STREPTOVIRUDINS, NEW ANTIBIOTICS WITH ANTIBACTERIAL AND ANTIVIRAL ACTIVITY

II. ISOLATION, CHEMICAL CHARACTERIZATION AND BIOLOGICAL ACTIVITY OF STREPTOVIRUDINS A_1, A_2, B_1, B_2, C_1, C_2, D_1 AND D_2

K. ECKARDT, H. THRUM, G. BRADLER, E. TONEW and M. TONEW

Akademie der Wissenschaften der DDR, Forschungszentrum für Molekularbiologie und Medizin, Zentralinstitut für Mikrobiologie und Experimentelle Therapie, DDR-69 Jena, Beuthenbergstraße 11

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Streptovirudin is a complex of antibiotics isolated from fermentation of a *Streptomyces* strain. Eight components have been isolated as pure substances, designated as streptovirudins A_1, A_2, B_1, B_2, C_1, C_2, D_1 and D_2. The streptovirudins are chemically and biologically related to each other and appear to be a new family of antibiotics exhibiting activity against a variety of Gram-positive bacteria, mycobacteria, and various DNA- and RNA-viruses. According to their physico-chemical properties these antibiotics have been classified in series I and II. The streptovirudins of series II (A_2, B_2, C_2, D_2) are related to the reported antibiotics tunicamycin, mycospocidin and 24010.

In the course of our screening for new antiviral antibiotics, a *Streptomyces* strain JA 10124 (described as a new variant of *Streptomyces griseoflavus* (KRAINSKY) WAKSMAN *et* HENRICI) was found to produce a new antibiotic[1]. This antibiotic, designated streptovirudin, was isolated from both mycelium and culture filtrate. The present communication deals with the isolation and chemical characterization of the antibiotic components $A_1 \sim D_2$. The two minor components E_1 and E_2 have not been isolated. Results of preliminary testing of the streptovirudins are also included in this paper. However, detailed discussion of the antiviral properties of streptovirudins will be the subject of a separate communication[2].

Experimental

Sephadex chromatography.

Sephadex LH 20 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) was mixed with methanol - water (1.5 : 8.5, v/v) and allowed to swell for 20 hours. The gel was then packed into a chromatographic column (3.2 cm internal diameter, constant height of 100 cm). Fifty to sixty mg of streptovirudin complex (obtained as described in the separate paper[1]) was dissolved in 5 ml of methanol and after addition of 30 ml of hot water applied on the top of the column. Precipitation was prevented by warming. The column was eluted with methanol - water (1.5 : 8.5, v/v) at a flow rate of 10 ml per 20 minutes. Selected fractions of 10 ml each were tested for bioactivity (agar diffusion method, paper discs, *Bacillus subtilis* ATCC 6633) and analyzed by paper chromatography (systems methanol - water (1.5 : 8.5, v/v) and *n*-propanol - water (1.5 : 8.5, v/v). Evaporation of the fractions containing the separated components yielded in each case a white gum. The antibiotics were then dissolved in small amounts of methanol. After filtration the solutions were evaporated to dryness.

Crystallization of streptovirudins.

One hundred eighty to two hundred mg of streptovirudins were dissolved in 2~5 ml of hot

methanol. The solutions were clarified by filtration. After addition of 10~20 ml of hot water the solutions were allowed to stand at 4°C for a few days. Then the precipitates were washed twice by decantation with acetone. The pure antibiotics were filtered off, washed with small amounts of acetone and dried. Yields, 80~100 mg.

Results and Discussion

Isolation

Streptovirudin complex was isolated from both mycelium and culture filtrate. As demonstrated in the separate communication[1], paper chromatograms of streptovirudin complex showed the presence of five bioactive components named streptovirudins A, B, C, D, and E. However, the presence of additional activities with Rf values identical to those of any of the five antibiotics could not be excluded. Interest in the separation of the antibiotic components was aroused by the observation that streptovirudin complex is highly active against various DNA- and RNA-viruses in cell cultures.

Further experiments were started with the material obtained by purification with charcoal followed by chromatography on a silica gel column[1]. The highly purified antibiotic complexes obtained by these procedures were used for separation experiments on Sephadex LH 20. This method, used under the conditions described in the experimental section, afforded eight components designated streptovirudins A_1, A_2, B_1, B_2, C_1, C_2, D_1 and D_2. Components E_1 and E_2 have not been isolated as yet. Crystallization from mixtures of methanol and water yielded the pure antibiotics as white needles or as white powders.

Fig. 1. Chromatographic elution pattern of streptovirudin complex isolated from culture filtrate.
Column: Sephadex LH 20

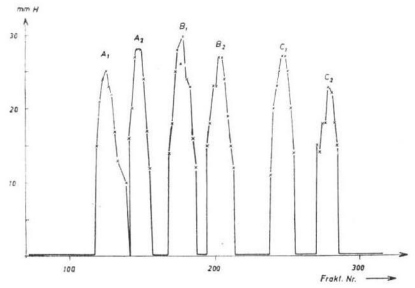


Fig. 1 illustrates a typical separation of streptovirudin complex isolated from extracts of culture filtrate. Selected fractions were tested against *B. subtilis* ATCC 6633. The components were well separated to each other.

Table 1 demonstrates that antibiotic complexes isolated from culture filtrate and mycelium are different from each other in the content of single components.

As shown in Table 2 the Rf values of streptovirudins A_1 and A_2 are nearly identical. The same is evident for the corresponding antibiotics B_1/B_2, C_1/C_2 and D_1/D_2. However,

Table 1. Composition of streptovirudin-complexes isolated from culture filtrate and from extracts of mycelium.
Separation of 700 mg of either material

	Streptovirudins (mg)									
	A_1	A_2	B_1	B_2	C_1	C_2	D_1	D_2	E_1	E_2
Complex from culture filtrate	85	109	85	73	28	22	<1	<1	—	—
Complex from mycelium	<1	6	16	20	40	60	26	24	<1	<1

Table 2. Physicochemical properties of streptovirudins

	Streptovirudins							
	A_1	A_2	B_1	B_2	C_1	C_2	D_1	D_2
	white needles	white needles	white needles	white powder	white needles	white powder	white needles	white powder
Melting point (°C)	263 ~265 (dec.)	250 ~252 (dec.)	254 ~256 (dec.)	249 ~251 (dec.)	263 ~265 (dec.)	251 ~253 (dec.)	252 ~253 (dec.)	255 ~256 (dec.)
uv: $\lambda_{max}^{CH_3OH}$(nm)	211 ~215	211 ~215, 259	211 ~215	211 ~215, 259	211 ~215	211 ~215, 259	211 ~215	211 ~215, 259
$[\alpha]_D^{22}$ *c* 0.5, CH_3OH	+55°	+69°	+55°	+67°	+54°	+79°	+46°	+55°
$[\alpha]_D^{22}$ *c* 0.2, water-saturated butanol	*	+76°	*	+70°	*	+70°	*	+59°
Elementary analysis (%) C	50.90	51.19	51.78	51.76	53.28	53.45	*	*
Elementary analysis (%) H	7.59	7.10	7.64	7.31	7.47	7.42		
Elementary analysis (%) N	6.62	6.69	6.58	6.61	6.70	6.51		
Rf**	0.74 ~0.79	0.68 ~0.73	0.55 ~0.62	0.48 ~0.53	0.25 ~0.30	0.22 ~0.27	0.18 ~0.23	0.13 ~0.19

* not determined

** PC: 2×developed, ascending. Bioautography with *B. subtilis*. Solvent system: methanol-water (1.5:8.5, vol/vol)

the physical properties of antibiotics of series I are different from those of series II. This may be important for identification of related antibiotics.

Characterization of Streptovirudins

The pure streptovirudins are soluble in methanol, less soluble in hot water, ethanol and butanol, and are insoluble in acetone, chloroform, benzene and most other common organic solvents. Streptovirudins are dextrorotatory. They gave negative ninhydrin test. Physicochemical properties are shown in Table 2.

According to their spectral properties streptovirudins belong to two subgroups. The ultraviolet absorption spectra of the streptovirudins A_1, B_1, C_1 and D_1 in methanol (Fig. 2) have only one peak at 211~215 nm (series I). The uv spectra of the streptovirudins A_2, B_2, C_2 and D_2 contain maxima at 211~215 nm and 259 nm (series II). As shown in Fig. 3, IR spectra of the streptovirudins of series I are very similar to each other, but they are clearly distinguishable from those of series II.

Average microanalytical figures are listed in Table 2. Attempts to derive a molecular formula based upon accurate molecular weight measurements by mass spectroscopy

Fig. 2. Ultraviolet spectra of streptovirudins in methanol

1 Streptovirudins A_1, B_1, C_1, D_1 (series I)

2 Streptovirudins A_2, B_2, C_2, D_2 (series II)

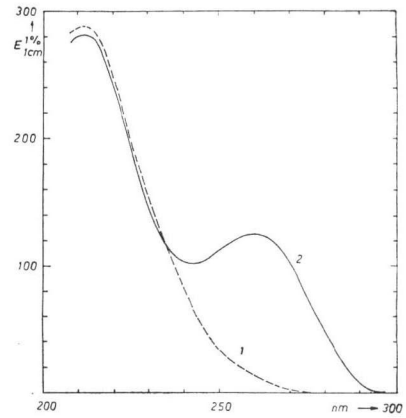
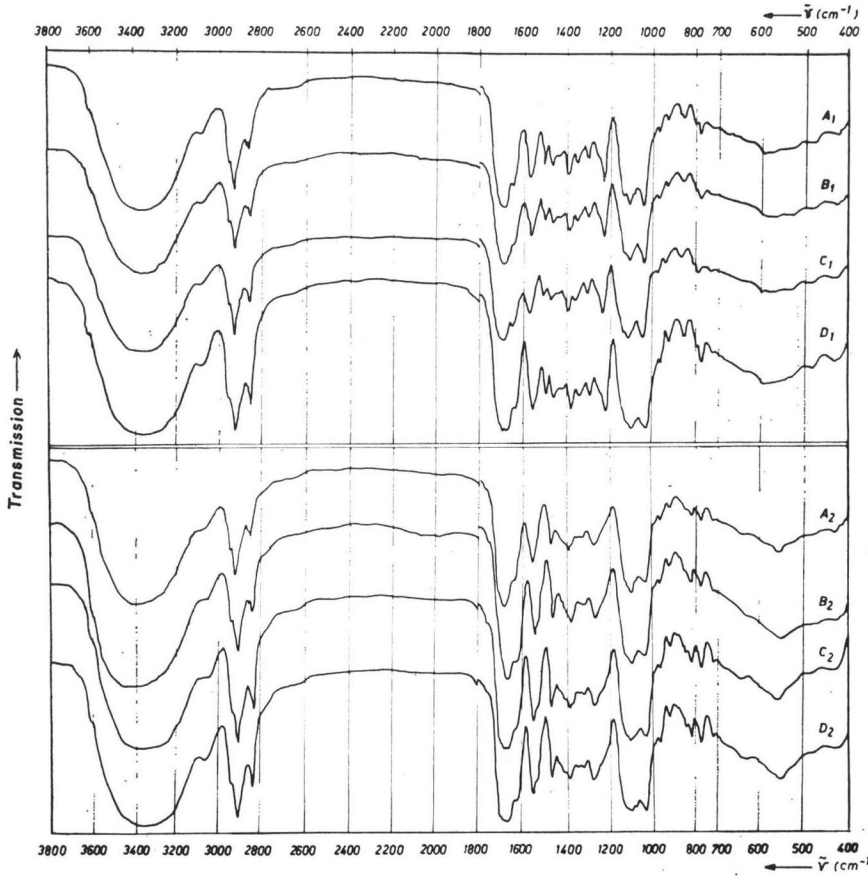


Fig. 3. Infrared spectra of streptovirudins (KBr pellets).



have been unsuccessful as yet.

Analysis of acid hydrolysates of streptovirudins (6 N HCl, 20 hours at 105°C) by thin-layer chromatography and with an automatic amino acid analyzer confirmed the existence of glucosamine as a constituent of all streptovirudins. It was determined in all hydrolysates, but no amino acid could be detected. Under these conditions an additional unidentified hydrolysis product with positive ninhydrin test (probably another amino sugar) was found in hydrolysates of streptovirudins A_1, B_1, C_1 and D_1. Similarly, hydrolysates of the streptovirudins A_2, B_2, C_2 and D_2 contained an unstable ninhydrin-positive degradation product when hydrolysis was performed under milder conditions. Detailed discussion of these studies will be the subject of a subsequent communication.

Antimicrobial Activity

The minimum inhibitory concentration (MIC) of streptovirudins for a number of microorganisms was determined by the agar diffusion method. Results obtained are summarized in Table 3. The antibiotics were found highly active against some Gram-positive bacteria, with the exception of *Micrococcus pyogenes* var. *aureus* SG 511 which was less sensitive to streptovirudins. Little activity was found against *Mycobacterium smegmatis* SG 987 and *Mycobacterium*

Table 3. *In vitro* antimicrobial spectrum of streptovirudins

Test organism	Streptovirudins (MIC, mcg/ml)							
	A_1	A_2	B_1	B_2	C_1	C_2	D_1	D_2
Bacillus subtilis SG 119	10	10	2.5	1.25	1.15	1.25	1.25	0.62
Bacillus subtilis ATCC 6633	5	5	2.5	1.25	1.25	1.25	1.25	0.62
Micrococcus pyogenes var. *aureus* SG 511	50	25	>50	25	50	25	100	50
Bacillus globifer OH 11	10	5	5	2.5	1.25	1.25	0.62	0.31
Bacillus mycoides SG 756	5	10	2.5	2.5	1.25	1.25	0.62	0.31
Sarcina lutea SG 125 A	>100	>100	>100	>100	>100	>100	50	100
Escherichia coli mutabile SG 458	>100	>100	>100	>100	>100	>100	>100	>100
Mycobacterium phlei SG 346	50	>100	25	25	12.5	50	6.25	12.5
Mycobacterium smegmatis SG 987	50	>100	25	50	12.5	25	3.1	6.25
Saccharomyces cerevisiae JH 1	>100	>100	>100	100	25	50	50	12.5
Kloeckera brevis JH 3	>100	>100	>100	100	50	>100	>100	25
Penicillium notatum JP 36	>100	>100	>100	>100	>100	100	100	50

phlei SG 346 or *Saccharomyces cerevisiae* JH 1 in the case of the streptovirudins C_1, C_2, D_1 and D_2. In general, the activity of streptovirudins increases from streptovirudin $A_1 \rightarrow D_2$.

All streptovirudins showed antiviral activity as determined by various methods. These results obtained in testing streptovirudins against DNA- and RNA-viruses will be reported in a separate communication[2].

Comparison of Streptovirudins to Related Antibiotics

The antibiotics described in this paper show differences from most antibiotics reported in the literature. Similar properties were described for the antibiotic tunicamycin[3]. From its published spectral data, tunicamycin is closely related to streptovirudins of series II, but differs clearly from streptovirudins of series I. The same is evident for mycospocidin[4] and antibiotic 24010[5]. But all three antibiotics can be distinguished from streptovirudins on the basis of the reported melting points. Furthermore, glycine was not detected in hydrolysates of streptovirudins as reported for mycospocidin. Antibiotic 24010 can be eliminated specifically by comparison of the uv spectra and by optical rotation. The specific rotation of antibiotic 24010 in water-saturated *n*-butanol is $[\alpha]_D^{20}+4.3°$ [5] The optical rotation of the streptovirudins A_2, B_2, C_2 and D_2 having the same uv spectrum as antibiotic 24010 is shown in Table 3. Differentiation by paper chromatography has not been done since reference samples were not available.

It would appear, then, that tunicamycin, mycospocidin, antibiotic 24010 and the streptovirudins A_2, B_2, C_2, D_2 as well as the streptovirudins of series I represent a new subclass of streptomycete antibiotics.

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