SEPARATION AND BIOLOGIC ACTIVITIES OF INDIVIDUAL COMPONENTS OF S15-1, A STREPTOTHRICIN CLASS ANTIBIOTIC

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A method is described for isolation of gram quantities of the components of the streptothricin complex S15-1 utilizing CM Sephadex column chromatography eluted with 10% acetic acid as an eluant followed by gradient elution with 10% acetic acid containing 0.02 N ~ 0.03 N HCl. Streptothricins F and E, as well as an unidentified component C^1, have been isolated and their comparative biological activities determined. Streptothricins F and E were comparable in taeniacidal activity in mice infected with *Hymenolepis nana* as feeding either one at 0.05% in the diet removed 92~100% of the adult tapeworms. The unidentified component C^1 was inactive at the levels tested. In contrast, component C^1 was the most active in antimicrobial activity against *Bacillus subtilis* and in inhibiting the urease activity of *Proteus mirabilis*. In the former test, the ratios of activity were; 1: 7: 30 for F: E: C^1 and in the latter; 1: 2: 4 for F: E: C^1.

Streptothricin antibiotic complex S15-1 (SQ 21,704)[1,2] has been reported to have taeniacidal activity when given orally to mice, cats, dogs, and sheep.[3,4,5,6] At the time, it was speculated that taeniacidal activity is a property common to many, but not all, members of the streptothricin family[3]. Since the S15-1 complex is known to contain at least three components, streptothricins F and E as well as an unidentified streptothricin type component, in the approximate proportions of 6: 3: 1[2,3], we were interested in determining the relative activities of the three components and to determine whether other *in vitro* biological systems could be used to predict the taeniacidal activity of mixtures.

Previously, it had been demonstrated that streptothricin F isolated from the S15-1 fermentation broth as well as from other sources was taeniacidal[3]. At that time, testing of the other components of the S15-1 complex was not possible because of the lack of sufficient material, as the chromatographic procedures then available were not adequate for the preparation of gram quantities of the lesser fractions. Consequently, a modified column chromatographic separation was developed that has general applicability to streptothricin mixtures and the potencies of the isolated fractions determined in appropriate biological systems.

Experimental

Separation of Individual Components

For separation of the components, a column, 18 cm long and 8 cm in diameter was packed by gravity with CM Sephadex C-25 (particle size 50~120 μ, from Pharmacia, Uppsala, Sweden) previously soaked in 10% aqueous acetic acid for about 15 hours to insure proper swelling. A nine gram sample of crude S15-1 (Batch NX005, estimated 80% purity) was dissolved in 40 ml of 10% aqueous acetic acid and carefully charged on top of the column without disturbing the surface. After the solution containing the crude S15-1 went into the column, it was eluted with 10% acetic acid.

Flow rates as fast as 20 ml per minute were used and usually 500 ml were collected in each fraction.

During development of the separation technique, it was learned that the volume of 10% acetic acid required to elute the slower moving components was excessive. To overcome this, gradient elution with dilute hydrochloric acid was used, starting after fraction No. 90, as follows: A one liter Erlenmeyer flask containing 800 ml of 10% aqueous acetic acid and a magnetic stirring bar was connected to the top of the column with a magnetic stirrer mounted underneath the flask. A one liter separatory funnel containing 0.02 N HCl in 10% aqueous acetic acid, replenished as required, was mounted over the flask. After fraction No. 110, the solvent was changed to 0.03 N HCl in 10% aqueous acetic acid. As the column was eluted, the contents of the flask were mixed by stirring as the contents of the separatory funnel dropped into the flask. The flow rates of the column, flask, and funnel were approximately the same.

Column fractions and separated components were analyzed by paper chromatography using Whatman No. 1 paper, and the solvent system of TANIYAMA.[7] The chromatogram was developed for 40~44 hours, air dried and then placed on an agar plate (pH 8) seeded with *Escherichia coli* SC2927. After overnight incubation at 37°C, the chromatogram was examined and photographed. The fractions containing the same component, were pooled and concentrated. The purities of the individual preparations used for biological testing were estimated to be greater than 90%.

Biological and Biochemical Activity

Antimicrobial Activity: The relative antimicrobial activities and dose response slopes of the three components isolated from S15-1 were determined using *Bacillus subtilis* ATCC6633 in an agar diffusion assay system similar to that for streptomycin[8] except for the standard used. Assay sensitivity was about 0.2 unit/ml.[8]

Inhibition of Urease Activity: Since the streptothricins act on bacteria by inhibiting protein synthesis,[9,10] inhibition of urease activity by *Proteus mirabilis* was a simple assay that could be adopted for routine use. The assay, similar to that described by PATTISON, *et al*,[11] depends on the induction of urease synthesis by *P. mirabilis* at 37°C by the addition of urea to the culture broth, the urease in turn hydrolyzes the urea to ammonia and water. The release of ammonia results in a change in pH of the broth with time, the change being dependent on the quantity of urease produced. The pH measurements were made 110 minutes after inhibitor and inducer were added. Control tubes were run without inhibitor and each component was tested at concentrations from 0.3 to 20 μg/ml. Each result represents the average of six individual assays run at six different times.

Taeniacidal Activity: With certain modifications, the method described by STANDEN[12] was used to determine the taeniacidal activities of the various components. Carworth CF_1 female mice were each infected *per os* with approximately 500 viable embryonated *Hymenolepis nana* eggs. Evaluation for the presence or absence of adult *H. nana* was done 16 days post-infection.

Feed containing various concentrations of the individual components was administered for four days in two separate tests. Following treatment each intestine was examined for the presence or absence of *H. nana*.

Results and Discussion

Separation of Individual Components

Table 1 shows the pattern of components eluted from the column while Fig. 1 shows the high quality of the separation of the separate components as determined by bioautography. From the nine gram sample of S15-1, approximately four grams of streptothricin F, two grams of streptothricin E, and 0.9 grams of a third unidentified component (component C^1) were obtained for testing. The identities of streptothricins F and E were confirmed by paper chromatographic comparison with authentic samples. In contrast, the third component C^1, proved not to be identical with fraction C of KAWAMURA *et al.*[2] The slight difference in Rf values observed in Fig. 1 between the isolated C^1 and the C^1 in the mixture was due to overloading of the chromatographic system with the S15-1 mix-

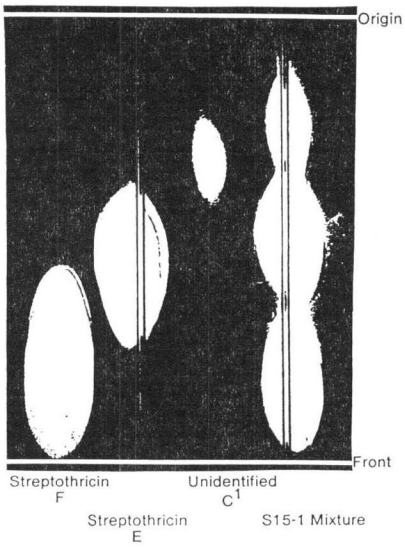
Table 1. CM Sephadex column of the S15-1 complex.*

Fraction No.	Component	Approximate vol. of eluate (liter)	Sample weight (g)
1~ 17	None	8	—
18~ 38	Streptothricin F	10	4
39~ 62	Streptothricins F, E	10	—
63~ 90	Streptothricin E	14	2
91~ 98	Streptothricin E & component C^1	4	—
99~127	Component C^1	9	0.9

* Column charged with nine grams of crude S15-1. (Batch NX005).

ture, a procedure followed to avoid missing any component present in limited quantity. In other chromatograms when systems were not overloaded the Rf values were identical.

Fig. 1. Bioautograph of CM Sephadex column fractions of the streptothricin antibiotic S-15-1.



The success of a preparative separation technique is primarily measured by the degree of resolution and loading capacity. With most techniques this represents a conflict, for resolution is lost as the charge increases in size. However, the technique described appears to be an exception. Using identical columns, a nine gram charge had the same resolution as a three gram charge of S15-1. The procedure has the drawback of requiring large volumes of eluant, but offers the advantages of large capacity, the use of non-toxic and readily available solvents, and the potential of scale up.

Biological and Biochemical Activity

Antimicrobial Activity

The relative antimicrobial activities and dose response slopes of the three isolated components, determined using a *B. subtilis* agar diffusion test, are summarized in Table 2. The dose response slopes of all the components differed from the reference mixture which was intermediate in potency among those of the three components. The relative microbiological activities, in comparison with the reference mixture show that streptothricin E and component C^1 are about equal and both are more active than streptothricin F. The extrapolated minimum detectable concentration (EMDC), a more accurate representation of activity, shows that component C^1 is about four times more active than streptothricin E and about 30 times more active than streptothricin F. The activity of the reference sample was between those of streptothricin E and C^1 suggesting that streptothricin E and component C^1 contributes more microbiological activity of the crude isolate S15-1 than does streptothricin F. The EMDC is greatly influenced by the dose response slope and the true activity of the antibiotic, hence the greater the slope the greater the apparent activity. Since the slopes of streptothricins F and E are essentially equal, the EMDC values are good estimates of these microbiological activities, whereas, the EMDC probably overestimates the activity of component C^1.

Urease Activity

The results on the inhibition of urease activity are given in Fig. 2 with the values for each com-

Table 2. Antimicrobial activities of S15-1 components.

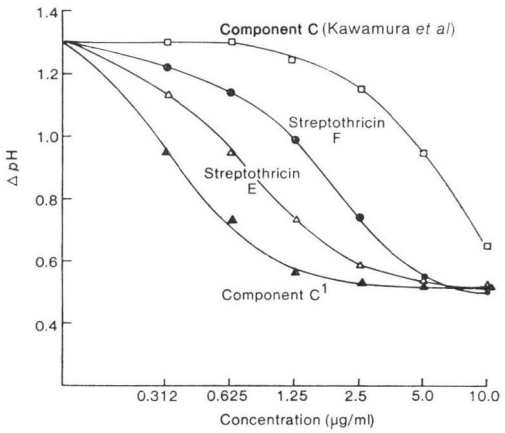
Component	Activity*		Slope function***
	Relative	EMDC**	
Streptothricin F	26	0.13	0.023
Streptothricin E	93	0.019	0.026
Component C^1	101	0.0042	0.042
Mixture (Analytical reference)	98	0.0084	0.034

* Determined with *B. subtilis* ATCC 6633 in an agar diffusion assay system.

** Extrapolated concentration equivalent to minimum readable zone size expressed as the antilogarithm of the dose response curve intercept.

*** Natural logarithm dose response.

Fig. 2. Effect of S15-1 components on inhibition of urease activity of *P. mirabilis*.



ponent plotted as Δ pH in comparison with the log dose. Based on the comparison of the plotted results, it appears that component C^1 is twice as active as streptothricin E and about four times more active than streptothricin F. The lack of identity of component C^1 with component C (Asahi)[2] is supported by the comparison of the inhibiting activities of the two preparations where C^1 is approximately ten times more active than component C.

Taeniacidal Activity

In the first test (Table 3), since 10 of 57 of the control animals did not become infected, the data were analyzed statistically using the formula $P=P^1-C/1-C$ where P=calculated proportion of animals cleared, P^1=observed proportion of animals cleared and C=natural frequency of cleared animals in control groups. At 0.05% in the diet both streptothricins F and E cleared 94~100% of the animals and at 0.025% cleared 24 and 79%, respectively. Lower levels of streptothricins F and E were not active and none of the levels of component C^1 demonstrated taeniacidal activity at the concentrations tested.

In the second test (Table 3), with concentrations selected to give a better dose response, mice were used that were shown to be infected with *H. nana* immediately prior to treatment by fecal examination. Because of the limited quantities of the components, exact dose comparisons could not be made between streptothricins F and E, while component C^1 was not available in sufficient quantity for testing. The data, plotted in Fig. 3 confirm the results of the first test. Streptothricins F and E are concluded to be approximately equal in activity against *H. nana* in the mouse.

Fig. 3. Comparative taeniacidal activities of streptothricins F and E against *H. nana*.

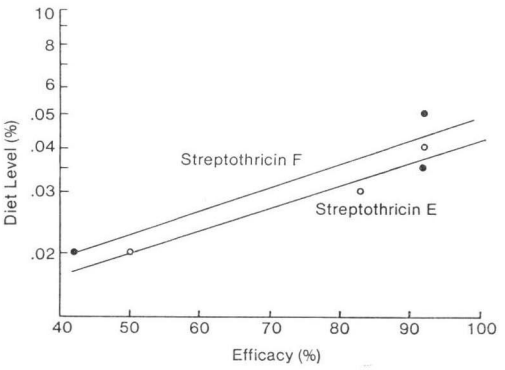


Table 3. Taeniacidal activities of S15-1 components in mice infected with *H. Nana*.

Component	Level in diet (%)	No. cleared/No. infected		Efficacy* (%)	
		Test #1	Test #2	Test #1	Test #2
Streptothricin F	0.05	19/20	11/12	94	92
	0.035		11/12		92
	0.025	23/60		24	
	0.02		5/12		42
	0.0125	6/60		0	
	0.00625	7/40		0	
Streptothricin E	0.05	20/20		100	
	0.04		11/12		92
	0.03		10/12		83
	0.025	43/52		79	
	0.02		6/12		50
	0.0125	10/60		0	
	0.00625	4/38		0	
Component C^1	0.05	3/38		0	
	0.025	4/39		0	
	0.00625	4/20		0	
Infected controls		10/57	0/12		

* Calculated efficacy P, corrected for failure to infect all animals, according to the formula:

$$P=\frac{P^1-C}{1-C}$$

where P=calculated efficacy, P^1=observed efficacy, C=frequency of clearance in infected untreated controls.

Comparative Biologic Activity

Our experiments have demonstrated unequivocally that both streptothricins F and E contribute to the taeniacidal activity of the S15-1 complex. A more precise measurement of the relative activities of these two compounds can only be obtained by significantly larger experiments involving large numbers of mice, experiments that were not possible with the materials available. The experiments have also shown that for all practical purposes, the third component, designated as component C^1, is without taeniacidal activity. This lack of activity is somewhat surprising because of its high activity in both the *in vitro* systems and because of its presumed identity as a streptothricin.

Table 4. Relative biologic activities of the S15-1 components.*

Component	β-Lysine units	Antimicrobial	Urease inhibition	Taeniacidal	LD_{50}**
Streptothricin F	1	1	1	1	1
Streptothricin E	2	7	2	1	12
Streptothricin D	3	—	—	—	30
Component C^1	>2***	~30	4	<1	

* Relative comparisons against value of 1 assigned to streptothricin F.

** LD_{50} values based on report by TANIYAMA *et al.*[14]

*** Component C^1 presumed to contain more than two β-lysine units.

In Table 4 the relative biologic activities of the three isolated components are given. It is interesting to examine these data from the point of view of earlier investigators, who observed *in vitro* antimicrobial activity and toxicity increased with the number of β-lysine units in the streptothricin side chain.[13,14] Our results confirm this relationship for both antimicrobial activity and for inhibition of urease activity, particularly if we assume C^1 is a streptothricin with more than two β-lysine units. The taeniacidal activities obviously are not consistent with the hypothesis, suggesting that some other factor comes into play.

Our results further demonstrated that the simple *in vitro* tests utilized are not satisfactory measurements of taeniacidal activity in S15-1 mixtures. The markedly higher activities of C^1 and to a lesser extent streptothricin E would result in very misleading estimations of the taeniacidal activity of the complex.

Acknowledgements

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