Paper Chromatography of Antifungal Antibiotics

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Paper chromatography is very helpful in the identification and comparison of antibiotics, but very few results from the application of this technique have been published so far. The awakening of interest in the search for antibiotics which are active against fungi pathogenic to plants and animals makes the investigator aware of the scant information that is available to help characterize such agents. A knowledge of the R_f values of the known antifungal agents would help advance the search for such therapeutic materials. Often these values, in conjunction with the biological inhibitory activities, are the only criteria by which one can determine whether the unknown agent appears to be a new material. When the R_f data are obtained for shake cultures, one can eliminate many previously described antibiotic-producing organisms from further study, thus allowing more research to be applied to antibiotics which are new and different. The studies reported in this paper should serve as a guide to the identification of some of the known antifungal antibiotics.

MATERIALS AND METHODS

The one-dimensional, ascending paper chromatogram technique was used. The paper strips (18 x 35 cm) were cut from large sheets of Whatman filter paper No. 1 (47 x 57 cm) by dividing the long dimension into widths of 18 cm and then cutting these sections to a length of 35 cm. Solutions of the antibiotic were placed 3 cm from the lower edge of the strip with a micropipette in 5-lambda (0.005-ml) portions until the desired quantity of substance had been added. The amounts placed on the paper varied with the sensitivity

TABLE 1. *Details of Pyrex dish technique*

Test Organism	Antibiotics	Agar	Incubation Time	Incubation Temp	Spore Suspension
			hr	*C*	
Penicillium oxalicum 99*	Filipin Nystatin Fradicin Ascosin Thiolutin Rimocidin sulfate Trichomycin	Trypticase soy agar†	18–24	26	5 ml with a light transmission of 50% in 120 ml agar
Glomerella cingulata 100‡	Antimycin A	Trypticase soy agar†	40	26	5 ml with a light transmission of 50% in 120 ml agar
Bacillus subtilis Cohn 23	Actinomycin Gliotoxin Nigericin Streptothricin sulfate	Emerson's agar§	18	30	400,000 spores per ml agar
Candida albicans 40	Candicidin A Endomycin	Emerson's agar	18	30	1 ml 1-day-old Emerson's broth culture in 120 ml agar
Saccharomyces sp. 45¶	Cycloheximide	Emerson's agar	18–24	30	1 ml 1-day-old Emerson's broth culture in 120 ml agar

* Numbers for test organisms correspond to numbers used in the collection of D. Gottlieb, Horticulture Field Laboratory, University of Illinois, Urbana, Illinois.

† Baltimore Biological Laboratories, Baltimore, Md.

‡ To prevent bacterial contamination 100 μg/ml Chloramphenicol were included in the agar.

§ Yeast extract, 1 g; bactopeptone, 4 g; beef extract, 4 g; NaCl, 2.5 g; cerelose, 10 g; agar, 15 to 20 g; distilled water, 1000 ml

¶ Probably *S. pastorianus*.

of the test organism, the solvent for the antibiotic, and the solvent system used for developing the chromatogram. Four materials were tested on each strip. Eight hundred ml of solvent mixture were placed in the bottom of cylindrical glass jars (25 x 45 cm). After the spots of solution on the paper strip had dried, the sheet was suspended in the jar so that approximately 1 cm dipped into the solvent system. The papers were then developed at 26 C until the solvent front had moved up about 27 cm. The strips were then air-dried and placed on seeded assay plates for 10 to 15 minutes.

TABLE 2. *Solvent systems and their development time*

Number	Solvent Systems	Development Time
		hr
I	Water saturated *n*-Butanol	15–16
II	*n*-Butanol:acetic acid:H_2O (2:1:1)	15–16
III	*n*-Butanol:pyridine:H_2O (1:0.6:1)	15–16
IV	3% Ammonium chloride	2–3
V	50% hydrous acetone	6–7
VI	Benzene:acetic acid:H_2O (2:2:1) (organic layer)	7–10
VII	*n*-Butanol saturated water	8–10

The position of the antibiotic on the paper was determined in the following manner: The Pyrex dish technique (Peterson and Reineke, 1950) was used and 120 ml of a seeded agar particularly suited to each organism were placed in the sterile dish. All plates were prepared immediately before putting the paper strip on the seeded agar. After incubation, clear zones occurred wherever the antibiotic had diffused from the paper into the agar. The distance that the solvent system had moved in the same time was measured on the original

TABLE 3. *Amount of antibiotics used on paper strips*

Antibiotic	Purity	Concentration and Solvent of Stock Solution	Minimum and Maximum Amounts in μg Brought on Paper Strips for Solvent System No.:*						
			I	II	III	IV	V	VI	VII
Actinomycin	Crystals	1 mg/ml in acetone	2–3	2–3	2–3	2–50	2–3	2–50	2–10
		100 μg/ml in acetone							0.2–0.5
Antimycin A	Crystals	1 mg/ml in methanol						5–100	
		100 μg/ml in methanol	0.2–0.25	0.3	0.3	0.3			
		10 μg/ml in methanol					0.05		0.1
Ascosin	Crude	1 mg/ml in methanol	75	25	25–75	75	25–75	75	
Candicidin A	Crude, 6000 u/mg	1 mg/ml in 50% ethanol	100–150	100	300	400	75–100	150	
Cycloheximide	Crystals	100 μg/ml in distilled water	0.2–1	0.2–1	0.2–1	0.2–1	0.2–0.5	0.2–1	
Endomycin	Crude 1000 u/mg	1 mg/ml in methanol	200	200	200	200	200	200	
Filipin	Crude 500 u/mg	1 mg/ml in methanol	75–100	75–100	75	75	50–75	50–75	50–100
Fradicin	Crystals	1 mg/ml in ethylene dichloride	40–75	40–75	40–75	50–75	25–100	40–75	75–100
Gliotoxin	Crystals	1 mg/ml in acetone	15–50	15–50	15–50	10–15	15–50	15–50	
Nigericin	Crystals	1 mg/ml in methanol	25–50	25–50	25–50	25–50	5–25	50	
		100 μg/ml in methanol							1
Nystatin	Crude	400 μg/ml in methanol	30–40	30–40	40	20–40	20–40	40	
Rimocidin sulfate	Crystals	1 mg/ml in methanol	25	25–100	25–100	75–100	25–75	50–75	
Streptothricin sulfate	Crystals	1 mg/ml in 50% methanol	50	50	20–50	50	20	50	
Thiolutin	Crude, 660 μg/mg	1 mg/ml in methanol	50–75	50–75	50–75	25–75	75	75	25–35
Trichomycin	3200 u/mg	1 mg/ml in ethylene	5–50	5	2–5	5–25		10	
		100 μg/ml in ethylene					1–2		

* Composition of these systems is given in table 2.

TABLE 4. *R_f values* of 15 antibiotics*

Antibiotic	R_f Value in Solvent System No.:†						
	I	II	III	IV	V	VI	VII
With antifungal and antibacterial activity							
Actinomycin	0.96	0.94	0.96	0.00–0.21	0.96	0.99	0.53‡ or 0.70§
Endomycin	0.69	0.93	0.86	0.00	0.88	0.00	
Gliotoxin	0.90	0.95	0.96	0.54	0.88	0.98	
Nigericin	0.95	0.98	0.98	0.00	0.86	0.00 & 1.00	0.28
Streptothricin sulfate	0.00	0.13	0.09	0.94	0.18	0.00	
Thiolutin	0.83	0.90	0.93	0.28	0.77	1.00	0.48
With antifungal activity only							
Antimycin A	0.93	0.98	0.97	0.00	0.86	1.00	0.20
Cycloheximide	0.90	0.96	0.96	0.87	0.91	0.96	
Filipin	0.91	0.95	0.96	0.00	0.72	0.18	0.00
Fradicin	0.84	0.85	0.94	0.00	0.36–0.67	0.89	0.07
Ascosin	0.33	0.88	0.79	0.00	0.43–0.73	0.00	
Candicidin A	0.44	0.86	0.80	0.00	0.38–0.69	0.00	
Trichomycin	0.35	0.85	0.76	0.00	0.68	0.00	
Nystatin	0.25	0.76	0.73	?	0.82	0.00	
Rimocidin sulfate	0.39	0.84	0.77	?	0.91	0.00	

* Average of at least two determinations.
† Composition of these systems is given in table 2.
‡ With amounts of 2 to 10 μg.
§ With amounts of 0.2 to 0.5 μg.

paper strip. An R_f value was calculated as the ratio of movement of the antibiotic to that of the solvent front.

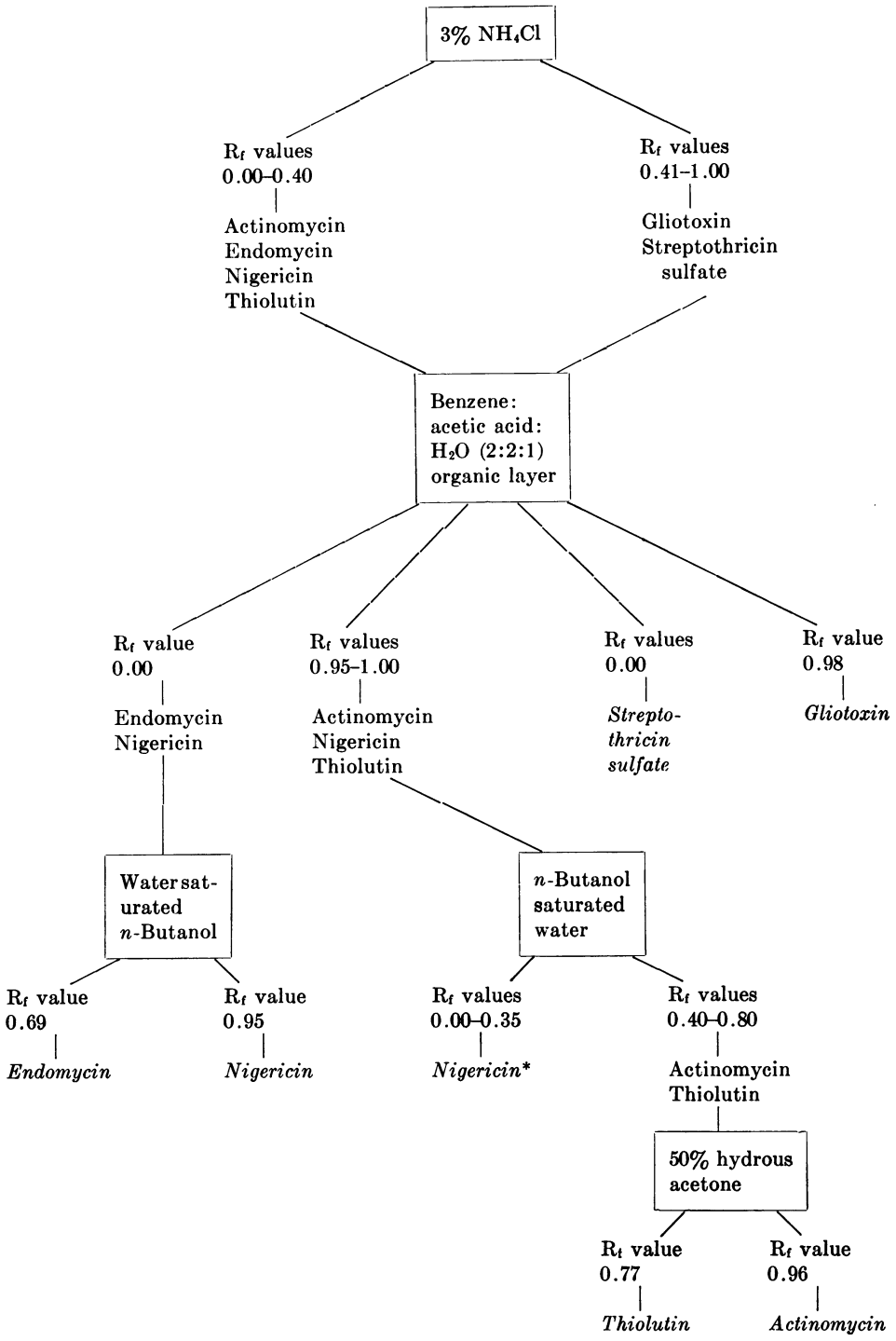
Seven solvent systems were used for 15 different antifungal agents, 6 of which also show antibacterial activity. These antibiotics were: actinomycin (Waksman and Tishler, 1942; obtained from Hoffmann-La Roche Inc., Nutley, New Jersey); antimycin A (Dunshee *et al.*, 1949; obtained from University of Wisconsin, Madison); ascosin (Hickey *et al.*, 1952; obtained from Commercial Solvents Corp., Terre Haute, Indiana); candicidin A (Lechevalier *et al.*, 1953; obtained from Rutgers University, New Brunswick, New Jersey); cycloheximide (Whiffen, 1948; obtained from Upjohn Co., Kalamazoo, Michigan); endomycin (Gottlieb *et al.*, 1951; obtained from Upjohn Co., Kalamazoo, Michigan); filipin (Gottlieb *et al.*; produced at the University of Illinois, Urbana, Illinois); fradicin (Swart *et al.*, 1950; obtained from Commerical Solvents Corp., Terre Haute, Indiana); gliotoxin (Brian and Hemming, 1945; obtained from the University of Illinois, Urbana, Illinois); nigericin (Harned *et al.*, 1951; obtained from Commercial Solvents Corp., Terre Haute, Indiana); nystatin (Hazen and Brown, 1951; obtained from E. R. Squibb & Son, New Brunswick, New Jersey); rimocidin sulfate (Davisson *et al.*, 1951; obtained from Chas. Pfizer & Co., Inc., Brooklyn, New York); streptothricin sulfate (Waksman and Woodruff, 1942; obtained from Upjohn Co., Kalamazoo, Michigan); thiolutin (Seneca *et al.*, 1952; obtained from Chas. Pfizer & Co., Inc., Brooklyn, New York); trichomycin (Hosoya *et al.*, 1952; obtained from National Institute of Health, Tokyo, Japan).

All further information concerning the test organisms, solvent systems, concentrations of different antibiotics, and so forth, is given in tables 1, 2, and 3.

RESULTS AND DISCUSSION

The R_f values of the 15 antibiotics are given in table 4. In a few cases certain solvent systems were unsatisfactory for specific antibiotics. For example, ascosin, candicidin A, and fradicin in 50 per cent acetone gave R_f values which varied over a rather wide range. Nystatin and rimocidin sulfate were inactive when placed in 3 per cent ammonium chloride. Nigericin, even though crystalline, always gave two spots in the benzene-acetic acid-water system.

The results in table 4 provided the information for preparing two flow sheets (figures 1 and 2), on which most of the 15 antibiotics can be separated on the basis of their R_f values. In some cases the differences are not very pronounced and other properties of the material must also be considered in order to determine the identity of the antibiotic. Separation of the ascosin-candicidin-trichomycin and nystatin-rimocidin groups (Okami *et al.*, 1954; Vining *et al.*, 1954) was very difficult with the solvent systems used in this study and ascosin could not be separated from trichomycin. Further research with other solvent systems will certainly lead to a better separation of these two groups and their members.



* In 50% hydrous acetone R_f value is 0.86.

FIG. 1. Flow sheet for the separation of six antibiotics with antifungal and antibacterial activity on the basis of their R_f values in five solvent systems.

In general, the paper chromatography technique has been helpful in establishing the identity of these antibiotics. But it is even more important that the information can be used to establish differences. This is especially necessary in the search for new antibiotics, for all other known materials must be "ruled out." Consistent differences among known materials and the unknown, when run under the same conditions, would indicate the presence of a new material. While paper chromatography is a useful technique, it is not a specific criterion, for it sometimes fails to differentiate some closely related antibiotics. It can best be used in combination with the other methods of differentiation available to the microbiologist and chemist.

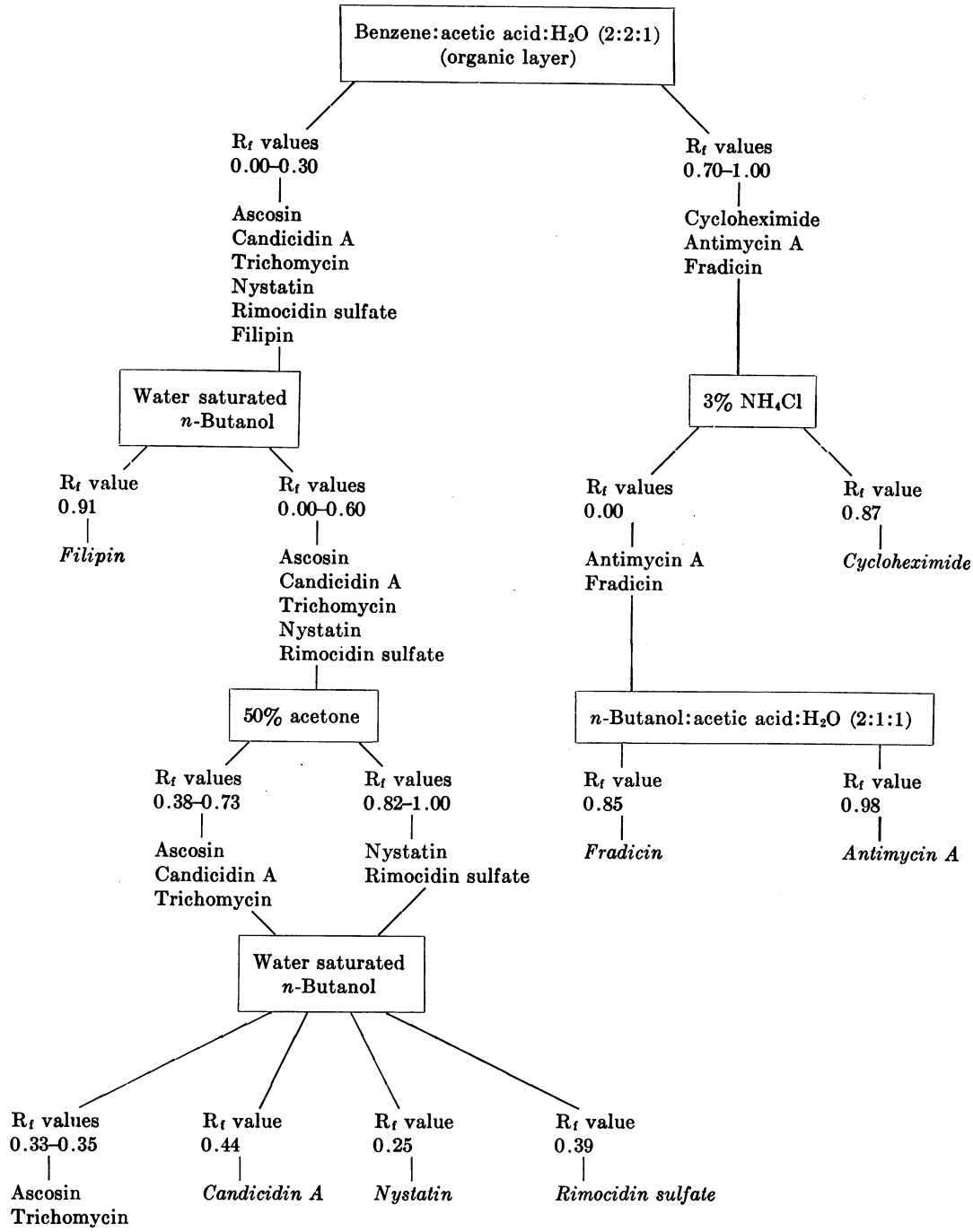


FIG. 2. Flow sheet for the separation of nine antibiotics with antifungal activity on the basis of their R_f values in five solvent systems.

SUMMARY

The R_f values of 15 antifungal antibiotics (6 of which show also antibacterial activity) in various solvent systems have been described. Flow sheets have been constructed for the separation of most of these agents on the basis of these data.

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Sorbic Acid as a Preservative for Sweet Cucumber Pickles[1]

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The preservation of sweet cucumber pickles may be accomplished by maintaining sugar and acetic acid concentrations at sufficient levels to inhibit all organisms. However, this is not possible in the manufacture of low Baumé, low acid sweet pickles, and therefore it is necessary either to pasteurize such products or to add sodium benzoate.

Recent work by Gooding (1945), Smith and Rollin (1954) and Deuel *et al.* (1954a, b) indicates that sorbic acid (2,4-hexadienoic acid) may be more efficient and less toxic as a food preservative than sodium benzoate. Melnick *et al.* (1954) found that sorbic acid would not prevent the growth of molds in cheese when the initial contamination was great. Therefore, it could not be used to preserve cheese prepared under unsanitary conditions. These studies have resulted in the tentative acceptance of this agent as a harmless food preservative by the U. S. Food and Drug Administration.

Phillips and Mundt (1950) and Jones and Harper (1952) reported that 0.1 per cent sorbic acid would prevent the growth of surface yeasts in cucumber fermentations. Emard and Vaughn (1952) found sorbic acid to inhibit many species of microorganisms, including yeasts, molds and bacteria. A concentration of 0.07 per cent at a pH of 5.0 to 5.5 was sufficient to inhibit the catalase positive organisms but allowed the catalase negative group to grow rapidly. The inhibitory action was related to the pH of the medium; as the pH decreased, the efficiency of inhibition increased.

Phillips and Mundt (1950) reported that the use of 0.1 per cent sorbic acid in cucumber fermentations had no adverse effect on the flavor of the finished pickles. Smith and Rollin (1954) noted that concentrations much larger than 0.1 per cent were necessary to be detected in the flavor of cheese.

Due to the apparent desirability of sorbic acid over sodium benzoate as a food preservative, a study was instigated to determine the efficiency of this compound in the preservation of sweet cucumber pickles. This paper presents the results of studies of the inhibitory effect of sorbic acid in combination with sucrose and acetic acid on yeasts from spoiled pickles and on lactobacilli. Experiments were conducted both in laboratory media and in sweet pickles.

Experimental Methods

The yeast cultures used in this study were three strains of "spoiled pickle yeasts": SPY-15, SPY-21 and SPY-29. They were isolated and described by Bell and

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