Synthesis and *In Vitro* Fungistatic Evaluation of Some *N*-Substituted Amides and Amine Salts of Sorbic Acid

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Abstract □ Four *N*-substituted amide derivatives of sorbic acid and two amine salts of sorbic acid were synthesized and tested for fungistatic activity against *Microsporum canis*, *Trichophyton mentagrophytes*, and *Candida albicans*. The compounds were tested by the disk-plate method initially and then followed by incorporation into polyethylene glycol ointment USP and tested on Sabouraud's dextrose agar culture plates. Results were compared with undecylenic acid and sorbic acid controls. The disk-plate method showed activity greater than sorbic acid, but less than undecylenic acid only for *N*-methylfurfurylsorbamide when tested against *Microsporum canis* and *Trichophyton mentagrophytes*. When incorporated into ointment, both *N*-methylfurfurylsorbamide and *t*-octylamine sorbate showed activity comparable to the controls against *Microsporum canis* and *Trichophyton mentagrophytes*. None of the new compounds were as effective against *Candida albicans* as were the controls.

Keyphrases □ Sorbic acid, *N*-substituted amide, amine salts—synthesis □ Antifungal screening—sorbic acid amide, amine salts, *N*-substituted □ Disk-plate method—antifungal analysis □ Ointments, sorbic acid derivatives—antifungal activity

Although great strides have been made during the last twenty years there is still no completely satisfactory treatment of either the superficial or the systemic mycoses. As Ainsworth and Sussman (1) point out, there is yet much to be learned about the requirements of the organisms; also how they vegetate between cases of human parasitism as well as how they affect human tissue as it is being invaded.

Much of the preliminary screening of new antifungal agents must resort to *in vitro* testing methods since *in vivo* methods are not readily available. Even though a good *in vitro* test may show activity there is no assurance that the same activity can be shown *in vivo*, since activity can be modified by changes in pH, solubility of agent in the surrounding media, diffusibility, and/or penetrability of the infected tissue.

According to Ainsworth and Sussman (2), antifungal activity may be due to enzyme inhibition within the fungal cell, hence penetration of the cell wall is essential. However, not all antifungal activity is demonstrated as intracellular enzyme inhibition. Some activities may be alteration of cell membrane permeability, alteration of some morphological form, or simply an interference with some extracellular enzyme which is necessary for food utilization. In these latter cases lipoid solubility would not be the principal criterion for activity.

In some early antifungal studies Peck (3) and Wyss *et al.* (4) found that such fatty acids as C_{11} and C_{13} chain lengths showed antifungal activity.

In more recent tests (5) it was found that vapors of volatile and partially volatile fatty acids up to C_7 inhibited mycelial growth while longer chains did not. The fatty acids up to C_{11} in an emulsion form with a nonionic detergent inhibited mycelial growth.

The mechanisms by which the fatty acids act as fungistatics have been investigated by Melnick *et al.* (6) and Deuel *et al.* (7) with a special emphasis on sorbic acid as a food preservative. In the presence of an excess α,β-unsaturated fatty acid, such as sorbic acid, the activity of the dehydrogenase systems of the fungi can be inhibited. It is this inhibition which is believed to be responsible for the antifungal activity of sorbic acid and the degree of activity appears to be a direct function of dosage.

Whitaker (8) has postulated that the enzyme inhibition by sorbic acid is due to sulfhydryl enzyme inhibition through the formation of a thiohexenoic acid derivative. This was demonstrated in the laboratory by inhibition of the sulfhydryl enzyme ficin. Substantiation of this postulate has been done by York and Reese (9) in recent work; certain compounds containing the $-C=C-NO_2$ grouping have shown antifungal activity.

McGowan (10) found that compounds in which the electrophilic NO_2 group was attached to an alkene side chain appeared to be several times as effective as when it was attached to an unsaturated ring.

Edwards and Pianka (11) in tests on ethylenic compounds, found that ethoxy carbonyl and methyl carbonyl groups attached to ethylenic bonds were less active than nitro groups.

Goettsch and Wiese (12) prepared several amide derivatives of thionaphthene-2-carboxylic acid and they were found to have equal or better fungistatic action than undecylenic acid. Two of the more effective compounds were the morpholine and cyclohexylamine derivatives.

Cronk *et al.* (13) synthesized and tested a number of derivatives of sorbic acid for antifungal activity. Two of the salts, *N*-isobutylamine sorbate and morpholine sorbate, exhibited a greater antifungal activity than did sorbic acid.

Goettsch *et al.* (14) prepared and tested a number of amide and salt derivatives of sorbic acid. They found that *N*-(2-methylpiperidyl) sorbamide had the greatest activity.

Since some compounds are not water-soluble, Hillegas and Camp (15) found alcohol, acetone, and propylene glycol to be efficient vehicles for agar plate testing. It was noted however, that the solvent itself may affect agar penetration and thus affect sizes of inhibition zones.

Zheutlin and Fox (16) in testing sulfonamides *in vitro* were able to demonstrate poor release of drug into aqueous media from greasy-type bases while much better release was achieved from water-miscible bases.

Hopkins (17) has proposed that polyethylene glycol bases are more advantageous than emulsion-type

bases since they bring the dissolved chemicals in contact with the applied surface in a known concentration rather than as droplets dispersed in an emulsion.

Since sorbic acid has been shown to possess antifungal properties, the objectives of this investigation were the syntheses of certain amide derivatives and amine salts of sorbic acid and the comparison of their *in vitro* antifungal properties on certain pathogens with that of the free acid.

EXPERIMENTAL PART I

Sorbyl Chloride—The chloride intermediate used in the synthesis of some of the derivatives was prepared by the direct action of thionyl chloride on sorbic acid (2,4-hexadienoic acid) and then purified by vacuum distillation as previously reported (14).

Method I—The amide derivatives were prepared by the reaction of sorbyl chloride with the respective amine as illustrated by the following process, using piperonylsorbamide (3,4-methylenedioxybenzylsorbamide).

A 1-l. 3-necked flask was assembled with a mechanical stirrer, a dropping funnel, and an upright condenser. In the reaction flask was placed 25 g. (0.16 mole) of piperonylamine in 100 ml. of anhydrous ether. A solution of 10.5 g. (0.08 mole) sorbyl chloride in 50 ml. of anhydrous ether was added dropwise to the amine solution over a period of 2 hr. A white precipitate began to form immediately as the reaction proceeded exothermically. Stirring was continued for 1 hr. after the addition of the sorbyl chloride solution. About 100 ml. of distilled water was added to the reaction flask and a part of the precipitate dissolved. The ether was removed from the remaining precipitate. The precipitate was then washed successively with numerous portions of aqueous 2% hydrochloric acid, aqueous 5% sodium carbonate, and finally with distilled water. Several recrystallizations from ethanol yielded white fluffy needle-like crystals which melted at 137–139°. This product weighed 6.14 g. or 30.2% of the theoretical yield.

Method II—The amine salts were prepared by the reaction of sorbic acid with the appropriate amine as illustrated by the following process, using *t*-octylamine sorbate (1,1,3,3-tetramethylbutylamine sorbate).

A 1-l. 3-necked flask was assembled as above. Into the reaction flask was placed 11.2 g. (0.10 mole) of sorbic acid in 800 ml. of ether. A solution of 13.0 g. (0.10 mole) of *t*-octylamine in 50 ml. of ether was added dropwise to the acid solution over a period of 1 hr. A clear solution resulted. This was allowed to stand undisturbed for 1 hr. with little change taking place. Vigorous agitation initiated the formation of a precipitate of the amine salt. The ether was removed leaving a white, fluffy precipitate. Two recrystallizations from ether produced white, fluffy, needle-like crystals which melted at 132–136°. The product weighed 9.92 g. or 41.2% of the theoretical yield. The recrystallization solvents, yields, melting points, boiling points, and analyses are given in Table I.

EXPERIMENTAL PART II

For the antimycotic studies, three species of fungi were used which are representative of the growth patterns of a number of the pathogenic fungi encountered by man. The species used were: *Microsporum canis* No. 650, *Trichophyton mentagrophytes* No. 9129, and *Candida albicans* No. 10231. The fungi were obtained from the American Type Culture Collection, Rockville, Md.

The compounds which were synthesized and tested are indicated by name and number in Table I.

The culture media were prepared with Sabouraud's dextrose agar (Difco) 65 g./l., pH approximately 5.6. The plates were prepared by the aseptic addition of 20 ml. of the sterile culture media to each 9-cm. diameter sterile disposable Petri dish by means of an automatic pipet (Brewer). The process was carried out in a laminar flow hood.

Ten-day old cultures of the test organisms grown on Sabouraud's dextrose agar slants were used to prepare the suspensions that were to be used for plate inoculation.

The suspensions for the *Trichophyton mentagrophytes* and the *Microsporum canis* were prepared by cutting approximately 4-cm. square plugs of the entire colony growth from the agar slant and adding this to 60 ml. of sterile distilled water in a sterile 120-ml. (4-oz.) wide mouth bottle. The bottle contained small chips of glass rod which, upon agitation of the contents, facilitated breaking up the mycelial mat and releasing the spores. The suspension of the *Candida albicans* was prepared by scraping two 4-mm. loopfuls of the colony growth from the agar slant, adding this to 60 ml. sterile distilled water, and then agitating vigorously. Each plate was inoculated with 0.5 ml. of the spore suspension. The plates were rotated gently to spread the suspension over the surface of the agar.

The compounds used for the first series of tests were prepared as 2% w/v solutions in acetone, with two exceptions. The 2-sorbamido-5-nitropyridine was not soluble to the extent of 2% in acetone, therefore a 1% solution was prepared. The *t*-octylamine sorbate was prepared as a 2% w/v solution in 90% acetone and 10% water to facilitate solution. The average amount of drug per disk was estimated through numerous weighings to be 1.2 mg.

Sterile disks (Bacto, 13 mm.) were dipped into the test solutions, touched to the side of the container to drain off the excess, then placed in the center of the inoculated plates. Five plates were impregnated with each test solution for each organism. The plates were incubated at 28 ± 2°. They were examined at the end of 7 days. The results are recorded in Table II as zones of inhibition in millimeters as measured by the clear zone from the outer periphery of the disk to the inner periphery of the nearest growth. The average of five plates is recorded.

The second series of tests, intended to measure possible synergistic activity, utilized the same techniques as before. In this case, the test compounds were prepared as 2% w/v solutions in combination with 2% w/v of undecylenic acid in acetone. The same exceptions due to solubility problems were made in preparing these solutions. The suspension of the organisms were prepared from an 11-day old agar slant culture. The plates inoculated with the organisms

Table I—Amides and Amine Salts of Sorbic Acid[a] $CH_3CH{=}CHCH{=}CHCOOH$

No.	Compd.	Recrystallization Solvent	Yield, %	M.p. or B.p., °C.	Formula	Anal., % Calcd.	Found
1	*N*-Methylfurfurylsorbamide	None	16.6	178–185 (10 mm.)	$C_{12}H_{15}NO_2$	C, 70.21 H, 7.36	C, 69.88 H, 7.03
2	*t*-Octylsorbamide (1,1,3,3-tetramethylbutyl sorbamide)	Ethanol	41.4	121–123	$C_{14}H_{25}NO$	C, 75.28 H, 11.28	C, 75.43 H, 10.92
3	2-Sorbamido-5-nitropyridine	Chloroform	9.0	202–205	$C_{11}H_{11}N_3O_3$	C, 56.66 H, 4.72	C, 56.12 H, 4.55
4	Piperonylsorbamide (3,4-methylenedioxybenzylsorbamide)	Ethanol	30.2	137–139	$C_{14}H_{15}NO_3$	C, 68.55 H, 6.16	C, 68.19 H, 5.84
5	*t*-Octylamine sorbate (1,1,3,3-tetra-Methylbutylamine sorbate)	Ethyl ether	41.2	132–136	$C_{14}H_{27}NO_2$	C, 69.66 H, 11.28	C, 70.00 H, 11.09
6	*N*-Methylfurfurylamine sorbate	Ethyl ether	42.2	84–86	$C_{12}H_{17}NO_3$	C, 64.55 H, 7.68	C, 64.56 H, 7.44

[a] All analyses were made by Clark Microanalytical Laboratory, Urbana, Ill.

Table II—Width of Zone of Inhibition in Millimeters[a] After 7-Day Incubation Period at 28 ± 2°C.

Compd. No.[b]	Organisms: *Microsporum canis*	*Trichophyton mentagrophytes*	*Candida albicans*
1	7	10	2
2	0	0	0
3	0	0	0
4	0	0	0
5	0	0	0
6	0	0	0
7 (sorbic acid control)	5	8	5
8 (undecylenic acid control)	8	13	10
9 (acetone)	0	0	0

[a] Measured from the outer periphery of the disk to the inner periphery of the nearest growth. [b] Compounds at 2% w/v concentration. Compound No. 3 prepared as 1% w/v concentration.

and impregnated with the paper disks containing the test solutions were incubated at 28 ± 2°. The results at the end of 7 and 14 days are recorded in Table III.

The third series of tests was made with the test compounds incorporated into an ointment base. The culture plates were prepared and inoculated as before. In this case the 0.5 ml. of culture suspension was spread over the surface of the agar plate with the aid of a sterile cotton swab. The ointments were made by preparing polyethylene glycol ointment USP and addding 0.3 mole/kg. of the compound to the fused base at 60°, then stirring until the base congealed. The ointments were added to the inoculated plates in two thin strips delivered from a syringe tip (Luer-Lok). Each strip was about 2 mm. in diameter, 4 cm. in length, and placed 3 cm. apart. An average of 15 such strips indicated that the average weight of each strip was 230 mg. Three plates were impregnated with each compound for each organism. The plates were incubated at 28 ± 2° for 7 days. The results were read as zones of inhibition in total area measured as approximate square millimeters. The zones of inhibition were read by placing the plate on a piece of graph paper which was divided into 10-mm. squares, counting the number of squares of cleared area and then calculating the total area in terms of square millimeters. A partially cleared square was counted as one if 50% or more was clear and as zero if less than 50% was clear. The results of the tests are recorded in Table IV, as an average of three plates.

DISCUSSION

Other amides not listed in the *Experimental Section* were synthesized but not tested due to a lack of stability or problems with purification. They were dicyclohexylsorbamide, 2-sorbamido-5-nitrothiazole, and tetrahydrofurfurylsorbamide. The dicyclohexyl sorbamide was a waxy, pale yellow solid which, within a few days,

Table III—Width of Zone of Inhibition in Millimeters[a] After 7- and 14-Day Incubation Periods at 28 ± 2°C.

Compd.[b]	Organism: *Microsporum canis* Days 7	*M. canis* 14	*Trichophyton mentagrophytes* 7	*T. mentagrophytes* 14	*Candida albicans* 7	*C. albicans* 14
1	13	0	12	0	10	10
2	10	0	8	0	8	0
3	5	0	10	0	10	10
4	10	0	10	0	7	4
5	10	0	10	0	5	5
6	10	0	10	0	12	12
7 (sorbic acid)	10	0	12	0	12	8
Undecylenic acid alone	10	0	10	0	10	8

[a] Measured from the outer periphery of the disk to the inner periphery of the nearest growth. [b] Compounds at 2% w/v concentration combined with 2% w/v undecylenic acid. Compound No. 3 prepared as 1% w/v concentration combined with 2% w/v undecylenic acid.

Table IV—Zones of Inhibition in Number of Square Millimeters of Area After 7-Day Incubation Period[a]

Compd. No.	*Microsporum canis*	*Trichophyton mentagrophytes*	*Candida albicans*
1	5300	6358	2033
2	0	0	0
3	0	130	0
4	467	0	0
5	5733	5050	350
6	3967	2867	833
7 (sorbic acid)	5922	4750	3200
8 (undecylenic acid)	6358	6358	6219
Polyethylene glycol base	0	0	0

[a] Compounds tested at a concentration of 0.3 mole/kg. of polyethylene glycol ointment.

became a dark, sticky mass having a consistency of honey. There was but little visible reaction when the 2-sorbamide-5-nitrothiazole was prepared and no suitable solvent was found to separate the amide from the unreacted amine. No suitable recrystallization solvent was found for the tetrahydrofurfurylsorbamide.

The purified *t*-octylsorbamide did not appear to be stable at room temperature. After 2 months' storage, the compound developed a yellow coloring and the melting point was no longer sharp. Recrystallization from alcohol restored the original characteristics of the compound. The other three amides prepared appeared to be quite stable, although the liquid *N*-methylfurfurylsorbamide was not subjected to room storage. This compound was packaged immediately after vacuum distillation in sealed glass ampuls and stored at 10° away from light. The two salts showed little change in physical appearance after 6 weeks' storage at room temperature, but a strong amine odor had developed indicating possible breakdown. The melting points did not change during this period, however. A more prolonged period of storage would be suggested to establish stability. The oxidizing tendency of the fatty acid side chain could contribute to the instability of this group of compounds. This has been reported for the amine soaps in a technical bulletin (18).

The techniques employed in the *in vitro* analyses for antifungal activity are modifications of the commonly used disk-plate methods for evaluating linear growth in the presence of toxicants. Undecylenic acid and sorbic acid were used as controls since both are known to possess antifungal activity and much information on their *in vitro* testing has already been published. Since the results of *in vitro* testing are difficult to evaluate quantitatively, it is necessary to evaluate the results as a comparison of activity to known compounds using similar experimental conditions.

Of the new compounds tested in Test Series 1, only *N*-methylfurfurylsorbamide showed any activity (see Table II). This compound appeared to be slightly more active against the *Microsporum canis* and *Trichophyton mentagrophytes* than was the sorbic acid, but not as active as undecylenic acid. Although the results are not included in the table, the plates were examined at the end of 14 days and neither the sorbic acid nor the *N*-methylfurfurylsorbamide exhibited any persisting activity. The undecylenic acid exhibited the only activity against *Candida albicans* after 14 days' incubation. Three out of five of the *N*-methylfurfurylsorbamide plates inoculated with *Candida albicans* remained clear in the 2-mm. zone. The *N*-methylfurfurylsorbamide activity appeared to be only static at the concentration tested against the first two species mentioned, with some doubt as to the interpretation of the third.

In the second series of tests (Table III) the *N*-methylfurfurylsorbamide demonstrated a very small advantage in combining this compound with undecylenic acid when tested against *Microsporum canis* and *Trichophyton mentagrophytes*. The amine salt, *N*-methylfurfurylamine sorbate, in combination appears to be slightly better than undecylenic acid on *Candida albicans* although this salt exhibited no activity when used alone.

The third series of tests in which the compounds were incorporated into a water-soluble base, polyethylene glycol ointment USP, tended to indicate considerably more activity for several of the compounds (Table IV). The *N*-methylfurfurylsorbamide appears to be better than any of the other new compounds tested, although several of them exhibited activity which was not revealed in the

disk-plate testing. *N*-Methylfurfurylsorbamide exhibited activity which approaches that of undecylenic acid on *Trichophyton mentagrophytes* and exceeds that of sorbic acid. It demonstrated considerable activity against *Microsporum canis* as well as *Candida albicans*. The *t*-octylamine sorbate also exhibited activity against *Trichophyton mentagrophytes* and *Microsporum canis* which approaches that of the controls. The polyethylene glycol ointment control indicated no activity on any of the three species. Although the results are not recorded in the tables, observation of the plates at the end of 14 days' incubation indicated that the activity of undecylenic acid, *N*-methylfurfurylsorbamide, and *t*-octylamine sorbate persisted. Sorbic acid and the other compounds which exhibited any activity showed a decrease of zone size to almost one-half of what was shown at the end of 7 days.

A part of the difference in activity in the disk-plate method results, and the results when the compounds were incorporated into the ointment base can be explained by the increase in concentration of drug available. Approximately 460 mg. of base containing 0.3 mole/kg. of compound was applied to each plate. This represents a total concentration per plate from 10 to 25 times that of the disk-plate method, depending upon the molecular weight of the compound.

The essential interest, however, remains in the activity of the compounds with respect to the controls under the same conditions of testing. There is perhaps a better relationship of activity when compared on a mole-to-mole basis rather than on a weight-to-weight basis as was done in the disk-plate method.

One factor which is limiting in the interpretation of zone size as related to antifungal potential is the lack of diffusion of the compound into the agar media. It was hoped that the surface activating and the solubilizing properties of the polyethylene glycol base would overcome a part of this problem. Inspection of some of the plates indicated that the compounds which were soluble in the polyethylene glycol base were apparently dispersed over the surface of the plate in the very thin superficial film of the spore suspension. It is recommended that further studies be conducted in the presence of serum to determine any possible antifungal inhibition by the presence of protein matter.

REFERENCES

(1) G. C. Ainsworth and A. S. Sussman, "The Fungi," vol. I, Academic Press, New York, N. Y., 1965, pp. 525–527.

(2) *Ibid.*, pp. 525–526.

(3) S. M. Peck, H. Rosenfeld, W. Leifer, and W. Bierman, *Arch. Dermatol. Syph.*, **39,** 126(1939).

(4) O. Wyss, B. J. Ludwig, and R. R. Joiner, *Arch. Biochem.*, **7,** 415(1945).

(5) R. H. Thornton, *New Zealand J. Agr. Res.*, **6,** 469(1963); through *Chem. Abstr.*, **60,** 12416(1964).

(6) D. Melnick, F. H. Luckmann, and C. M. Gooding, *Food Res.*, **19,** 44(1954).

(7) H. J. Deuel, Jr., C. E. Calbert, L. Anisfield, H. McKeehan, and H. D. Blunden, *ibid.*, **19,** 13(1954).

(8) J. R. Whitaker, *ibid.*, **24,** 37(1959).

(9) G. K. York and H. V. Reese, *J. Bacteriol.*, **88,** 411(1964).

(10) J. C. McGowan, *Ann. Appl. Biol.*, **35,** 25(1948).

(11) J. D. Edwards and M. Pianka, *J. Sci. Food Agr.*, **14,** 55 (1963); through *Chem. Abstr.*, **59,** 5048(1964).

(12) R. W. Goettsch and G. A. Wiese, *J. Am. Pharm. Assoc., Sci. Ed.*, **47,** 319(1958).

(13) D. H. Cronk, L. C. Zopf, and J. W. Jones, *ibid.*, **48,** 455 (1959).

(14) R. W. Goettsch, A. G. Danti, and D. H. Cronk, *J. Pharm. Sci.*, **51,** 728(1962).

(15) A. B. Hillegas and E. Camp, *J. Invest. Dermatol.*, **6,** 217 (1945).

(16) H. E. C. Zheutlin and C. L. Fox, *ibid.*, **5,** 161(1945).

(17) J. G. Hopkins, *ibid.*, **7,** 171(1946).

(18) "Emulsions and Detergents," 8th ed., technical bulletin, Carbide and Carbon Chemicals Corp., New York, N. Y., 1949.

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