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Antimicrobial Activity of Hydroxamic Acids

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o-, *m*- and *p*-alkyloxybenzohydroxamic acids were synthesized and subjected to the examination for antibacterial and antifungal activity. All the hydroxamic acids tested were almost ineffective for pathogens examined of Enterobacteriaceae, but some of alkyloxybenzo- and fatty acyl-hydroxamic acid were found to be as highly effective for pathogenic fungi as butyl *p*-hydroxybenzoate being used as a comparison. The increase of carbon number of *p*-alkyloxybenzo- and fatty acyl-hydroxamic acid led to the increase in their antifungal activity, reached to the maximum at C_6 in alkyloxy moiety and C_{10} in fatty acyl derivatives, and then the gradual decrease in both series.

Considering the inhibitory power of hydroxamic acid on plant and bacterial urease, we discussed the possible correlation between antimicrobial activity and inhibitory powers on urease activity of the compounds.

It has already been reported that several hydroxamic acids have antibacterial and antifungal activity: Urbanski and his colleagues[2–5] observed salicylohydroxamic acid and its some derivatives to be effective as antitubercular agents and aryloxyacetohydroxamic acid[6] besides to be effective against some pathogenic fungi. Gale, *et al.*[7] reported that decanoic hydroxamic acid showed more potent antifungal activity than the parent acid, and Dudman[8] described the antifungel properties of sorbic hydroxamic acid over a wider pH range than sorbic acid. Zayed, *et al.*[9–11] have recently reported that (phenylthio)-acetohydroxamic acid possessed fungicidal properties against phytopathogenic moulds.

In the proceeding papers,[12–15] K. Kobashi and J. Hase reported that hydroxamic acid is a potent and specific inhibitor on both plant and bacterial urease (urea amidohydrolase, EC 3.5.1.5) and described the relation between chemical structure of various hydroxamic acids and their inhibitory effects on the activity of urease, and presented the evidence for the formation of an enzyme–inhibitor complex. In this report, we investigated the antibacterial and antifungal activity of urease inhibitors, hydroxamic acids, against pathogenic bacteria and fungi.

1) Location: *Gofuku, Toyama-shi, Toyama.*
2) T. Urbanski, *Nature*, **166**, 267 (1950).
3) T. Urbanski, S. Horung, S. Slopek and J. Venulet, *Nature*, **170**, 753 (1952).
4) H. Halweg and P. Krakowa, *Bull. Acad. Polon. Sci.*, Cl. III, **3**, 437 (1955).
5) M. Buracyewska and W. Manowska, *Bull. Acad. Polon. Sci.*, Cl. III, **3**, 487 (1955).
6) Z. Eckstein and T. Urbanski, *Bull. Acad. Polon. Sci.*, Cl. III, **4**, 627 (1956).
7) G.R. Gale, F. Bernheim and A.M. Welch, *Proc. Soc. Exp. Biol. Med.*, **109**, 188 (1962).
8) W.F. Dudman, *Appl. Microbiol.*, **11**, 362 (1963).
9) S.M.A.D. Zayed, A.F. Aboulezz, A.M. Salama and W.S. El-Hamouly, *J. Pharm. Pharmacol.*, **17**, 809 (1965).
10) S.M.A.D. Zayed, I.Y. Mostafa and M. Farghaly, *Z. Naturforsch.*, **216**, 180 (1966).
11) I.Y. Mostafa, S.M.A.D. Zayed and M. Farghaly, *Arch. Microbiol.*, **55**, 342 (1967).
12) K. Kobashi, J. Hase and K. Uehara, *Biochem. Biophys. Acta*, **65**, 380 (1962).
13) K. Kobashi, K. Kumaki and J. Hase, *Biochem. Biophys. Acta*, **227**, 429 (1971).
14) K. Kobashi, J. Hase and T. Komai, *Biochem. Biophys. Res. Commun.*, **23**, 34 (1966).
15) J. Hase and K. Kobashi, *J. Biochem.*, **62**, 293 (1967).

Experimental

Methyl or Ethyl Alkyloxybenzoate——As a typical procedure, the alkylation of ethyl *p*-hydroxybenzoate (I) with hexylbromide (II) was carried out as follows. To a solution of I (8.0 g) in acetone (10 ml) were added 2.0 g of K_2CO_3 and 0.5 g of KI, which had previously been powdered sufficiently, and then added 6.0 g of II. The mixture was refluxed on a water bath for 8 hr and filtered to remove the salt and acetone was distilled off and to the residue was added a small amount of H_2O. The mixture was extracted with ether and the ether layer was washed twice with 2% NaOH, subsequently with H_2O, dried with anhyd. Na_2SO_4 overnight and distilled *in vacuo* to give 3.0 g of pale yellow liquid of ethyl *p*-hexyloxybenzoate (III), 194—196° (16 mmHg). Yield, 46%. Other methyl or ethyl alkyloxybenzoates were obtained in a similar way.

Alkyloxybenzohydroxamic Acid——As a typical procedure, the substitution of III with hydroxylamine (IV) in alkaline solution was carried out as follows. 2.6 g of IV in MeOH (28 ml) and 3.1 g of KOH in MeOH (12 ml) was mixed, cooled on an ice bath, and KCl precipitated was discarded by filtration. To the solution was added 4.0 g of III and stand in a sealed tube for 2 days, until 1.5 g of K salt of *p*-hexyloxybenzohydroxamic acid (V) was obtained. Yield, 37.5%. K salt of V was dissolved in 1.25M hot acetic acid and 0.9 g of V was crystallized by cooling in colorless plates, mp 155—158°. Yield, 62%. Other alkyloxybenzohydroxamic acid were obtained in a similar way.

Antibacterial Test——A sample was dissolved in a modified medium of Knight (pH 7.0), which contained ten times more casamino acid than the original Knight's medium, with the aid of MeOH in a final concentration of 3%. The solution was diluted to a desired concentration with the medium into test tubes to make a series of dilution. About 10^9 cells of micro-organism which had been preincubated for 6 hr at 37° in the modified medium of Knight were inoculated into 5 ml of the test tubes mentioned above. After incubation for 8 hr at 37°, minimum concentration for complete inhibition of growth was measured. Chloramphenicol was used as a control sample for comparison.

Antifungal Test——A sample was dissolved in a small amount of MeOH, and diluted to a desired concentration with 4% glucose containing Sabouraud agar (pH 7.0) into petri dishes. The spores of each fungus, which was obtained by preculture for 4 days at 27° in a liquid medium of Sabouraud, were suspended in sterilized distilled H_2O. Disks of 5.1 mm in diameter were immersed in the spore suspension of *Microsporium gypseum*, *Aspergillus oryzae* or *Trichophyton interdigitale* and about 5×10^4 spores per disk were inoculated on the agar plates mentioned above. About the same number of spores of *Candida albicans* or *Saccharomyces sake* were streaked on the agar plates. After incubation at 27° for 6 days in the cases of Microsporium, Aspergillus and Trichophyton, and for 2 days in the cases of Candida and Saccharomyces, minimum concentration for complete inhibition of growth was measured. Butyl *p*-hydroxybenzoate and benzalkonium chloride were used as control samples for comparison. MeOH itself used as an aid of dissolution of the sample neither inhibited the growth of these fungi nor affected on the value of minimum growth inhibitory concentration of these control antifungal agents.

Result

Synthesis of Alkyloxybenzohydroxamic Acids

Alkyloxybenzohydroxamic acids were synthesized by the reaction shown in Chart 1. Properties of hydroxamic acids tested in this report are listed in Table I, among which thirteen derivatives have not been reported in the literature. The results of analysis of unknown derivatives are shown in Table II.

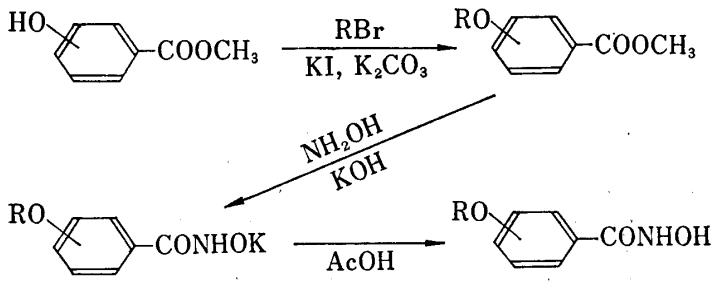


Chart 1

Antibacterial Activity

Antibacterial activity of hydroxamic acids was tested against *Escherichia coli* O-55, *Shigella flexneri* 2a, *Shigella flexneri* EW-10, *Salmonella typhi* S-57 and *Staphylococcus aureus* 209 P by measuring their minimum concentration for complete inhibition of growth (μg/ml). Hydroxamic acids tested were aceto-(No. 1),[25] benzo-(No. 9), *o*- and *p*-

25) Number in parentheses corresponds to that in Table I.

Table I. A List of Hydroxamic Acids Tested

No.	Hydroxamic Acid	mp[a] (°C)	Appearance (Recryst. solvt.)	Reference
1	aceto	88	prisms (acetic acid–H_2O)	16)
2	capro	64	plates (benzene)	16)
3	caprylo	79	plates (benzene)	16)
4	caprino	88	plates (benzene)	16)
5	undercano	90	plates (benzene)	—
6	lauro	93	plates (benzene)	16)
7	palmito	99	plates (benzene)	16)
8	adipino mono	100	powder (acetic acid–H_2O)	—
9	benzo	125	plates (ethyl acetate)	17)
10	*o*-carboxylbenzo	189	plates (acetic acid–H_2O)	18)
11	picolino	121	prisms (EtOH)	19)
12	nicotino	168	prisms (EtOH)	19)
13	isonicotino	160	prisms (EtOH)	19)
14	furan-2-carbo	123	needles (EtOH)	20)
15	thiophene-2-carbo	123	prisms (EtOH)	21)
16	*o*-hydroxybenzo	170	plates (MeOH)	22)
17	*p*-hydroxybenzo	168	needles (H_2O)	—
18	*o*-methoxybenzo	130	plates (benzene)	23)
19	*m*-methoxybenzo	80	plates (H_2O)	—
20	*p*-methoxybenzo	162[b]	needles (H_2O)	24)
21	*o*-butoxybenzo	105	needles (MeOH)	23)
22	*m*-butoxybenzo	108	plates (benzene)	—
23	*p*-butoxybenzo	162	plates (acetone)	—
24	*o*-hexyloxybenzo	89	needles (MeOH)	23)
25	*m*-hexyloxybenzo	115	plates (H_2O)	—
26	*p*-hexyloxybenzo	158	plates (acetone)	—
27	*o*-octyloxybenzo	74	needles (MeOH)	23)
28	*m*-octyloxybenzo	86	plates (petroleum ether)	—
29	*p*-octyloxybenzo	154	plates (acetone)	—
30	*o*-dodecyloxybenzo	83	plates (MeOH)	—
31	*m*-dodecyloxybenzo	102	plates (benzene)	—
32	*p*-dodecyloxybenzo	132	plates (MeOH)	—

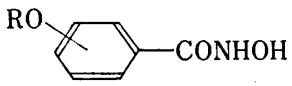
a) mp were uncorrected. *b*) lit.[24] mp 157°

hydroxydenzo-(No. 16, 17), *o*-, *m*- and *p*-methoxybenzo-(No. 18, 19, 20) hydroxamic acid, and heterocyclic hydroxamic acids which involved α-, β- and γ-hydroxamic acid (No. 11, 12, 13) of pyridine derivatives, and furan-2- and thiophene-2-carbohydroxamic acid (No. 14, 15). All the hydroxamic acids mentioned above, however, were found to be almost ineffective against all the test-bacteria with 320 μg/ml.

Antifungal Activity

Antifungal activity of hydroxamic acids was tested against *Candida albicans, Saccharomyces sake, Microsporium gypseum, Aspergillus oryzae* and *Trichophyton interdigitale* by the disk–dilution method. Antifungal activity of alkyloxybenzohydroxamic acid is shown in Table

16) Y. Inoue and Y. Yukawa, *Nogei Kagaku Zasshi*, **16**, 504 (1940).
17) C.R. Hauser and W.B. Renfrow Jr., *Org. Syn.*, **XIX**, 15 (1939).
18) L. Bauer and Y.M. Stanley, *J. Am. Chem. Soc.*, **79**, 1983 (1957).
19) B.B. Hacklby Jr., R. Plapinger, M. Stobberg and T.W. Jauregg, *J. Am. Chem.*, **77**, 3651 (1955).
20) P.H. Pichard and A. Neville, *J. Chem. Soc.*, **79**, 847 (1901).
21) L.W. James and C.D. Hurd, *J. Am. Chem. Soc.*, **43**, 2422 (1921).
22) A. Jeanrenaud, *Ber.*, **22**, 1273 (1889).
23) K. Takahashi, Y. Shiobara and S. Ishida, Japan Patent 37—5178.
24) W. Lossen, *Ann.*, **175**, 284 (1875).

Table II. 

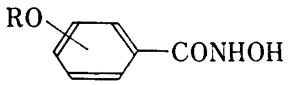
No.[a]	RO-	Formula	Analysis (%) Calcd. C	H	N	Found C	H	N
30	*o*-$C_{12}H_{25}O$	$C_{19}H_{31}O_3N$	70.99	9.72	4.36	70.95	9.75	4.74
19	*m*-CH_3O	$C_8H_9O_3N$	57.48	5.43	8.38	56.24	5.36	8.34
22	*m*-C_4H_9O	$C_{11}H_{15}O_3N$	63.14	7.23	6.69	62.91	6.96	6.85
25	*m*-$C_6H_{13}O$	$C_{13}H_{19}O_3N$	65.80	8.07	5.90	65.96	8.08	6.02
28	*m*-$C_8H_{17}O$	$C_{15}H_{23}O_3N$	67.89	8.74	5.28	67.61	8.41	5.12
31	*m*-$C_{12}H_{25}O$	$C_{19}H_{31}O_3N$	70.99	9.72	4.36	72.35	9.56	4.39
17	*p*-HO	$C_7H_7O_3N$	54.90	4.61	9.15	54.75	4.71	8.96
23	*p*-C_4H_9O	$C_{11}H_{15}O_3N$	63.14	7.23	6.69	63.40	7.27	6.92
26	*p*-$C_6H_{13}O$	$C_{13}H_{19}O_3N$	65.80	8.07	5.90	65.57	8.00	5.88
29	*p*-$C_8H_{17}O$	$C_{15}H_{23}O_3N$	67.89	8.74	5.28	67.99	8.86	5.40
32	*p*-$C_{12}H_{25}O$	$C_{19}H_{31}O_3N$	70.99	9.72	4.36	72.00	9.80	4.73

a) Number in this table corresponds to that in Table I.

III and that of fatty acyl- and heterocyclic hydroxamic acid in Table IV. Butyl *p*-hydroxybenzoate, which is applied as a preservative, and benzalkonium chloride, an external disinfectant, were used as control.

As shown in Table III, the increase of carbon number of alkyloxy group of the hydroxamic acid led to gradual increase in antifungal activity and to the maximum activity at C_6 derivative:

Table III. Antifungal Activity of Alkyloxybenzohydroxamic Acids



(Minimum Growth Inhibitory Concentration: μg/ml)

R	*Trichophyton interdigitale*	*Microsporium gypseum*	*Candida albicans*	*Saccharomyces sake*	*Aspergillus oryzae*
o-H	320	320	1000	>1000	1000
p-H	1000	>1000	>1000	>1000	>1000
o-CH_3	1000	320	1000	>1000	>1000
m-CH_3	320	320	1000	>1000	>1000
p-CH_3	100	100	1000	>1000	1000
o-C_4H_9	32	100	1000	320	1000
m-C_4H_9	100	100	320	320	320
p-C_4H_9	100	100	320	320	1000
o-C_6H_{13}	32	32	320	320	320
m-C_6H_{13}	32	32	320	320	320
p-C_6H_{13}	32	32	320	320	>1000
o-C_8H_{17}	32	100	320	320	>1000
m-C_8H_{17}	320	320	320	320	>1000
p-C_8H_{17}	>1000	1000	1000	>1000	>1000
m-$C_{12}H_{25}$	1000	1000	1000	>1000	1000
p-$C_{12}H_{25}$	>1000	1000	1000	>1000	>1000
Butyl *p*-hydroxybenzoate	32	32	100	100	100
Benzalkonium chloride	1000	10	1000	100	1000

o-, *m*- and *p*-hexyloxybenzohydroxamic acids (No. 24, 25, 26) were the most effective against pathogenic fungi, *Trichophyton interdigitale* and *Microsporium gypserum* with minimum growth inhibiting concentration of 32 μg/ml, which was almost the same value as that of butyl *p*-hydroxybenzoate. More increase in carbon number than six in alkoxy group led to decrease in antifungal activity against all the fungi tested.

Position of substitution of alkyloxy group did not significantly influence the antifungal activity. Alkyloxybenzohydroxamic acids showed moderate effect against *Candida albicans* and *Saccharomyces sake*, and lower activity against *Aspergillus oryzae*.

TABLE IV. Antifungal Activity of Some Hydroxamic Acids
(Minimum Growth Inhibitory Concentration: μg/ml)

Hydroxamic acids	*Trichophyton interdigitale*	*Microsporium gypseum*	*Candida albicans*	*Saccharomyces sake*	*Aspergillus oryzae*
Aceto	>320	>320	>320	>320	>320
Capro	32	100	100	100	>320
Caprylo	32	32	320	100	320
Caprino	32	100	100	10	320
Undecano[a)]	32	32	320	10	320
Lauro	100	32	320	10	>320
Palmito	>320	>320	>320	>320	>320
Adipino mono[a)]	>320	>320	>320	>320	>320
Benzo	320	>320	320	>320	>320
o-Carboxybenzo	>320	320	>320	>320	320
Picolino	320	100	>320	320	>320
Nicotino	>320	100	>320	>320	>320
Isonicotino	>320	320	>320	>320	>320
Furan-2-carbo	>320	>320	>320	>320	>320

a) Unknown compounds in the literature. *Anal.* Calcd. for $C_{11}H_{23}O_2N$ (undecanohydroxamic acid): N, 6.96. Found: N, 7.03. *Anal.* Calcd. for $C_6H_{11}O_4N$ (adipino monohydroxamic acid): N, 8.69. Found: N, 8.85.

Similar results were obtained in the series of aliphatic hydroxamic acids as shown in Table IV. Increase in carbon number led to increase in antifungal activity and reached to the maximum activity near C_{10} derivative. Caprino- (No. 4), undecano-(No. 5) and lauro-(No. 6) hydroxamic acid showed excellent antifungal activity against yeast, *Saccharomyces sake*, with minimum growth inhibiting concentration of 10 μg/ml and caprylo-(No. 3) and undecano-(No. 5) derivatives against *Tricophyton interdigitale* and *Microsporium gypseum* with minimum growth inhibiting concentration of 32 μg/ml. Palmitohydroxamic acid (No. 7) was almost ineffective. δ-Carboxyl group lowered antifungal activity as shown in the case of adipino monohydroxamic acid (No. 8). Heterocyclic derivatives were almost ineffective, although picolino-(No. 11) and nicotino-(No. 12) hydroxamic acid were some what effective against *Microsporium gypseum*.

Discussion

Our previous report[15)] described the inhibition by hydroxamic acid on urease extracted from *Proteus vulgaris* and on ureolytic activity of the living cell. J. Yoshida, R. Nakamura and one of the authors[26)] found that hydroxamic acid have highly protective effect against urea toxication in the ruminantia by inhibiting the urea splitting activity of microflora in the rumen. From these results and those of our proceeding papers[12–14)] on plant urease, all the hydroxamic acids which have previously been reported as antimicrobial agents by Urbanski,

26) J. Yashida, R. Nakamura and K. Kobashi, *Japan. J. Zootechnical Sci.*, **34**, Extra No. 28 (1964).

et al.[2-6] Gale, *et al.*[7] and Dudman[8] are supposed to have considerable inhibitory powers on both plant and microbial urease activity. Seneca, *et al.*[27] discussed the significance of bacterial urease through the studies on classification of micro-organism on the basis of urease production. Hence antimicrobial activity of these hydroxamic acids might be partly due to the inhibition and disturbance of urea metabolism caused by these compounds.

The results obtained in this paper suggest that the correlation between antibacterial activity and inhibitory effect on urease activity of hydroxamic acid seems unlikely, because highly inhibitory hydroxamic acids on bacterial urease did not show any antibacterial activity against such Enterobacteriaceae as *Escherichia coli*, *Shigella flexneri* and *Salmonella typhi*, although these genus happened to be classified in the group of urease positive in lysate of the cell and of non-urea splitters in living culture according to H. Seneca, *et al.*[27]

On the other hand, some derivatives of fatty acyl- and alkyloxybenzo-hydroxamic acids showed excellent antifungal activity. The maximum effectiveness was observed at C_6-C_8 for urease inhibition and at C_8-C_{10} for antifungal activity in the series of fatty acyl derivatives. In the series of alkyloxybenzo-derivatives, the maximum effectiveness in carbon number of alkyloxy group was observed at C_1 for urease inhibition and at C_6 for antifungal activity.

With regard to effect of various substituents and their position of aromatic hydroxamic acid on their inhibitory effect on urease from both plant and bacterial origin, we already reported[13] that *m*- and *p*-substituted derivatives were powerful inhibitors with the same I_{50} values, while *o*-substituted ones were markedly less or non-inhibitory. However, we could not observed such significant *ortho*-effect of substitution of alkyloxy moiety on the antifungal activity.

For urease inhibition, –CONHOH group was found to be the absolutely necessary structure and alkyl moiety influenced its effectiveness with secondary significance.[12,13] However, for antifungal activity, alkyl group and its length may play a major role and –CONHOH group may function as an ion, in the similar fashion as in the case of butyl *p*-hydroxybenzoate.

These differences between antimicrobial and urease–inhibitory activity did not support the above mentioned possibility that growth inhibition of micro-organism by hydroxamic acid may be due to the disturbance of urea metabolism. Definite conclusion can not be made as yet concerning this hypothesis until more knowledge is gained on the distribution of fungal urease and its physiological role.

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27) H. Seneca, P. Peer and R. Nally, *Nature*, **192**, 1106 (1962).