Fungistatic Activity of Cations of Nonaromatic Amines

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3-Methylpyrrolidine and *sec*-butylamine were the most active of 50 nonaromatic amines tested in their cationic form for inhibition of germination of *Penicillium digitatum* spores. Pyrrolidine, 2-methylpyrrolidine, isopropylamine, 1-methyl-2-propenylamine, and cyclobutylamine showed lesser activity, but all other simple amines tested were not inhibitory. Replacement of the C-1 or C-4 methyl group of *sec*-butylamine with CF_3, CCl_3, COOH, OCH_3, CH_2OH, Cl, NH_2, or OH resulted in compounds which were not active. (−)-*sec*-Butylamine was considerably more active than the (+) enantiomer, both in preventing spore germination and in inhibiting mycelial growth of three species of fungi which were sensitive to racemic *sec*-butylamine. (−)-*sec*-Butylamine and 3-methylpyrrolidine were uniquely effective in preventing infection of citrus fruits by *P. digitatum*. The receptor site for inhibitory amines on the fungus cell appears to consist of an anionic component which binds the $-NH_3^+$ group and a hydrophobic area which is complementary to the *sec*-butyl radical as spatially oriented in (−)-*sec*-butylamine.

The antifungal properties of aliphatic amines, guanidines, and quaternary ammonium compounds with more than ten carbon atoms are well known (Byrde, 1969), but lower aliphatic amines in their cationic form are not generally considered to be fungitoxic by usual standards of comparison. *sec*-Butylamine (2-aminobutane) was found to be anomalous in this respect, however, being inhibitory to several species of fungi at a concentration of 1 m*M* (Eckert and Kolbezen, 1963, 1964). The antifungal spectrum of this amine was relatively narrow and no activity was found against bacteria. Despite these limited antimicrobial properties, *sec*-butylamine was shown to be effective in controlling several postharvest diseases when applied either in a neutral water solution or as a gas. The practical applications of this compound for postharvest treatment have been reviewed elsewhere (Eckert, 1969).

The unique fungistatic activity of the *sec*-butylammonium cation and its practical applications prompted a systematic evaluation of the antifungal properties of a number of aliphatic amines in an effort to find other active compounds and to establish a relationship between structure and activity in this group. One phase of this investigation has been reported in abstract (Eckert and Kolbezen, 1967).

MATERIALS AND METHODS

(+)- and (−)-*sec*-butylamine were resolved as salts of tartaric acid (Bruck *et al.*, 1956); $[\alpha]^{25}D$ (neat) = +8.216 and −7.744.

1-Methyl-2-propenylamine (bp 61° C/737 mm) was prepared as described by Roberts and Mazur (1951). The amine was purified by recrystallization of the picrate to constant melting point, 156.5–158° C, the literature value.

2-Methylpyrrolidine (bp 95–96° C/730 mm) was obtained by reduction of 5-methyl-2-pyrrolidone prepared as described by Karrer and Ehrhardt (1951). Several derivatives had melting points corresponding to literature values (Fenner and Tafel, 1898).

3-Methylpyrrolidine (bp 101–102° C/731 mm) was made by reduction of 3-methylsuccinimide by the technique of McCasland and Proskow (1954) for the analogous reduction of 2,3-dimethylsuccinimide. The 3-methylsuccinimide was prepared by the method of Sircar (1927). The picrate of 3-methylpyrrolidine crystallized from benzene melted at 107–108.5° C. The (+) isomer was partially resolved by four crystallizations of the (+)-3-methylpyrrolidine (+)-bicamphorate from ether:ethanol (82:18). The resulting amine was estimated to contain 76% (+) isomer by comparing its rotation in ethanol with the value $[\alpha]^{20}D = -15.5°$ (c 0.586 in ethanol) reported by Fleš and Ghyczy (1964) for the (−) isomer obtained by asymmetric synthesis (Figure 1). The amine recovered from the liquors of the (+) (+) salt was estimated in like manner to contain 60% (−) isomer.

N-1-Dimethylpropylamine (bp 76–79° C/730 mm) was made by catalytic hydrogenation of the imine obtained by reaction of methylethylketone with methylamine.

N,N-1-Trimethylpropylamine (bp 92–96.2° C/730 mm) was prepared by the Leuckart reaction as described by Icke and Wisegarver (1955).

N,N,N-1-Tetramethylpropylammonium chloride was made by silver chloride treatment of the quaternary iodide prepared as described by Cope *et al.* (1957). The chloride had a melting point of 259–260° C with evolution of a fishy odor.

N-*sec*-Butylacetamide (bp 215–219° C/736 mm) was obtained from the reaction of *sec*-butylamine with acetic anhydride.

Cyclobutylamine (bp 78.8–81.0° C/742 mm) was prepared from cyclobutane carboxylic acid by the Schmidt reaction (Wolff, 1946).

1-Methyl-2-propynylamine (bp 82–83° C/736 mm) was prepared by the method of Marszak-Fleury (1958). The absolute configuration (Fig. 1) was deduced by reduction of the (+) isomer to (−)-*sec*-butylamine.

1-Methyl-3,3,3-trichloropropylamine was prepared by stannous chloride reduction of 1,1,1-trichloro-3-nitrobutane made by sodium borohydride reduction of 1,1,1-trichloro-3-nitro-2-butene. The butene was prepared by the method of Bluestone (1959) from 1,1,1-trichloro-3-nitro-2-butanol which was obtained by condensation of chloral hydrate with nitroethane as described by Chattaway *et al.* (1936). 1,1,1-Trichloro-3-nitro-2-butene, 80 g, was added dropwise over a period of 30 min to a solution of 80 g of sodium borohydride in 2 l. of water. The mixture was agitated vigorously with a Vibro-

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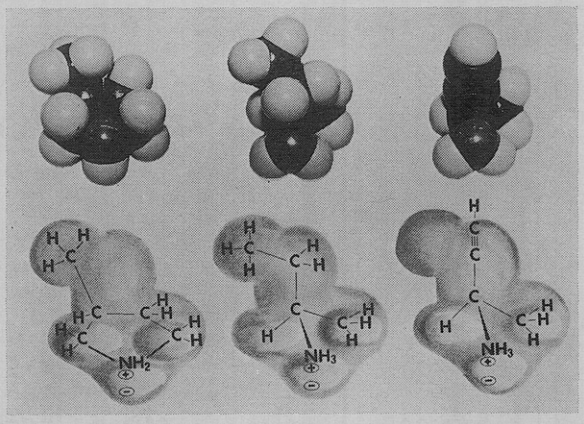


Figure 1. Molecular models of (−)-3-methylpyrrolidine, (−)-*sec*-butylamine, and (+)-1-methyl-2-propynylamine (left to right). Bottom row of illustrations indicates how the first two amines could fit the hypothetical receptor on *P. digitatum* whereas the unsaturated amine would not

mixer and was maintained at 27.5–29.0° C by external cooling. The yield was very adversely affected when temperature exceeded 30° C during the reduction. The mixture was then poured into 800 ml of ice-cold urea–acetic acid solution (Kornblum and Graham, 1951). The crystalline product was filtered, washed with water, and dried over calcium chloride; yield: 1,1,1-trichloro-3-nitrobutane, 66 g, 81%. After several recrystallizations from ethanol the melting point was 50–51° C. The infrared spectra of the nitroolefin and the saturated nitro compound showed the expected differences. The olefin showed =C—H str 3080 cm^{-1}, =C—H out-of-plane bending 810 cm^{-1}, C–Cl str 760 cm^{-1}, NO_2 asym str 1535 cm^{-1}. The 1,1,1-trichloro-3-nitrobutane had C–Cl str 782 cm^{-1} and NO_2 asym str 1543 cm^{-1}.

A solution of 10 g of 1,1,1-trichloro-3-nitrobutane in 20 ml of ethanol was added slowly to a stirred solution of 40 g of stannous chloride dihydrate in 30 ml of concentrated hydrochloric acid at 80° C. The temperature of the reaction mixture was kept at 90–95° C by slow addition of the nitro compound. When the addition was complete the mixture was evaporated under vacuum to remove excess hydrochloric acid. The residue was dissolved in water, cooled, made basic with sodium hydroxide, and extracted three times with 50-ml portions of ether. The amine was extracted from the ether with dilute hydrochloric acid. The solution of amine hydrochloride was treated with charcoal, filtered, and evaporated to dryness; yield: 1-methyl-3,3,3-trichloropropylamine hydrochloride, 7.7 g, 75%. After recrystallization from chloroform–ethanol and vacuum sublimation, the amine hydrochloride, mp 200–201° C, gave the following elemental analysis. % Calcd: C = 22.55, H = 4.26, N = 6.62, Cl = 66.57; % Found: C = 22.37, H = 4.34, N = 6.64, Cl = 64.96. The picrate had mp 188–190° C. The infrared spectrum of the free base showed N–H str 3360 cm^{-1} and 3280 cm^{-1}, NH_2 scissoring 1590 cm^{-1}, C–Cl str 760 cm^{-1}. The hydrochloride showed NH_3^+ 3000 cm^{-1} (broad) and C–Cl str 767 cm^{-1}.

1-Methyl-3,3,3-trifluoropropylamine was prepared by stannous chloride reduction of 1,1,1-trifluoro-3-nitrobutane by the same general procedure as the preceding compound. The amine was distilled from the basified reaction mixture. The nitrobutane was made by sodium borohydride reduction of 1,1,1-trifluoro-3-nitro-2-butene, which was prepared as described by McBee *et al.* (1956). 1-Methyl-3,3,3-trifluoropropylamine hydrochloride, mp 185–188° C, gave the following elemental analysis. % Calcd: C = 29.37, H = 5.54, N = 8.56, F = 34.85; % Found: C = 29.04, H = 5.83, N = 8.69, F = 32.59.

1-(Trifluoromethyl)propylamine was prepared by catalytic hydrogenation of the oxime of 1,1,1-trifluoro-2-butanone. Synthesis of the ketone was described by Sykes *et al.* (1956). Hydroxylamine hydrochloride, 91 g, was added to a solution of 57 g of 1,1,1-trifluoro-2-butanone in 850 ml of methanol. After the solid dissolved, 51.2 g of sodium hydroxide in 100 ml of water was added. The mixture was placed under reflux for 24 hr. The solid sodium chloride was removed and the solution was distilled to dryness in vacuum. Most of the methanol was removed from the distillate under vacuum and the resulting two-phase mixture was extracted with ether. The combined ether extracts were dried with $MgSO_4$ and distilled. The oxime was collected at 48–51° C/21–28 mm; yield: 31 g, 49%.

The 31 g of oxime was dissolved in 150 ml of diethyl ether and 15 g of 5% palladium on charcoal was added. The suspension was hydrogenated while shaking at 31 lb/in.^{2} at room temperature for 60 hr. The mixture was cooled in ice, shaken with 40 ml of 6 *N* hydrochloric acid, and filtered. The filtrate was concentrated under vacuum and the amine was liberated with potassium hydroxide. After drying over solid KOH the amine distilled at 65–66° C as reported by Raasch (1962) who prepared this amine by another method. Conversion to the hydrochloride gave 9.9 g, 28% (the yield could be improved by recovery of unreacted oxime from the hydrogenation mixture). After sublimation the hydrochloride had mp 278–280° C in a sealed capillary. % Calcd: C = 29.37, H = 5.54, N = 8.56, F = 34.85; % Found: C = 30.38, H = 5.29, N = 8.34, F = 34.83.

meso-2,3-Diaminobutane was obtained from a sample of 2,3-diaminobutane, furnished by the Wyandotte Chemical Co., of which the meso isomer was the main component. It was purified by recrystallization of the hydrochloride.

Racemic 2,3-diaminobutane was prepared by reduction of dimethylglyoxime according to Strack and Schwaneberg (1934).

1-Methyl-2-chloroethylamine was prepared by the reaction of propylene imine with hydrochloric acid as described by Smith and Platon (1922).

All other amines were obtained from commercial sources and distilled prior to use.

Spore Germination Test. Dry conidia of *Penicillium digitatum* Sacc. were suspended in glass-distilled water in a glass tissue homogenizer and 1 ml of the suspension was added to a solution of the amine hydrochloride in 1% (v/v) orange juice at pH 6. The orange juice, a commercial concentrate of Valencia oranges, was diluted to natural strength and clarified with Celite 545 before use. The assay mixture, 0.2 ml, containing 10^6 conidia per ml, was added to each of three glass microbeakers (0.5 ml volume) coated with a silicone (Siliclad, Clay-Adams Co., New York) to minimize the meniscus on the surface of the culture. The microbeakers were placed in a water saturated atmosphere at 25° C and germination of the conidia was evaluated after 16 hr. At least four concentrations of an amine and a control with KCl equivalent to the highest concentration of amine hydrochloride were run on each occasion. The spore germination data were subjected to probit analysis as described by Finney (1952).

Mycelium Growth Test. Sterile neutral solutions of the optical isomers and racemic *sec*-butylamine were added to sterile liquid medium of the following composition (per

Table I. Inhibition of Germination of *Penicillium digitatum* Spores by Amine Cations[a]

Amine	R_1	R_2	R_3	ED_{50} ($M \times 10^4$)	5% fiducial limits[b] (±)	Probit regression line: Slope	Probit regression line: Intercept
1. Ammonia				>1000			
A. Primary aliphatic monoamines							
2. Methylamine	H	H	H	296	14	6.59	−4.69
3. Ethylamine	H	CH_3	H	247	22	4.42	−1.16
4. Propylamine	H	C_2H_5	H	135	11	4.78	−0.42
5. *n*-Butylamine	H	*n*-C_3H_7	H	227	16	6.75	−4.16
6. *iso*-Butylamine	H	*iso*-C_3H_7	H	94.9	6.2	4.01	1.08
7. 2-Methylbutylamine	H	*sec*-C_4H_9	H	189	11	4.49	−0.72
8. Isopropylamine	CH_3	CH_3	H	18.4	1.5	3.48	4.08
9. *sec*-Butylamine	CH_3	C_2H_5	H	0.989	0.24	4.47	9.49
10. (+)-*sec*-Butylamine	CH_3	C_2H_5	H	23.5	3.1	1.77	4.34
11. (−)-*sec*-Butylamine	CH_3	C_2H_5	H	0.356	0.019	4.39	11.3
12. 1-Methyl-2-propenylamine	CH_3	CH_2=CH—	H	6.43	0.55	3.04	5.58
13. 1-Methyl-2-propynylamine	CH_3	CH≡C—	H	>1000			
14. 1,2-Dimethylpropylamine	CH_3	*iso*-C_3H_7	H	407	15	7.14	−6.50
15. 1-Methylbutylamine	CH_3	*n*-C_3H_7	H	377	23	4.40	−1.94
16. 1-Methylpentylamine	CH_3	*n*-C_4H_9	H	206	15	5.28	−1.93
17. 1-Methylhexylamine	CH_3	*n*-C_5H_{11}	H	62.3	16.4	4.42	1.49
18. 1,4-Dimethylpentylamine	CH_3	*iso*-C_5H_{11}	H	75.0	37.2	5.92	−0.190
19. 1-Ethylpropylamine	C_2H_5	C_2H_5	H	637	124	2.18	1.06
20. *tert*-Butylamine	CH_3	CH_3	CH_3	165	12	4.67	−0.68
21. *tert*-Pentylamine	CH_3	C_2H_5	CH_3	314	25	5.38	−3.06
B. Substituted primary monoamines							
	R_1	X					
22. 1-Methyl-3,3,3-trifluoropropylamine	CH_3	CF_3		556	43	3.91	−1.84
23. 1-Methyl-3,3,3-trichloropropylamine	CH_3	CCl_3		80.7	3.2	5.98	−4.25
24. 3-Aminobutyric acid	CH_3	COOH		86.8	22.8	1.31	3.77
25. 1-Methyl-2-methoxyethylamine	CH_3	—OCH_3		332	41	2.78	0.769
26. 3-Amino-1-butanol	CH_3	CH_2OH		378	45	2.58	0.930
27. 1-Methyl-2-chloroethylamine	CH_3	Cl		*ca.* 1000			
28. 1,2-Propanediamine	CH_3	NH_2		64.7	4.5	4.55	1.30
29. 2-Aminobutyric acid	COOH	CH_3		>1000			
30. 2-Amino-1-butanol	CH_2OH	CH_3		121	8	5.44	−9.15
31. 2-Amino-1-propanol	CH_3	OH		139	11	5.37	−1.14
32. 1-(Trifluoromethyl) propylamine	CF_3	CH_3		>1000			
C. N-Substituted *sec*-butylamines							
33. *N*-1-Dimethylpropylamine				525	39	4.19	−2.20
34. *N*,*N*-1-Trimethylpropylamine				1020	130	3.20	−1.44
35. *N*,*N*,*N*-1-Tetramethylpropylammonium chloride				195	4	12.4	−10.9
36. *N*-*sec*-Butylacetamide				468	23	5.64	−4.43
37. di-*sec*-Butylamine				487	22	6.22	−5.50
D. Di- and polyamines							
38. 1,2-Propanediamine				64.7	4.5	4.55	1.30
39. 1,3-Butanediamine				140	42	1.37	3.43
40. 1,4-Butanediamine				103	9	2.62	2.34
41. racemic 2,3-Butanediamine				200–300			
42. *meso*-2,3-Butanediamine				286	52	2.63	1.17
43. 2,4-Pentanediamine				500–1000			
44. racemic 2,5-Hexanediamine				500–1000			
45. *N*-(3-Aminopropyl)-1,4-butanediamine (spermidine)				0.490	0.080	2.31	8.03
46. *N*,*N*′-Bis(3-aminopropyl)-1,4-butanediamine (spermine)				0.0282	0.0012	11.8	35.2
E. Cyclic amines							
47. Pyrrolidine				1.51	0.12	3.52	7.89
48. 2-Methylpyrrolidine				15.9	0.3	2.61	4.47
49. 3-Methylpyrrolidine				0.440	0.060	2.39	8.23
50. Cyclobutylamine				9.69	0.96	2.83	5.03

[a] The solution of amine hydrochloride was added to the assay mixture and pH adjusted to 6.0 before addition of spores. [b] Expressed as mean deviation from ED_{50}.

liter): 10 g of glucose, 1 g of yeast extract, 2 g of DL-asparagine, 1 g of KH_2PO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, pH 6.0 before autoclaving. Ten-milliliter portions of medium containing the amine hydrochloride or an equivalent quantity of NaCl were dispensed into triplicate 125-ml Erlenmeyer flasks with loose-fitting stainless steel closures to minimize evaporation during incubation. Three flasks with each amine were seeded with mycelial fragments of the three test fungi: *Penicillium digitatum* Sacc., *Monilinia fructicola* (Wint.) Honey, and *Phomopsis citri* Fawc. The cultures were incubated without shaking at 25° C for 7 days and the fungus mats were filtered, dried at 90° C overnight, and weighed.

Fruit Decay Tests. Valencia oranges were inoculated with *P. digitatum* by making two punctures 180° apart on the equator of the fruit with a steel needle 1 mm diam × 2 mm long which had been dipped in a suspension of 10^6 conidia/ml. The inoculated fruit were incubated at 20° C for 18 hr before application of the solution of amine salt. One drop (*ca.* 30 mg) of neutral solutions of the amines in 0.1% (w/v) aqueous Triton X-100 (a nonionic surfactant of the general type alkylphenoxy poly(ethyleneoxy)ethanol, Rohm & Haas Co., Philadelphia) was deposited with a Pasteur pipette on top of each inoculation site and dried in a draft of cool air. The fruit were stored at 20° C for 10 days.

sec-Butylamine, pyrrolidine, and 2- and 3-methylpyrrolidines were evaluated in a similar fashion except that lemons were inoculated with a rotary saw as described previously (Eckert and Kolbezen, 1964). The inoculated lemons were incubated at 20° C for 18 hr and then dipped individually for 5 sec in 0.03 *M* solutions of the amine hydrochlorides pH 7 containing 0.01% Triton X-100. Ninety lemons were treated with each solution and the fruit were stored as three replications of 30 fruit each. Decay was evaluated after 9 days storage at 20° C and the mean differences were evaluated statistically by Duncan's multiple range test (Duncan, 1955).

RESULTS

Spore Germination Tests. A comparison of the antifungal activity of the aliphatic monoamines (Table I, compounds 2 to 21) revealed that *sec*-butylamine isopropylamine, and 1-methyl-2-propenylamine were most effective in preventing spore germination. Furthermore, (−)-*sec*-butylamine was about 2.8 times more active than racemic *sec*-butylamine and approximately 65 times more active than its enantiomer. Modification of any substituents bonded to the asymmetric carbon of *sec*-butylamine (R_1, R_2, or R_3 in Table I, A) resulted in loss of fungistatic activity. Replacement of the R_1 methyl group of *sec*-butylamine (No. 9) with H (propylamine, No. 4) or $-C_2H_5$ (1-ethylpropylamine, No. 19) abolished antifungal activity. Substitution of the R_1 methyl group by COOH (No. 29), CH_2OH (No. 30), or CF_3 (No. 32) resulted in compounds which were 200 to 300 times less active than racemic *sec*-butylamine (Table I, B).

tert-Pentylamine (No. 21), in which the H on the asymmetric carbon of *sec*-butylamine (R_3 in Table I, A) is replaced by a $-CH_3$, was approximately 300 times less active than racemic *sec*-butylamine. Similarly, *tert*-butylamine (No. 20), which bears the same relationship to isopropylamine (No. 8), was much less active than the latter.

The ethyl group (Table I, A, R_2) attached to the asymmetric carbon of *sec*-butylamine appeared to be a less rigid structural requirement for antifungal activity. Although ethylamine (R_2 = H) was completely inactive, isopropylamine (R_2 = CH_3) demonstrated the second highest activity of the saturated primary aliphatic amines. Increasing the length of the R_2 beyond ethyl (*sec*-butylamine) drastically reduced activity, irrespective of the configuration of the chain (Nos. 14, 15, 16, 17, 18). 1-Methyl-2-propenylamine (No. 12) (R_2 = CH_2 = CH–) possessed about one-sixth the activity of *sec*-butylamine, but further unsaturation at this position (R_2 = CH≡C— 1-methyl-2-propynylamine, No. 13) resulted in total loss of antifungal activity.

All substitutions and alterations of the R_2 ethyl group resulted in compounds with greatly diminished antifungal activity compared to *sec*-butylamine. Substitution of the terminal CH_3 (X in Table I, B) by any of the groups present in this position in compounds 22–28 abolished the activity characteristic of *sec*-butylamine. All N-substituted derivatives of *sec*-butylamine (compounds 33 to 37) were inactive also. Several simple diamines tested (compounds 38 to 44) were feebly active, especially in comparison to the polyamines, spermine and spermidine (Nos. 45 and 46), which were reported to possess strong antifungal properties (Razin *et al.*, 1958).

Pyrrolidine (No. 47), previously reported to control Penicillium decay of oranges (Winston and Meckstroth, 1953), was almost as effective as *sec*-butylamine in inhibiting spore germination. 3-Methylpyrrolidine, which was synthesized because of conformational similarities to (−)-*sec*-butylamine (Figure 1), proved to be the most active monoamine tested. However, in contrast to *sec*-butylamine, the optical isomers of 3-methylpyrrolidine did not exhibit significantly different fungistatic activity. Partially resolved mixtures of 3-methylpyrrolidine isomers, 76% (+) and 60% (−), respectively, showed the same degree of fungistatic activity as the racemic mixture when tested at the ED_{50} concentration of the latter. Synthetic mixtures of *sec*-butylamine isomers of the same percent composition as above were substantially different in fungistatic activity at the ED_{50} concentration as predicted from earlier tests with the pure (+) and (−) isomers. Therefore, the difference in activity, if any, between (+)- and (−)-3-methylpyrrolidine is much smaller than that of (+)- and (−)-*sec*-butylamine. 2-Methylpyrrolidine and cyclobutylamine were considerably less effective than 3-methylpyrrolidine, but possessed activity of the same magnitude as isopropylamine and 1-methyl-2-propenylamine.

Table II. Inhibition of Mycelial Growth of Three Fungi in Liquid Culture by Optical Isomers of *sec*-Butylamine

	Percent of dry weight of controls		
	1 m*M* (−) isomer	10 m*M* (+) isomer	2 m*M* racemic mixture
P. digitatum	44 ± 18[a]	105 ± 24	53 ± 12
P. citri	9 ± 2	74 ± 20	12 ± 5
M. fructicola	27 ± 12	114 ± 3	27 ± 20

[a] Mean and standard deviation of three experiments.

Inhibition of Mycelial Growth by Optical Isomers of *sec*-Butylamine. The dry weight of mycelial mats produced by *P. digitatum*, *P. citri*, and *M. fructicola* in liquid cultures was substantially reduced by racemic *sec*-butylamine or by the (−) optical isomer, whereas the (+) isomer produced at most a slight inhibition of growth (Table II). Furthermore, the fungistatic activity of the racemic mixture was approximately one-half that of the (−) enantiomer, indicating that the latter accounted for a major portion of the fungistatic activity of the racemate.

Effect of Antifungal Amines on Infection of Citrus Fruits by *P. digitatum*. Earlier experiments revealed that there was

Table III. Prevention of Infection of Citrus Fruits by *P. digitatum* by Treatment with Neutral Solutions of Fungistatic Amines

Test A. Valencia oranges

Treatment[a]	Number of infections[b]
0.05 *M* NaCl (control)	63 ± 4
0.01 *M* (−)-*sec*-butylamine	4 ± 3
0.05 *M* (+)-*sec*-butylamine	20 ± 6
0.01 *M* racemic *sec*-butylamine	10 ± 4

Test B. Lemons

Treatment[c]	% Decay[d]
NaCl (control)	75.0[a]
2-Methylpyrrolidine	64.5[b]
Pyrrolidine	52.5[c]
3-Methylpyrrolidine	21.5[d]
sec-Butylamine (racemic)	25.8[d]

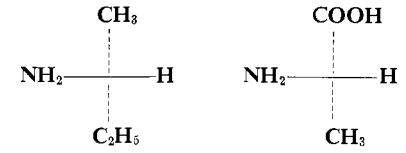
[a] Approximately 30 mg of treatment solution applied on each inoculation site. [b] Number of visible decay lesions per 80 inoculations after 10 days at 20° C. Mean and standard deviation of three experiments. [c] Lemons dipped for 5 sec in 0.03 *M* solutions. Each treatment applied to three 40-fruit replications. [d] Lower case letter superscript denotes statistical population at the 0.05 level of probability.

little correlation between *in vitro* fungistatic action of amine salts and disease control (Eckert and Kolbezen, 1963, 1964). The effectiveness of the intrinsically fungistatic amines (*in vitro*) in preventing infection of oranges was therefore evaluated. Table III reveals that (−)-*sec*-butylamine is more effective than (+)-*sec*-butylamine in preventing decay of oranges, as might be predicted from the performance in the spore germination tests. (+)-*sec*-Butylamine showed some activity but was considerably weaker than the racemate. Pyrrolidine and the methyl pyrrolidines likewise showed the same pattern of effectiveness in preventing fruit infection (Table III) as in the *in vitro* spore germination test. In contrast spermine and spermidine did not prevent infection of oranges in preliminary tests and were not evaluated further.

DISCUSSION

The most fungistatic amines found in this study and their ED_{50} values ($M \times 10^4$) were 3-methylpyrrolidine (0.440), *sec*-butylamine (0.989), pyrrolidine (1.51), 1-methyl-2-propenylamine (6.43), cyclobutylamine (9.69), 2-methylpyrrolidine (15.9), and isopropylamine (18.4). Since (−)-*sec*-butylamine was as effective as 3-methylpyrrolidine and (+)-*sec*-butylamine possessed only 1/70th of this level of activity, some deductions may be made regarding the receptor site on the sensitive fungus for inhibitory amines.

The absolute configuration of (−)-*sec*-butylamine (Kjaer and Hansen, 1957) and its relationship to the natural amino acids is shown in Figure 2. The (−)-*sec*-butylammonium cation is actively absorbed and concentrated, but not metabolized in 8 hr, by hyphal cells of *P. digitatum* (Eckert *et al.*, 1971). Furthermore, *sec*-butylamine cations inhibit the uptake of several amino acids by this fungus and their incorporation into the protein fraction of treated hyphae (Bartz and Eckert, 1971). The fungistatic action of *sec*-butylamine against spores is strongly antagonized by many inorganic and organic cations including amino acids and homologs of *sec*-butylamine which are not fungistatic. These observations suggest that the *sec*-butylammonium cation interacts reversibly



(−)-*sec*-butylamine L-(+)-alanine

Figure 2. Configuration of (−)-*sec*-butylamine (Kjaer and Hansen, 1957) and L-(+)-alanine

with an immobile anion associated with the fungus cell. An alternate possibility is that *sec*-butylamine forms an imine bond with a carbonyl receptor, since Metzler (1957) has demonstrated that this amine reacts readily with pyridoxal under physiological conditions.

Figure 1 depicts a hypothetical receptor which is structurally specific for the (−)-*sec*-butylammonium cation and closely related compounds. Stability of the amine–receptor complex would be conferred primarily by an ionic bond between the amine cation and an immobile anionic group (*e.g.*, carboxyl or phosphate). This bond could be reinforced by one or more hydrogen bonds arising through the $-NH_3^+$ protons (Hall, 1957). Albert (1968) has pointed out that H bonds can explain the stability of an amine–carboxylate receptor complex in an environment of competing cations. Finally, the anionic component of the site must be freely accessible and relatively nonselective with regards to structures attached to the $-NH_3^+$ group in order to explain antagonism of the (−)-*sec*-butylammonium cation by organic cations which are not fungistatic. The *sec*-butyl radical must interact with the receptor in some manner also, since this portion of the molecule is the primary determinant of specific fungistatic activity. The most probable mechanism for this interaction is through hydrophobic bonding, which has been shown by Inagami (1964) to contribute substantially to the binding of the *n*-butylammonium cation to the enzyme trypsin.

The hypothetical receptor must also accommodate 3-methylpyrrolidine, pyrrolidine, 1-methyl-2-propenylamine, and isopropylamine, all of which possess antifungal activity. The optical isomers of 3-methylpyrrolidine were equally active, suggesting that the spatial orientation of H and CH_3 bonded to C-3 of this compound is less critical than the configuration of the substituents adjacent to the asymmetric carbon of *sec*-butylamine. The inactivity of 1-methylbutylamine, 1-ethylpropylamine, and *tert*-pentylamine indicates that the receptor site cannot accommodate one additional methyl group at any position adjacent to the asymmetric carbon of *sec*-butylamine. Analogs of *sec*-butylamine with substituents of volume greater than CH_3, *i.e.*, $-C_2H_5$, $-CF_3$, $-CCl_3$ (Kodolov, 1966), would also be excluded from the receptor site. Aliphatic amines of smaller molecular volume would be free to approach the receptor but would not be bound as tightly as *sec*-butylamine because the former lack one or more groups which could participate in hydrophobic bonding (Inagami, 1964).

The reduction in activity which results from substitution of a methyl group of *sec*-butylamine with a hydroxyl or amino group, both of which have smaller van der Waals radii, may be due to hydration of these groups, resulting in an effective volume greater than $-CH_3$. Intra- or intermolecular H bonding are considered less likely possibilities. The inactivity of 1-methyl-2-chloroethylamine is difficult to rationalize on steric grounds because the $-CH_2Cl$ group is almost identical in size to the ethyl group on the asymmetric

carbon of *sec*-butylamine. The inductive effect of the chlorine atom is probably responsible for inactivity as discussed below for other analogs with electronegative substituents.

The reduction in activity associated with replacement of the ethyl group on the asymmetric carbon of *sec*-butylamine by $CH_2{=}CH-$ or $CH{\equiv}C-$ could be due to a reduction in bond length or the increase in bond angle. Bond length is an improbable factor since isopropylamine was much more active than 1-methyl-2-propynylamine. The increase in bond angle (C–C–C 109°; C=C—C 124°; C≡C—C 180°) appears to be the most important reason for loss of activity in unsaturated amines (Figure 1), but the inductive effects of unsaturation upon basicity could contribute to a lesser degree.

All unsubstituted saturated amines evaluated for antifungal activity have pK_a values between 10.0–11.5 (Hall, 1957; Perrin, 1965). Those amines with unsaturation or electronegative substituents are less basic and with one exception all were completely protonated under the conditions of the assay for fungistatic activity. The pK_a of 1-(trifluoromethyl)-propylamine was determined by titration to be 5.5, which agrees with the value reported by Raasch (1962). The inactivity of this compound cannot be attributed, however, to a lack of cations since it has an ED_{50} value greater than 0.1 *M* at pH 6, under which conditions 24% of the molecules were in a protonated state. All simple primary amines tested apparently had the same capacity for forming an ionic bond with the receptor. However, the hydrogen bond reinforcing the ionic bond would be considerably weaker in the less basic amines, *i.e.*, the unsaturated amines and the halogenated amines (Ferguson, 1963). This could be a major factor in the reduced activity of these compounds.

Inactivity of the N-substituted derivatives of *sec*-butylamine (compounds 33 to 37) suggests that the approach of the $-NH_3^+$ group to the anionic receptor is sterically hindered in these compounds, thereby weakening both the ionic bond and the potential for H bonding (Hall, 1957). *N,N,N*-1-Tetramethyl-propylammonium chloride, which is incapable of forming H bonds, is more active than *N*-1-dimethylpropylamine, but the unusual slope of the dosage response curve of the quaternary compound suggests that it interacts with a different receptor. The anionic portion of the site binding the *sec*-butylammonium cation would appear to possess little hydrophobic character since this property should favor binding of *N*-methyl derivatives, as does the active site of cholinesterase. Investigations are in progress to further elucidate the mechanism of antifungal action of the *sec*-butylammonium cation.

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