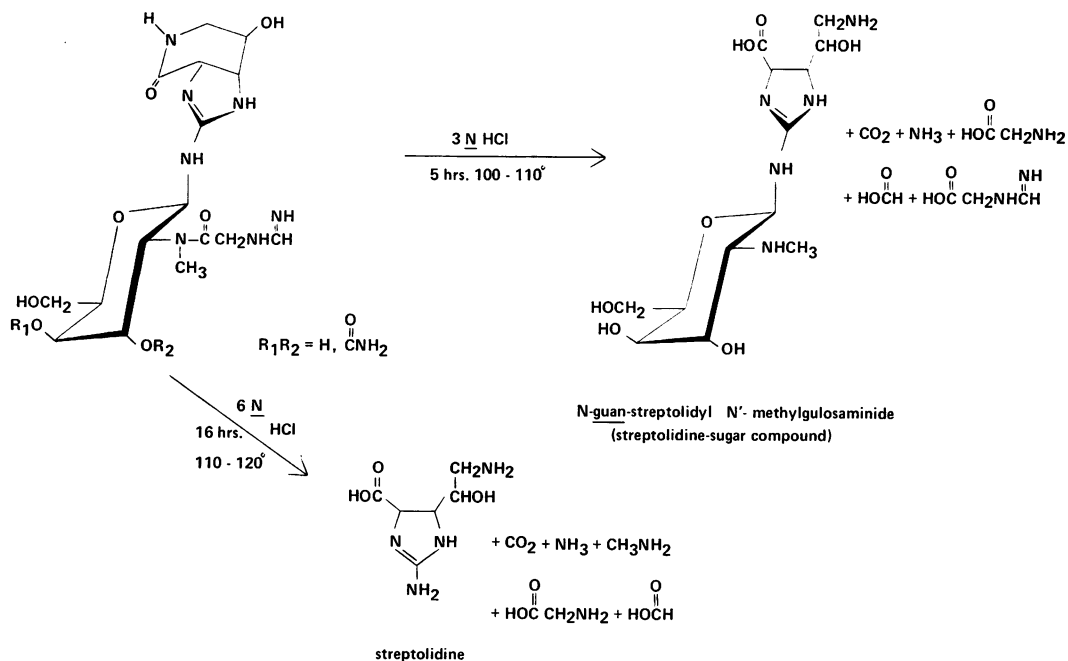


FIG. 2. Hydrolysis products of LL-AC541.

these fragments using ion exchange column chromatography was reported by Egorov and co-workers (2). A number of closely related streptothricin-type antibiotics studied by this column technique were shown to differ by the number of  $\beta$ -lysine units per molecule (3). Our initial attempts to resolve the hydrolysis fragments of streptothricin-type antibiotics by an amino acid autoanalyzer with a standard 3.5 to 5.0 pH gradient were unsuccessful. Some of the fragments were too basic to be eluted from the column of a styrene-sulfonic acid resin during a 24-hr run. However, when a pH 5.0 buffer was used to continuously elute the column, all of the fragments emerged and were well resolved.

The autoanalyzer determinations were conducted under two sets of conditions. Condition A required approximately 14 hr for completion, and condition B was conducted with a smaller column requiring only five hr. Different procedures were used for ninhydrin determinations; consequently, the color yields from conditions A and B were not equivalent. In most cases, the resolution provided by the smaller column was adequate and had the advantage of shorter run times. The results are given in Table 1.

For the analyzer studies, the antibiotics were hydrolyzed with 6 N HCl at 110 to 120 C for 16 hr and with 3 N HCl at 100 to 110 C for 5 hr. The more drastic conditions resulted in the destruction of the streptolidine-sugar compounds

with the formation of additional streptolidine and complete degradation of the sugar to yield ammonia or methylamine as the only other ninhydrin-positive fragment.

Of particular significance was the fact that the milder hydrolysis gave streptolidine-sugar compounds which could be analyzed by this procedure. Egorov and co-workers did not report data on these fragments (2). Our attempts to resolve the streptolidine-sugar compounds by paper chromatography or paper electrophoresis were only partially successful. However, some of these compounds were readily resolved by the amino acid analyzer with the pH 5.0 buffer system. For example, *N*-guan-streptolidyl gulosaminide and *N*-guan-streptolidyl *N'*-methylgulosaminide, obtained from streptothricin and LL-AC541, respectively, had retention times of 605 and 767 min when condition A was used; they were also well resolved by condition B.

A comparison of hydrolysates from various streptothricin-type antibiotics is given in Table 2 and illustrates various qualitative and quantitative differences that have been observed.

Application of this method to the study of LL-BL136, a basic-water soluble antibiotic discovered in our laboratories, differentiated it from all known antibiotics of the streptothricin class except antibiotic SF-701 reported by T. Tsuruoka et al. (6). Both LL-BL136 and SF-701 yielded, as ninhydrin-positive fragments, ammonia, strep-

TABLE 4. Comparison of nuclear magnetic resonance spectra of LL-BL136, LL-AC541 and desformimino LL-AC541<sup>a</sup>

Assignment	$\delta$ (Hz)		
	LL-BL136	LL-AC541	Desformimino LL-AC541
CH <sub>3</sub> N—	3.24 s		
CH <sub>3</sub> N—	3.50 s	3.53 s	3.53 s
—CH <sub>2</sub> —(sugar C <sub>6</sub> )	4.17 (6.0) d	4.17 (6.0) d	4.18 (6.2) d
—CH <sub>2</sub> —(glycyl)	4.71 s	4.92 s	4.56 s
Anomeric proton	5.99 (~10.5) d	6.10 (9.7) d	6.08 (10.2) d
Formimino proton		8.40 s	

<sup>a</sup> Spectra in D<sub>2</sub>O with tetramethylsilane as external reference. Multiplicity: d, doublet; s, singlet.

tolidyl *N'*-methylgulosaminide (1:1:1). A consideration of the data and an analogy to the closely related LL-AC541 antibiotic resulted in a structure proposal for LL-BL136 as shown in Fig. 1 (Ig).

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