A NEW POLYENE ANTIBIOTIC, FLAVOMYCOIN STRUCTURAL INVESTIGATIONS. I

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Flavomycoin is a polyene antibiotic isolated from the mycelium of *Streptomyces roseoflavus* ARAI 1951 var. *jenensis* nov. var. JA 5068 as yellow-green crystals. It shows inhibitive activities against yeasts and fungi as well as protozoa. It is optically active, exhibits an ultraviolet spectrum without fine structure with maxima at 363 nm and 263 nm. Analytical studies indicated that the molecular formula of the antibiotic is $C_{41}H_{68}O_{10}$ and the molecular weight 721. These results were confirmed by degradation to the parent hydrocarbon and by catalytic hydrogenation. As flavomycoin is different from the related substances mycoticin and flavofungin, it must be considered as a new antibiotic.

During the course of a screening program for antifungal antibiotics a new antibiotic, named flavomycoin, was obtained from the mycelium of *Streptomyces roseoflavus* ARAI 1951 var. *jenensis* nov. var. JA 5068[1]. Flavomycoin is active against pathogenic and nonpathogenic yeasts and fungi as well as protozoa. The antibiotic was isolated by extraction of the mycelia with organic solvents. After purification it was obtained as a yellow-green crystalline substance. Flavomycoin belongs to a new group of polyene antibiotics characterized by conjugation of the polyene chromophore with the lactone group of the macrolide ring[2]. The present paper deals with the physical and chemical properties of flavomycoin and some chemical investigations on its structure.

Physical and Chemical Properties of Flavomycoin

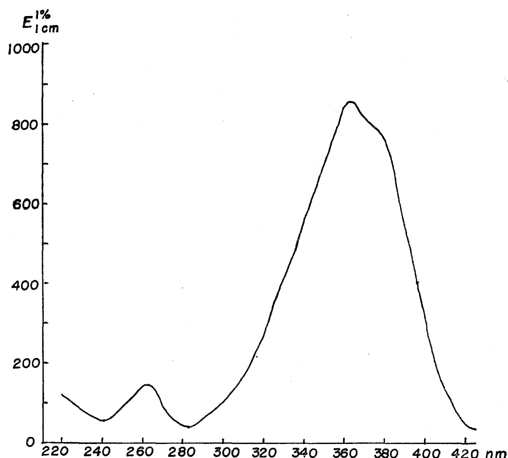
Flavomycoin crystallizes from 80 % methanol or ethanol as yellow-green needles which associate to bunches. The melting point is 161~163°C with decomposition. Flavomycoin is freely soluble in pyridine, glacial acetic acid, water-saturated butanol and dimethylformamide, moderately soluble in aqueous aliphatic alcohols, aqueous ethyl acetate and acetone, but insoluble in water, benzene, chloroform and petroleum ether.

The optical rotation of the antibiotic is the following: $[\alpha]_D^{23}=-4°$ (*c* 4, pyridine), $[\alpha]_D^{23}=-45°$ (*c* 1, 95 % dioxane), $[\alpha]_D^{25}=-60°$ (*c* 3, dimethylformamide), $[\alpha]_D^{27}=-53°$ (*c* 1.1, 95 % acetone), $[\alpha]_D^{25}=+109°$ (*c* 4, acetic acid), $[\alpha]_D^{23}=-16°$ (*c* 1, methanol).

With concentrated sulfuric acid flavomycoin affords a redviolet color. It decolorizes potassium permanganate and bromine solutions, but gives negative reactions to TOLLENS, MOLISCH, ninhydrin, ferric chloride and iodoform tests.

Flavomycoin does not contain nitrogen, phosphorus, sulfur or halogen. The results of elementary analysis of flavomycoin were as follows: C 64.49, H 9.35, O 26.16 % (difference). The determination of water according to KARL FISCHER showed that the crystalline substance contains 4~5 % water. From these analytical data one can calculate the following empirical formula for flavomycoin $C_{41}H_{68}O_{10} \cdot 2H_2O$.

Fig. 1. Ultraviolet absorption spectrum of flavomycoin in methanol.



The ultraviolet absorption spectrum of flavomycoin in methanol shows maxima at 363 nm ($E^{1\%}_{1cm}$ 860, lg ε 4,792), at 262 nm ($E^{1\%}_{1cm}$ 150, lg ε 4,033) and a shoulder at 377 nm ($E^{1\%}_{1cm}$ 790, lg ε 4,756), as shown in Fig. 1.

The infrared absorption spectrum in KBr tablet (Fig. 2) shows characteristic bands at 1705 cm^{-1} corresponding either to a carbonyl group or an unsaturated ester and lactone group; 1585 cm^{-1}, 1630 cm^{-1} and 3020 cm^{-1} to double bonds; 2880 cm^{-1} and 1940 cm^{-1} to methyl and methylene groups; 3400 cm^{-1} to hydroxyl functions. The band at 1100 cm^{-1} showing the highest extinction in the spectrum is attributed to a C-O valency-vibration of an ester or lactone group.

Stability: Flavomycoin is unstable in the solid state when exposed to air and light. A crystalline sample of flavomycoin placed in an aerated petri dish during 60

Fig. 2. Infrared absorption spectrum of flavomycoin (KBr).

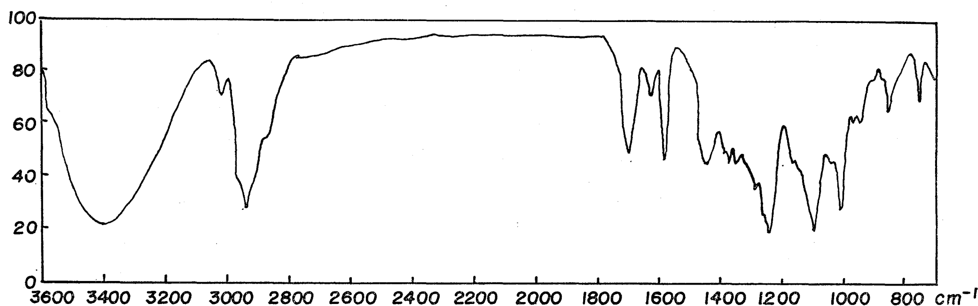


Fig. 3. Infrared absorption spectrum of perhydro-flavomycoin (KBr).

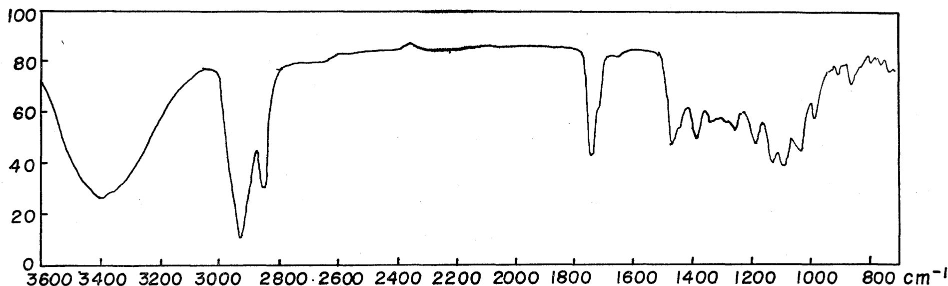


Fig. 4. Change of ultraviolet absorption of flavomycoin exposed to air.

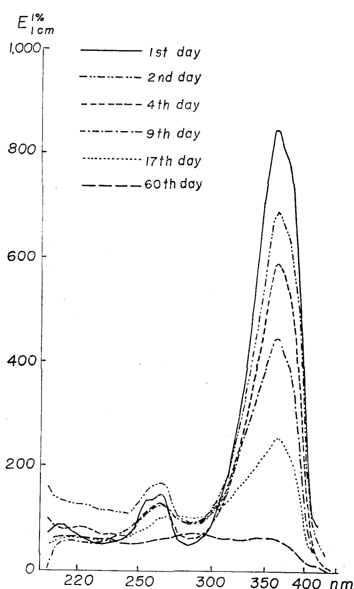
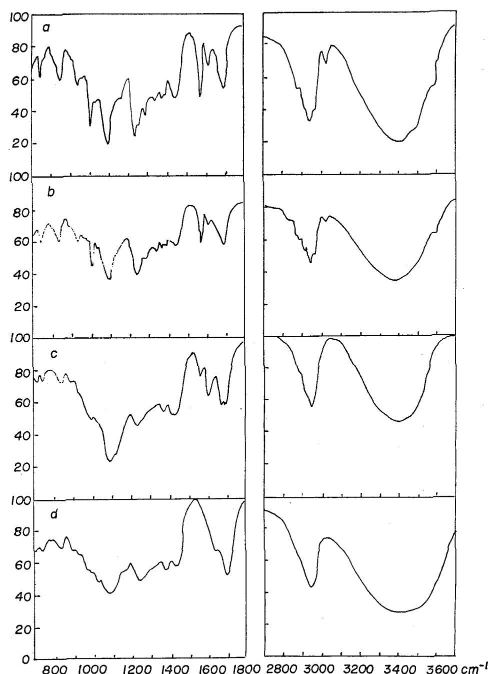


Fig. 5. Change of infrared absorption of flavomycoin exposed to air.
a=1 st day b=4 th day
c=17 th day d=60 th day



days, gave the following modifications in its ultraviolet and infrared spectra (Figs. 4 and 5). In the ultraviolet-range the maximum at 363 nm completely disappears, and in the infrared region the bands of the double bonds strongly decrease in intensity. The chemical destruction is accompanied by the loss of biological activity. Therefore, flavomycoin must be preserved in an inert gas atmosphere and in darkness. In aqueous solutions flavomycoin is readily destroyed at pH-values below 6 and above 10, but it is relatively stable in organic solvents.

Determination of the Molecular Weight of Flavomycoin

Because of the small stability and the existence of crystallization water in the molecule it was very difficult to determine the molecular weight with conventional methods. Therefore, the mass spectrometry was applied to estimate the molecular weight of flavomycoin. Fig. 6 shows the negative ion sprectrum of flavomycoin which was taken by using of low energy (2~4 eV)[3,4]. The highest peak at m/e 720 is identical with the molecular ion peak, while the next peaks are caused by cleavage of at least six moles H_2O from the molecule (720=M, 702=M-H_2O, 684=702-H_2O, 666=684-H_2O, 648=666-H_2O, 630=648-H_2O, 612=630-H_2O.

These results are confirmed by the mass spectrum of perhydro-flavomycoin, too (Fig. 7). The parent peak at m/e 729 arised from fragmentation of a H atom, so that the mass number of the molecular ion is 730. In comparison with the molecule peak of flavomycoin this means an uptake of 5 moles hydrogen per molecule which is

Fig. 6. Mass spectrum of flavomycoin (negative ion spectrum).

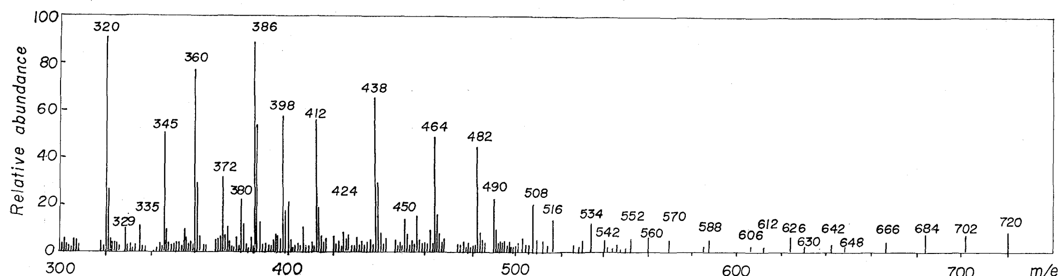
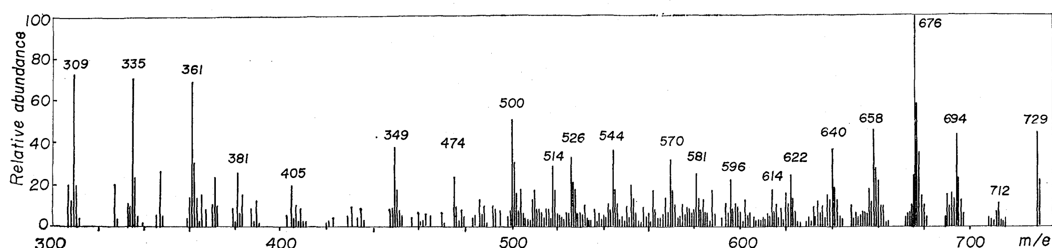


Fig. 7. Mass spectrum of perhydro-flavomycoin (negative ion spectrum).



corresponding to the values obtained by catalytic hydrogenation. Furthermore the cleavage of 6 mole H_2O can also be observed (729=M-1, 712=730-H_2O, 694=712-H_2O, 676=694-H_2O, 658=676-H_2O, 640=658-H_2O, 622=640-H_2O).

Comparing the mass numbers in the spectra of both compounds it is remarkable that the difference of 10 mass units appears at the molecular peaks as well as at the most of the following peaks. The results of the mass spectrometry and the quantitative elementary analysis indicated the chemical molecular weight of flavomycoin to be 721 and the molecular formula to be $C_{41}H_{68}O_{10}$.

A further proof for the size of the molecule afforded the degradation to the parent hydrocarbon according to COPE *et al.*[5] Flavomycoin was hydrogenated and the perhydro-flavomycoin reduced with lithium aluminum hydride in refluxing tetrahydrofuran to a polyol which was further treated with red phosphorus in refluxing 48 % hydriodic acid. The hydroxyl-free but iodine-containing product was reduced with lithium aluminum hydride and finally hydrogenated with ADAMS catalyst. The chromatographic purification of the hydrocarbon fraction on alumina yielded a colorless oil in an overall yield of 5 %. Its infrared spectrum was characteristic of a saturated hydrocarbon and its molecular weight was 576 as determined by negative ion mass spectrometry. Thus the molecular formula of the hydrocarbon is $C_{41}H_{84}$. This exactly confirms the former results of molecular weight determination.

Other Reactions

Flavomycoin was hydrogenated in glacial acetic acid with a platinum catalyst at atmospheric pressure. The uptake of hydrogen was five moles based on a molecular weight of 721. Perhydro-flavomycoin is a colorless substance without biological activity and shows only end absorption in the ultraviolet region. It can be obtained as needles by recrystallization from methanol-water mixtures. The infrared spectrum

is given in Fig. 3.

Perhydro-flavomycoin was acetylated using acetic anhydride and pyridine at room temperature. The product purified by chromatography showed an acetyl content of 28.9 % corresponding to seven hydroxyl groups. The infrared spectrum of the acetate and the test according to ZEREWITINOFF, however, suggest that a further hydroxyl group is present which cannot be acetylated. The presence of vicinal hydroxyl groups can be excluded because at the quantitative periodate oxidation of flavomycoin and perhydro-flavomycoin respectively less than 0.1 mole periodate was consumed.

Quantitative saponification of perhydro-flavomycoin required long refluxing in 3 % aqueous alcoholic potassium hydroxide. It consumed 0.95 mole alkali. During saponification no volatile product could be detected by 3,5-dinitrobenzoyl-chloride. Therefore, short chain alcohol as ester component can be excluded. The ZEISEL test was negative, too. The results and the proved size of the

Fig. 8. Comparison of flavomycoin and flavofungin in infrared spectrum (finger print region).

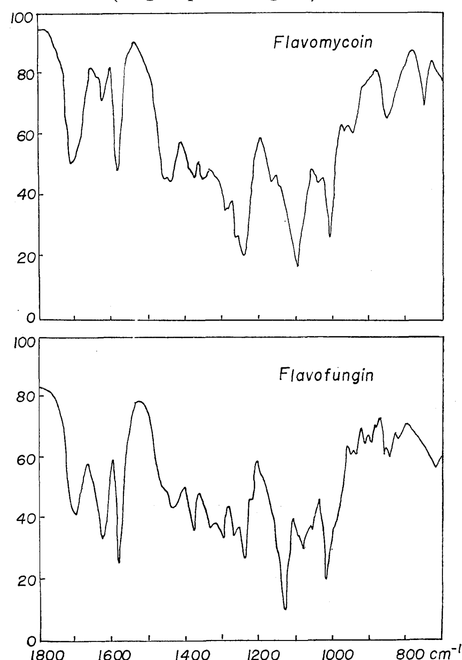


Fig. 9. Comparative paper-gram of flavomycoin with flavofungin.

Solvent system: chloroform - tetrahydrofuran - formamide (50 : 50 : 5)
Paper: Schleicher & Schüll 2043 b, 11 × 28 cm, ascending, 2.5 hours, 7°C.
Test organism: *Penicillium notatum* P 36

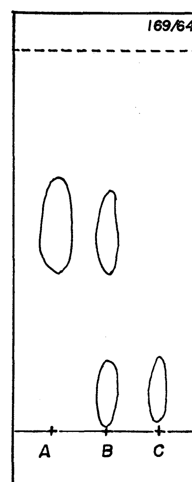


Table 1. Comparison of physico- chemical properties of flavomycoin, flavofungin and mycoticin

	Flavomycoin	Flavofungin	Mycoticin
UV-absorption maxima	263 nm ($E^{1\%}_{1cm}$ 150) 363 nm ($E^{1\%}_{1cm}$ 860)	263 nm ($E^{1\%}_{1cm}$ 126) 366 nm ($E^{1\%}_{1cm}$ 869)	262 nm ($E^{1\%}_{1cm}$ 79) 364 nm ($E^{1\%}_{1cm}$ 948)
IR-absorption, charact. band	1100 cm^{-1}	1130 cm^{-1}	1130 cm^{-1}
Specific rotation $[\alpha]^D$	−4° (4, pyridine) −45° (1, dioxane) −16° (1, methanol)	−93° (4, pyridine) −76° (0.2, methanol)	+63° (0.4, dioxane)
Melting point	161～163° (dec.)	210° (dec.)	221～222°
H_2-uptake	5 moles	6 moles	6 moles
Acetylation	heptaacetate	octaacetate	octaacetate
Deacetylation	impossible	possible	possible
$C-CH_3$	3	3～4	3～4
Mol. weight	721	A 650 B 664	A 650 B 664
Formula	$C_{41}H_{68}O_{10}$	A $C_{36}H_{58}O_{10}$ B $C_{37}H_{60}O_{10}$	A $C_{36}H_{58}O_{10}$ B $C_{37}H_{60}O_{10}$

parent hydrocarbon point to a lactone group in flavomycoin.

Comparison of Flavomycoin with Related Antibiotics

Flavomycoin was compared with the antifungal antibiotics mycoticin[6] and flavofungin[7,8] which show similar ultraviolet spectra (Table 1). Flavomycoin may be distinguished from flavofungin and mycoticin by its optical rotation, the infrared absorption, the melting point, the uptake of hydrogen, the molecular weight and the impossibility of reconverting the flavomycoin acetate to the original antibiotic by alkaline hydrolysis. The characteristic difference between flavomycoin and flavofungin in the infrared absorption at 1100 cm^{-1} and 1130 cm^{-1} respectively is shown in Fig. 8. The complete infrared spectrum of mycoticin is not published up to now. According to BOGNAR *et al.*[7] flavofungin and mycoticin are very similar substances. Both antibiotics are mixtures of the same two compounds in different proportions. A direct comparison of flavomycoin and flavofungin using paper chromatography is shown in Fig. 9. The Rf values in different solvent systems are summarized in Table 2.

Table 2. Paper chromatography of flavomycoin and flavofungin, Rf values in different solvent systems

Solvent system	Rf values	
	Flavomycoin	Flavofungin
I. Methanol - water-ammonia (80 : 16 : 4)	0.90	0.79
II. *n*-Butanol - methanol - water (4 : 1 : 2)	0.88	0.85
III. *n*-Propanol - acetic acid - water (60 : 4 : 4)	0.94	0.95
IV. Dimethylformamide - water (50 : 50)	0.77	0.72
V. Pyridine - butanol - water (4 : 6 : 5)	0.86	0.89
VI. Dimethylformamide - water - glacial acetic acid (50 : 45 : 5)	0.84	0.81
VII. Chloroform - tetrahydrofuran - formamide (50 : 50 : 5)	0.55	0.12
VIII. Chloroform - methylethylketone - tetrahydrofuran - formamide (60 : 20 : 20 : 4)	0.42	0.01
IX. Chloroform - methylethylketone - formamide (66 : 33 : 4)	0.33	0.02
X. Benzene - methylethylketone (50 : 50, formamide-saturated)	0.69	0.45
XI. Benzene - dioxane (50 : 50, formamide-saturated)	0.21	0.07

Conclusions

By reason of the physico- chemical properties and paper chromatographic investigations, flavomycoin is different from other similar substances. Therefore, it must be considered as a new antibiotic.

Experimental

Flavomycoin

A sample of the purified antibiotic was crystallized from 80 % methanol as needles, m.p. 161~163°C (dec.). The substance was dried at 22°C (0.1 mmHg) and immediately submitted for analysis.

Anal. Calcd. for $C_{41}H_{68}O_{10}\cdot 2H_2O$: C 65.05, H 9.58, H_2O 4.75 %
Found: C 64.49, H 9.35, H_2O 4.23 %

Perhydro-flavomycoin

A solution of 1.075 g flavomycoin was hydrogenated with 83 mg of platinum oxide in 50 ml of glacial acetic acid for 5 hours at room temperature. The catalyst was removed

by filtration, the solvent by freeze-drying. Two recrystallizations of the residue from a mixture of methanol - water (7 : 3) yielded 880 mg of colorless, fine needles, m.p. 80~82°C. $[\alpha]_D^{25}$ −11° (*c* 4, pyridine). For analysis the substance was dried *in vacuo* (0.1 mmHg) at room temperature over phosphorus pentoxide.

Anal. Calcd. for $C_{41}H_{78}O_{10}\cdot 2H_2O$: C 64.36, H 10.54, H_2O 4.7 %
Found : C 64.79, H 10.20, H_2O 3.9~4.5 %

Acetylation of perhydro-flavomycoin

Perhydro-flavomycoin (500 mg) was dissolved in 15 ml of acetic anhydride and 10 ml of pyridine. The mixture was allowed to stand for 48 hours at room temperature and then concentrated to 10 ml *in vacuo*. After 150 ml of ice-water was added, the mixture was allowed to stand overnight at room temperature and then extracted with chloroform three times. The combined chloroform extracts were washed with water, dried with anhydrous sodium sulfate, concentrated to about 5 ml, and chromatographed on alumina (neutral, activity II). The chloroform eluate was evaporated under reduced pressure to yield 190 mg of colorless glass, m.p. 60~62°C.

Anal. Calcd. for $C_{55}H_{92}O_{17}$: C 64.43, H 9.04, $7\times COCH_3$ 29.35 %
Found : C 64.18, H 8.96 $COCH_3$ 28.95 %

Quantitative saponification of perhydro-flavomycoin

Perhydro-flavomycoin (0.206 g) was dissolved in 20 ml of 70 % ethanol and 20 ml of 1 N ethanolic KOH. After refluxing for 4 hours the alkaline solution was titrated with 1 N HCl using a potentiometer. The consumption of 2.69 ml 1 N HCl corresponds to 0.95 mole KOH per mole perhydro-flavomycoin.

Conversion of perhydro-flavomycoin to parent hydrocarbon

A solution of 8.0 g of perhydro-flavomycoin in 400 ml of tetrahydrofuran was heated under reflux for 40 hours with an excess of lithium aluminum hydride. The unchanged hydride was destroyed with ethyl acetate and the mixture evaporated to dryness. The inorganic material was dissolved in dilute sulfuric acid and the polyol extracted with *n*-butanol. The combined extracts were washed with water and the butanol was removed *in vacuo*. The oily residue was dissolved in a little *t*-butanol and freeze dried to yield 7.1 g of white amorphous powder.

Seven grams of polyol were dissolved in 100 ml of glacial acetic acid and added to a refluxing suspension of 420 ml of 48 % hydriodic acid and 10 g red phosphorus. After 24 hours 170 ml hydriodic acid were removed by distillation, 350 ml of water added and the mixture was extracted with 100 ml of chloroform thrice. The extracts were washed with 2 % sodium thiosulfate solution, then with water, and dried over anhydrous sodium sulfate. Removal of solvent gave an oil (3.25 g) which was dissolved in tetrahydrofuran and refluxed for 10 hours with 3 g of lithium aluminum hydride. The unchanged hydride was destroyed with ethyl acetate. After evaporation of the solvent, the inorganic material was dissolved in 10 % sulfuric acid and organic material extracted with hexane. The hexane solution, after being washed with 5 % sodium carbonate solution and dried over sodium carbonate, was concentrated and hydrogenated using platinum oxide as catalyst. The catalyst was separated and the concentrated solution chromatographed on a column of neutral alumina (activity II). The first fractions eluted with hexane were combined and yielded 390 mg of hydrocarbon.

Paper chromatography of flavomycoin

Method: Ascending chromatography on Schleicher and Schüll 2043b paper. Temperature: 7°C. For the solvent systems VII~XI (Table 2) the paper sheets were impregnated with 20 % solution of formamide in acetone for 5 minutes. Then the sheets were pressed between filter paper and after air drying the solutions of antibiotics were applied.

Acknowledgements

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