ANTIBIOTICS PRODUCED BY *STREPTOMYCES*. VII*

CYTOTETRIN, A NEW ANTITUMOR ANTIBIOTIC

JÁNOS BÉRDY, JÓZSEF ZSADÁNYI, MÁRIA HALÁSZ
ISTVÁN HORVÁTH and KÁROLY MAGYAR

Research Institute for Pharmaceutical Chemistry, Budapest, Hungary

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From the culture broth of *Streptomyces griseoflavus* a new orange red basic antibiotic named cytotetrin complex was isolated by solvent extraction. The complex was fractionated into five active components, cytotetrins A, B, C, D and E, respectively. The physical, chemical and biological properties of each cytotetrin are discussed. They are active against Gram-positive microorganisms and some tumors. Cytotetrin is related to nogalamycin.

In the course of screening for new antitumor antibiotics, it has been found that a new orange red basic antibiotic complex is produced by a streptomycete isolated from a soil sample. The organism designated as S-191 proved to be identical with *Streptomyces griseoflavus* "CBS-Cifferi" (BUCSAV 7, 13).

The antibiotic complex produced by *S. griseoflavus* No. S-191 is especially active against Gram-positive bacteria and moreover exhibits some antitumor activity. The components of the antibiotic complex were separated, obtained in crystalline state and identified as new antibiotics of the anthracycline family. These antibiotics were named cytotetrin complex and its single components designated as cytotetrins A, B, C, D and E, respectively.

Experimental and Results

I. Production and Isolation

The antibiotic complex was produced in a complex medium (adjusted to pH 7.0) containing 8.0 % glucose, 2.5 % soybean flour, 0.5 % corn steep liquor, 0.1 % caseine hydrolysate, 0.5 % sodium chloride, 0.3 % potassium nitrate and 0.5 % palm oil. For a typical run, 300 liters of the sterilized medium in a stainless steel fermentor were inoculated with 30 liters of a seed culture. The cultivation was carried out at 28°C witha eration (300 liters/min.) and agitation (250 r.p.m.). About 800~1,000 mcg/ml of cytoterin were produced in 80~100 hours cultivation period. The activity of the culture broth was assayed against *Bacillus subtilis* ATCC 6633 by the agar diffusion method.

The extraction and purification procedure for cytotetrin is summarized in Chart 1.

The mycelial cake (47 kg) obtained by filtration of the culture broth (300 liters) at pH 4.0 (HCl), was extracted with 50 liters methanol with stirring for half an hour. The extraction was repeated three times and the combined extracts (142 liters, 750 mcg/ml)

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were concentrated *in vacuo* to about one-fifth of the original volume and added to the culture filtrate (285 liters, 360 mcg/ml). The combined solution was adjusted to pH 7.8 and was extracted three times with EtOAc (1/3 volume) in the presence of 10 % sodium chloride. The extract (280 liters) was reextracted three times with 0.5 M acetic acid (1/8 volume). The combined aqueous extract was adjusted to pH 7.8 and reextracted with EtOAc (3×20 liters) in the presence of 10 % sodium chloride. This procedure was repeated three times. The final EtOAc extract was washed with distilled water, dried and concentrated *in vacuo* almost to dryness. The residue, triturated with petroleum ether, represented an orange red precipitate. The active product obtained was recovered by filtration, dissolved in a small volume of acetone, filtered and finally precipitated with ten volumes of petroleum ether. Thus 125 g of crude cytotetrin complex was obtained as an orange red powder. In thin-layer chromatography the crude antibiotic complex showed about 7~8 orange to red spots. By paper and thin-layer chromatography, followed by bioassay, at least five antibiotically active orange-colored substances could be detected. These active substances were named cytotetrins A, B, C, D and E. Cytotetrin A is the major component, followed by B, C, D and E in decreasing order of abundance.

Chart 1. Isolation of cytotetrins

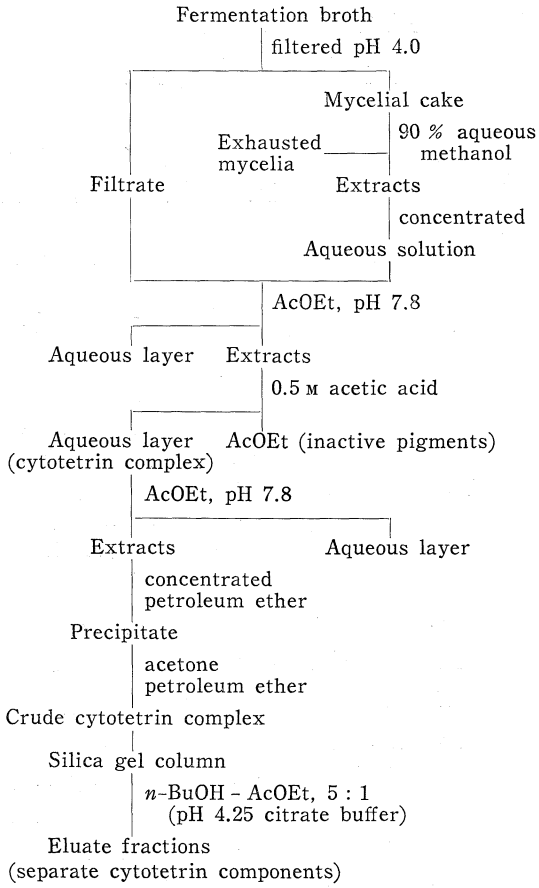
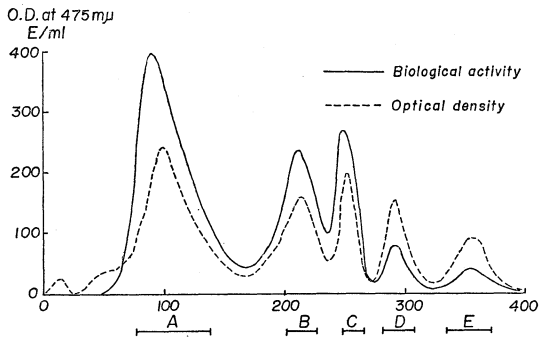


Fig. 1. Partitition chromatography of cytotetrin complex.

Sample : 10 g. column : Celite 535 (buffered with pH 4.25 citrate buffer), 60×850 mm, solvent : *n*-BuOH - AcOEt (5 : 1), saturated with the buffer. Flow-rate : 5 ml/minute. Fraction volume : 50 ml.



Partition chromatography on Celite 535 column, with pH 4.25 citrate buffer as stationary phase and *n*-butanol - EtOAc (5 : 1) mixture as the mobile phase was found to be suitable for purification and separation of the cytotetrin components. The elution diagram is presented in Fig. 1.

The fractions containing each type of cytotetrin were combined and extracted with

0.75 M acetic acid. The pure components were isolated as described above. The amorphous powders obtained were re-chromatographed in the same system and crystallized from various solvents or mixtures of solvents, *e.g.* methanol, aqueous methanol, benzene-methanol and benzene-cyclohexane.

The homogenity of each component was confirmed by thin-layer and paper-chromatography.

The separation of the cytotetrins could also be achieved by a two step countercurrent distribution procedure. The most promising results were observed in buffered systems. In the system *n*-butanol - 0.1 M pH 4.0 citrate buffer (1 : 1), cytotetrins A, B and C (I) were separated from the D and E (II) components. In a similar distribution between *n*-butanol and pH 5.5 or 7.7 buffer system the single components of I and II could be separated.

II. Physicochemical Properties

1. Cytotetrin complex

The cytotetrin complex represents an orange red amorphous powder. It melts with decomposition between 140~200°C. It is soluble in lower alcohols (methanol, ethanol, butanols), ketones (acetone, methyl-ethyl ketone), chlorinated solvents (chloroform, dichlorethane), esters (ethyl-acetate, butyl-acetate), dioxane and diluted acids, less soluble in benzene, toluene, diethylether, and practically insoluble in hexane, petroleum ether and water.

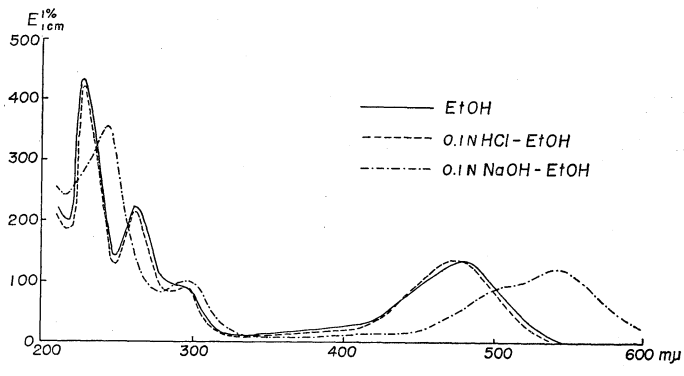
The optical rotation was found to be $[\alpha]_D^{25}$ +212° (*c* 0.1, methanol), and +136° (*c* 0.1, chloroform).

The ultraviolet and visible absorption spectra in ethanol, 0.1 N ethanolic hydrochloric acid and 0.1 N ethanolic sodium hydroxide are shown in Fig. 2. Maximum absorptions were found at 235 nm ($E^{1\%}_{1cm}$ 430), 261 nm ($E^{1\%}_{1cm}$ 218), 475~480 nm ($E^{1\%}_{1cm}$ 135) with a shoulder at 292 nm ($E^{1\%}_{1cm}$ 88) in ethanol; at 236 nm ($E^{1\%}_{1cm}$ 416), 262 nm ($E^{1\%}_{1cm}$ 206), 294 nm ($E^{1\%}_{1cm}$ 86), 470~473 nm ($E^{1\%}_{1cm}$ 132) in 0.1 N ethanolic hydrochloric acid, and at 241 nm ($E^{1\%}_{1cm}$ 355), 296 nm ($E^{1\%}_{1cm}$ 98), and 542 nm ($E^{1\%}_{1cm}$ 118) in 0.1 N ethanol sodium hydroxide.

The results of elementary analyses were as follows: C 58.13, 57.92; H 7.02, 7.09; N 3.36, 3.42 (per cent).

Cytotetrin is quite stable in 50 % aqueous methanolic solution between pH 3~11 at 25°C. In solution the cytotetrin is rapidly destroyed by ultraviolet light.

Fig. 2. Ultraviolet and visible absorption spectra of cytotetrin complex.



2. Cytotetrins A, B, C, D and E

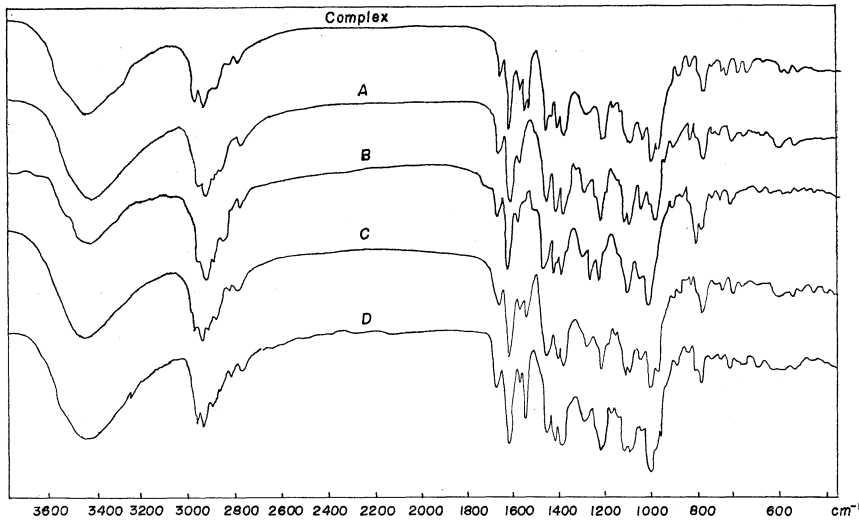
All the single components of the cytotetrin com-

Table 1. Properties of cytotetrins

		Cytotetrins				
		A	B	C	D	E
Melting point ℃		212~214	216~217	196~197	166~168	160~165 (d)
Optical rotation $[\alpha]_D^{25}$ c 0.1						
Methanol		+207	+245	+235	+292	+215
Ethanol		+127	+155	+143	+246	+102
Chloroform		+138	+162	+139	+262	+130
λ_{max}, nm ($E_{1cm}^{1\%}$) in ethanol		235 (460)	235 (472)	235 (432)	235 (426)	235 (420)
		261 (236)	261 (250)	262 (253)	261 (228)	260 (208)
		479 (138)	478 (150)	477 (139)	479 (128)	478 (121)
		288 (95)	288 (104)	286 (97)	295 (97)	290 (90)
$\lambda_{infl.}$						
Rf*	System I	0.65	0.42	0.35	0.20	0.10
	〃 II	0.55	0.35	0.22	0.16	0.07
	〃 III	0.25	0.13	0.10	0.07	0.04

* System I. Paper chromatography, Ederol 208 paper impregnated with pH 6 phosphate buffer, developing, solvent : *n*-butanol, saturated with pH 6.0 phosphate buffer.
System II. Thin-layer chromotography on Kieselgel-G ; developing solvent : *n*-butanol - acetic acid - water (3 : 2 : 2).
System III. Thin-layer chromatography on Kieselgel-G ; developing solvent : *n*-butanol - acetic acid - water (8 : 3 : 3).

Fig. 3. Infrared absorption spectra of cytotetrins (KBr).



plex have very similar physicochemical properties. Their solubility, the ultraviolet, visible and infrared spectra are practically identical, but cytotetrins D and E are somewhat less soluble and their infrared spectra slightly different (in the region of 1550 cm^{-1}) from the A, B, and C components. All cytotetrins (except E) were obtained as orange red crystalline substances. The physicochemical characteristics are shown in Table 1.

Fig. 3 shows the infrared spectra of cytotetrins.

The elementary analyses of cytotetrins are shown in Table 2.

For cytotetrin A the analytical data indicate the molecular formula $C_{38}H_{52}N_2O_{16}$ (calc.: C 57.59, H 6.56, N 3.54, O 32.31, MW 792), although slight deviations from this

formula are not excluded.

Experiments on the determination of the chemical structure of cytotetrin A are in progress.

III. Biological Properties

The cytotetrins exhibit antimicrobial activity against Gram-positive bacteria, and no or less activity against Gram-negative bacteria. The *in vitro* results, obtained by the serial tube-dilution method are summarized in Table 3.

The most active are the A, B and C components, the E and D components are less active.

Acute toxicity studies with CBA mice showed the intravenous toxicity values as follows: LD_{50}, i. v. in mg/kg 0.9, 1.2, 1.2, 1.9, 6.0 and 5.0 for the cytotetrin complex and the A, B, C, D and E components respectively.

The cytotetrins (especially A, B and C) exhibit a weak *in vivo* inhibitory activity against YOSHIDA subcutaneous sarcoma and S-180 i. m. sarcoma. In cell cultures the components (5 mg/ml) have a

Table 2. Elementary analysis of cytotetrins*

%	A	B	C	D	E
C	57.71	57.83	58.16	57.65	57.03
H	6.60	6.92	7.10	7.30	7.32
N	3.49	3.42	3.32	4.03	3.95
O (diff.)	32.10	31.83	31.42	31.00	31.70
C-CH_3	8.8	8.1	8.2	8.9	—
O-CH_3	5.0	3.2	1.3	0	2.2
-OH	14.0	15.2	—	—	—
Molecular weight (RAST)	823~916	—	1,584 ~1,646	660~695	—
Equivalent weight**	782				

* The results are averages of about 3~6 determlnations.
** By potentiometric titration.

Table 3. Minimum inhibitory concentration of cytotetrins (mcg/ml)

Test organisms	Complex	A	B	C	D	E
Staphylococcus aureus 53	2.5	10.0	1.2	1.2	10.0	0.6
S. aureus 80/81	0.6	0.3	0.6	1.25	2.5	2.5
S. aureus 1115	2.5	1.2	1.2	0.6	5.0	2.5
B. subtilis ATCC-6633	0.6	1.2	0.3	0.3	0.6	2.5
Sarcina subflava	0.3	0.3	0.16	0.3	5.0	0.6
Streptococcus faecalis	1.2	5.0	0.6	2.5	10.0	1.2
Pseudomonas aeruginosa 91085	100	100	100	100	100	100
E. coli 0-111	50	50	50	100	100	100
〃 4R	50	25	50	50	100	100
〃 6R	100	50	100	100	100	100
Shigella sonnei	100	50	50	50	100	100

Assay: Two-fold dilution method in peptone and heart infusion broth.

Table 4. Antitumor activity of cytotetrin A

Tumor	Dose mg/kg	Application No.	Application route	Inhibition %
YOSHIDA s. c. sarcoma	0.3	5	i. v.	43
S-180 i. m. sarcoma	0.15	4	i. p.	39
WALKER s. c. carcinoma	0.1	5	i. v.	16
NK/Ly lymphoma	0.1	4	i. p.	0
				Survival time %
YOSHIDA ascites sarcoma	0.15 0.3	5 2	i. p. i. p.	15 toxic
EHRLICH ascites carcinoma	0.2	5	i. p.	0
NK/Ly lymphoma	0.2	3	i. v.	12
Shay chloroleucemia	0.3	3	i. p.	10
LA-1210	0.2	3	i. v.	7

WALKER s.c. carcinosarcoma was implanted by the trocar method. All other tumors were implanted with ascitic fluid containing 10^7 tumor cells in a volume of 0.2 ml, 24 hours before treatment. Cytotetrin administered once a day to a group of 10 random bred Swiss albino mice, or C. B. Wistar rats respectively.
Inhibition %: Average tumor weight of treated /no treated (solid tumors).

strong *in vitro* cytotoxic effect against YOSHIDA sarcoma cells. Data on the antitumor activity of cytotetrin A are given in Table 4.

Discussion

Based on its characteristics, cytotetrin belongs to the anthracycline series[1]. The ultraviolet and visible spectra of cytotetrin show that it contains a 1,4-dihydroxy-anthraquinone chromophore, which is similar to those of nogalamycin[2,3], ruticulomycin[4] and antibiotic A-195[5]. Reticulomycin A has been proved to be identical with nogalamycin[2]. Furthermore, from data put together in Table 5, especially from the infrared spectra, it seems that the antibiotic A-195 is also identical with nogalamycin. Cytotetrin can easily be distinguished from other anthracycline antibiotics by spectroscopic properties. Despite certain similarities concerning the chromophores, the ultraviolet and visible spectra, the cytotetrins are definitely different from nogalamycin, ruticulomycin and antibiotic A-195 in regard to elementary analyses, optical rotations, melting points, infrared spectra (lack of 1740 cm^{-1} band), and some other properties. These differences are summarized in Table 5.

Therefore, cytotetrin was recognized to be a new, hitherto unknown antibiotic.

Table 5. Differences between cytotetrins and nogalamycins

Properties	Cytotetrins	Nogalamycin	Ruticulomycin		A-195
			A	B	
Melting point °C	A, B >210 C 196~197 D, E 170	195~196	183~184	179~180	194~196 (d)
$[\alpha]_D^{25}$ (chl)	+130~262°	+479°	—	—	—
N %	3.3~4.0	1.97	1.80	1.84	1.71
Band in i.r. at 1740 cm^{-1} (ester)	no	yes	yes	yes	yes
λ_{max} in alkaline ethanol	542	553	—	—	—

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