THERAPY FOR UROLITHIASIS BY HYDROXAMIC ACIDS. III.* UREASE INHIBITORY POTENCY AND URINARY EXCRETION RATE OF N-ACYLGLYCINOHYDROXAMIC ACIDS

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Hydroxamic acid, a potent urease inhibitor, having a high urinary excretion rate is expected to be a therapeutic agent for urolithiasis caused by urea-splitting bacterial infection of the urinary tract. Twenty-one new derivatives of N-aliphatic-acylglycinohydroxamic acids (GHAs) were synthesized, and their inhibitory potencies against the urease activity of sword bean in a phosphate buffer and against the ureolytic activity of *Proteus mirabilis* in human urine, and their urinary excretion rates in rats were also measured for this purpose. I_{50} values of most of GHAs against the urease activity of sword bean were about 1 to 10 μM and 2-ethyl-*n*-butyroyl GHA was the most potent inhibitor with the value of 0.79 μM . I_{50} values of most of the GHAs against the ureolytic activity of *Proteus mirabilis* were about 5 to 50 μM and *n*-nonaroyl GHA was the most potent inhibitor with the value of 3.6 μM . 2,2-Dimethylpropionyl GHA had the highest urinary excretion rate with the recovery of 11%. Routes of administration of 2,2-dimethylpropionyl GHA and sex of rats used did not affect the amount of urinary excretion at all. The results in this report suggest that DL 2-methyl-*n*-butyroyl, 2-ethyl-*n*-butyroyl and 2,2-dimethylpropionyl GHA are the most hopeful therapeutic agents for urolithiasis among them.

Keywords—N-acylglycinohydroxamic acids; urolithiasis; urease inhibitor; urinary excretion rate; *Proteus mirabilis*; sword bean

Since the alkalization of urine caused by the infection of urea-splitting bacteria in the urinary tract has been regarded as the most important etiological factor in the development of the phosphate stone, particularly struvite.¹⁾ Hydroxamic acid, a potent and specific urease inhibitor,²⁾ with a high urinary excretion rate, might be expected as the therapeutic agent for urolithiasis. Griffith and Musher³⁾ and Andersen⁴⁾ have also reported the possibility of the clinical usefulness of acetohydroxamic acid and *p*-chlorohippurohydroxamic acid, respectively. In our previous report,⁵⁾ some substituted hippurohydroxamic acids were found to have potent urease inhibitory

actions and high urinary excretion rates. However, acetohydroxamic acid and typical substituted hippurohydroxamic acids were discovered to have weak mutagenicity in the microbial screening systems.⁶⁾ Recently, Wang and Lee⁷⁾ have reported no mutagenicity of *p*-butoxyphenylacetohydroxamic acid and caprylohydroxamic acid was also demonstrated to be non-mutagenic.⁶⁾ These observations suggest that all hydroxamic acids do not induce mutagenesis. In order to find a suitable derivative of hydroxamic acid having the potent inhibitory action, high urinary excretion rate and no mutagenicity, we synthesized twenty-one new N-

* Part II: See Reference 5).

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aliphatic-acylglycinohydroxamic acids (acyl-GHAs) and measured their urease inhibitory potencies and urinary excretion rates in rats.

MATERIALS AND METHODS

Chemicals — Acyl-GHAs were synthesized according to the reaction process shown in Chart

1 by the method described previously.⁸⁾ Properties and results of elemental analysis of twenty-one new hydroxamic acids are listed in Table I.

Assay Methods — Preparation and assay methods of urease were the same as described in our previous paper.⁵⁾ The apparent I_{50} and I_{80} values were the final concentration (μM) of the

R: The same as those in Table I.

DMF: dimethylformamide

CHART 1. *The Synthetic Route of N-Acylglycinohydroxamic Acids*

TABLE I. *A List of New Hydroxamic Acids RCO-NHCH₂CONHOH*

Acyl residue (RCO-)	mp (°C)	Formula	Analysis (%)						Recryst. solvent
			Calcd			Found			
			C	H	N	C	H	N	
<i>n</i> -Dodecanoyl-	132–133	C ₁₄ H ₂₈ N ₂ O ₃	61.73	10.36	10.28	61.84	10.34	10.41	MeOH-H ₂ O
<i>n</i> -Decanoyl-	130–131	C ₁₂ H ₂₄ N ₂ O ₃	58.99	9.90	11.47	58.66	10.25	11.40	EtOH-H ₂ O
<i>n</i> -Nonanoyl-	128–129	C ₁₁ H ₂₂ N ₂ O ₃	57.36	9.63	12.17	57.18	9.96	12.19	MeOH
<i>n</i> -Octanoyl-	130–131	C ₁₀ H ₂₀ N ₂ O ₂	55.53	9.32	12.95	55.22	9.57	13.00	MeOH
<i>n</i> -Heptanoyl-	132–133	C ₉ H ₁₈ N ₂ O ₃	53.44	8.97	13.85	53.41	8.94	13.81	H ₂ O
<i>n</i> -Hexanoyl-	130–131	C ₈ H ₁₆ N ₂ O ₃	51.05	8.57	14.88	51.41	8.59	14.82	MeOH-H ₂ O
4-Methyl- <i>n</i> -pentanoyl-	129–130	C ₈ H ₁₆ N ₂ O ₃	51.05	8.57	14.88	51.02	8.83	14.95	MeOH-EtOH
3-Methyl- <i>n</i> -butyroyl-	146–148	C ₇ H ₁₄ N ₂ O ₃	48.26	8.10	16.08	48.25	8.18	16.03	EtOH
2-Ethyl- <i>n</i> -butyroyl-	169–170	C ₈ H ₁₆ N ₂ O ₃	51.05	8.57	14.88	50.97	8.77	15.02	MeOH
DL 2-Methyl- <i>n</i> -butyroyl-	153–154	C ₇ H ₁₄ N ₂ O ₃	48.26	8.10	16.08	48.26	7.86	16.09	EtOH
2,2-Dimethylpropionyl-	179–180	C ₇ H ₁₄ N ₂ O ₃	48.26	8.10	16.08	48.27	8.37	16.17	MeOH
Adamantylcarbonyl-	154–155	C ₁₃ H ₂₀ N ₂ O ₃	61.88	7.99	11.10	61.86	8.18	10.99	MeOH
3-Cyclohexylpropionyl-	149–150	C ₁₁ H ₂₀ N ₂ O ₃	57.87	8.83	12.27	57.92	8.60	12.26	MeOH
Cyclohexylacetyl-	155–156	C ₁₀ H ₁₈ N ₂ O ₃	56.05	8.47	13.08	56.42	8.28	13.03	MeOH
Cyclohexylcarbonyl-	159–160	C ₉ H ₁₆ N ₂ O ₃	53.98	8.06	13.99	53.51	7.97	13.89	EtOH
3-Phenylpropionyl-	145–146	C ₁₁ H ₁₄ N ₂ O ₃	59.45	6.35	12.60	59.45	6.34	12.48	EtOH
Phenylacetyl	134–135	C ₁₀ H ₁₂ N ₂ O ₃	57.68	5.81	13.46	57.34	5.77	13.61	EtOH
<i>p</i> -Methoxyphenylacetyl-	150–151	C ₁₁ H ₁₄ N ₂ O ₄	55.45	5.92	11.76	55.71	5.97	11.81	MeOH
<i>p</i> -Nitrophenylacetyl-	158–159	C ₁₀ H ₁₁ N ₂ O ₅	47.43	4.38	16.60	47.45	4.11	16.53	EtOH
<i>p</i> -Methylphenylacetyl-	159–160	C ₁₁ H ₁₄ N ₂ O ₃	59.45	6.35	12.60	59.24	6.13	12.75	EtOH
<i>p</i> -Chlorophenylacetyl-	160–161	C ₁₀ H ₁₁ ClN ₂ O ₃	49.50	4.57	11.54	49.00	4.40	11.51	MeOH

compounds, which gave 50% and 80% inhibition, respectively, in the preincubation mixtures. The specific activity of partially purified urease from sword bean was 71 IU/mg of protein. Strain B of *Proteus mirabilis* isolated from patients with the urinary tract infection was kindly provided by Dr. Takeuchi, Shiga Medical University, Ootsu. The ureolytic activities of the bacteria obtained by the cultivation method described previously⁵⁾ were 653 ± 247 mIU/ 10^9 viable cells. The meth-

ods of dissolution of hydroxamic acids and of oral administration to rats (female Wistar strain rats with 145 ± 25 g of body weight) were performed in the same way as described in our previous paper.⁵⁾ The hydroxamic acid unchanged in the urine specimens was determined enzymatically according to the method of Kobashi *et al.*⁹⁾ The measurement of the time course of the urinary excretion rate of 2,2-dimethylpropionyl GHA was performed as follows: 1 ml of the saline solu-

TABLE II. Inhibitory Potency against Ureolytic Activity of Sword Bean and *Proteus mirabilis* and Urinary Excretion Rate in Rats of *N*-Acylglycinohydroxamic Acids [R CO-NHCH₂CONHOH]

Acyl residue	Inhibitory potency (μ M) against the ureolytic activity of			Urinary excretion rate				
	Sword bean I ₅₀ ^{a)}	I ₈₀ ^{b)}	<i>P. mirabilis</i> I ₅₀ ^{a)}	Number of rat	Dose (mg/kg)	0–6 hr (%)	6–24 hr (%)	Total (%)
<i>n</i> -Dodecanoyl	1.5	— ^{d)}	8.6	3	100	0.06	0.10	0.16
<i>n</i> -Decanoyl	2.1	6.0	5.8	3	100	0.14	0.12	0.26
<i>n</i> -Nonanoyl	1.3	3.8	3.6	3	100	0.19	0.07	0.26
<i>n</i> -Octanoyl	1.6	5.4	7.8	3	100	0.30	0.14	0.44
<i>n</i> -Heptanoyl	1.5	8.0	—	3	100	3.6	—	—
<i>n</i> -Hexanoyl	1.3	8.0	9.2	3	100	0.85	0.28	1.1
4-Methyl- <i>n</i> -pentanoyl	1.9	6.0	—	2	100	1.9	—	—
3-Methyl- <i>n</i> -butyryl	1.5	5.0	18	3	100	1.9	0.22	2.1
2-Ethyl- <i>n</i> -butyryl	0.79	1.9	5.3	3	108	—	—	2.1 ^{c)}
DL 2-Methyl- <i>n</i> -butyryl	1.3	4.4	6.2	3	108	—	—	5.3 ^{c)}
2,2-Dimethylpropionyl	4.4	19	45	13	125	—	—	11 ^{c)}
Adamantyl carbonyl	14	—	—	3	83	1.2	—	—
3-Cyclohexyl-propionyl	9.2	—	41	3	100	0.71	0.88	1.6
Cyclohexylacetyl	4.7	27	94	3	100	0.37	0.24	0.61
Cyclohexyl carbonyl	2.1	6.0	17	3	100	0.95	0.10	1.1
3-Phenylpropionyl	0.9	3.8	9.5	3	100	2.5	0.38	2.9
Phenylacetyl	1.3	4.0	20	3	100	2.7	0.59	3.3
Benzoyl	0.77	2.0	6.6	3	100	3.7	0.34	4.04
<i>p</i> -Chlorophenylacetyl	0.94	10	—	3	83	0.58	—	—
<i>p</i> -Methylphenylacetyl	7.2	—	—	3	83	2.5	—	—
<i>p</i> -Nitrophenylacetyl	11	—	—	3	83	6.5	—	—
<i>p</i> -Methoxyphenylacetyl	13	—	—	3	100	6.8	—	—

a) and b) represent an inhibitor concentration in the preincubation mixture which gave 50 and 80% inhibition, respectively. The inhibitory potency of the hydroxamic acids against the urease activity of sword bean was measured in 0.1 M phosphate buffer (pH 7.7), and that against the ureolytic activity of intact *P. mirabilis* was measured in human urine.

c) Recovery percent in urine for 24 hr after administration.

d) Not determined.

tion containing 20 mg of the hydroxamic acid was administered orally in a single dosage (20 mg/rat) to a group of five female Wistar rats (180 to 186 g of body weight) and 1 ml of the saline was orally given three times at an interval of 1, 3 and 5 hr and the excreted urine was collected at 2, 4, 6 and 24 hr after the oral administration.

RESULTS

Urease Inhibitory Potency of Hydroxamic Acids

Among linear and branched aliphatic acyl GHAs, the most potent inhibitor against the urease activity of sword bean was 2-ethyl-*n*-butyryl-GHA, whose apparent I_{50} value was $0.79 \mu\text{M}$ and was almost the same as that of hippurohydroxamic acid.⁵⁾ Nine of the others had I_{50} values of 1.7 ± 0.4 regardless of the carbon number and branched structure of the acyl residues, though 2, 2-dimethylpropionyl GHA was less inhibitory ($4.4 \mu\text{M}$). Among ω -cyclohexylacyl derivatives, cyclohexyl carbonyl GHA had the I_{50} value of $2.1 \mu\text{M}$, and its higher homologues showed less inhibitory potency with increase in the carbon number. Adamantyl carbonyl GHA was far less inhibitory ($14.0 \mu\text{M}$). Three ω -phenylacyl GHAs had relatively potent and similar inhibitory actions. Among *p*-substituted phenylacetyl GHAs, chloro-derivative, the parent compounds, methyl-, nitro- and methoxy-deriva-

tive were arranged in the descending order of the inhibitory potency.

As mentioned in our previous report,⁵⁾ the apparent I_{50} values of the hydroxamic acids have been employed to represent the inhibitory potency on urease activity. However, a correlation curve between the inhibition percentages and the logarithmic concentration of the hydroxamic acids, in general, is sigmoidal. Since complete inhibition of the urease activity in the urinary tract is required for the prevention of urinary stone formation, the apparent I_{80} value causing 80% inhibition was measured as shown in Table II. The I_{80} values of most of the hydroxamic acids tested were about 3 to 5 times higher than their I_{50} values, but the inhibition slopes of several derivatives at higher concentrations were too low to measure the I_{80} values.

The inhibitory potencies of *n*-aliphatic acyl GHAs against the ureolytic activity of intact *P. mirabilis* in human urine were arranged according to the carbon number on a gentle arc with the maximum inhibition at *n*-nonanoyl GHA whose I_{50} value was $3.6 \mu\text{M}$. The I_{50} values of branched aliphatic acyl GHAs varied between 5.3 and $45.0 \mu\text{M}$ in spite of a little difference in the carbon number: 2-ethyl-*n*-butyryl GHA had the most potent inhibitory action. On the other hand, the I_{50} values of three ω -cyclohexylacyl derivatives

TABLE III. *Effect of Sex and Route of Administration on Urinary Excretion Rates of N-2, 2-Dimethylpropionylglycinohydroxamic Acid*

Route of administration	Rat				Urinary excretion rate within 6 hr ^{a)}			
	Sex	Number	Body weight (g)	Dose (mg/rat)	Hydroxamic acid in urine			Urinary excretion rate (%)
					Urine volume (ml/rat)	Amount (mg/rat)	Concentration (mM)	
Oral	Male	5	161 ± 11 ^{b)}	15	6.0 ± 1.3	1.72 ± 0.51	1.64 ± 0.10	11.5 ± 0.31
Oral	Female	13	120 ± 7	15	7.1 ± 1.8	1.64 ± 0.65	1.32 ± 0.21	10.9 ± 1.59
Intra-peritoneal ^{c)}	Female	5	186 ± 6	20	5.2 ± 1.1	2.34 ± 0.35	2.58 ± 0.15	11.7 ± 1.70

a) No hydroxamic acid was detected within 6–24 hr after administration.

b) Mean $\pm$ S. D.

c) 1 ml of hydroxamic acid in saline solution (20 mg/ml) was administered.

were higher than those of the others. Among ω -phenylacyl GHAs, hippurohydroxamic acid and 3-phenylpropionyl GHA were potent inhibitors with I_{50} values of 6.6 and 9.5 μM , respectively, but phenylacetyl GHA was markedly less inhibitory, though its carbon number is between those of these homologues.

Urinary Excretion Rates of Hydroxamic Acids in Rats

The recoveries in urine of *n*-aliphatic acyl GHAs orally administered were very small, showing recoveries of 0.16 to 3.60% of the dose. The recoveries of branched aliphatic acyl GHAs was relatively high, due to the branched structure of the acyl residues: those of 2,2-dimethylpropionyl and DL 2-methylbutyryl GHA were 11.0 and 5.3%, respectively, while the others were about 2%. Urinary excretion rates of all the ω -cyclohexyl acyl GHAs tested were low, showing recoveries of 0.61 to 1.59% of the dose. In the ω -phenyl acyl GHAs, recoveries of three non-substituted derivatives were found in the descending order with the increase in the carbon number of acyl residues, and nitration and methoxylation on the benzene ring of phenylacetyl GHA increased the recovery by three times. Furthermore, 2,2-dimethylpropionyl GHA was found to be excreted in unchanged form, with the concentrations of 6.07, 0.61 and 0.21 mM within 0 to 2, 2 to 4 and 4 to 6 hr, respectively, and not detected in urine within 6 to 24 hr after the oral administration. Its total recovery in urine was 9.38% of the administered dose.

Effect of difference in sex and the route of administration on the urinary excretion of 2,2-dimethylpropionyl GHA was examined (Table III). No significant difference was observed between male and female or between oral and intraperitoneal administration.

DISCUSSION

In the previous report,¹⁰⁾ the inhibitory potencies of *n*-aliphatic acyl hydroxamic acids against the urease activity of sword bean varied parabolically with the hydrophobic characters of acyl residues with the maximum in case of hep-

tano- and octano-hydroxamic acid, which was well fit with the parabolic function calculated according to Hansch-Fujita method. On the other hand, the I_{50} values of ten aliphatic acyl GHAs tested resembled each other averaging $1.5 \pm 0.6 \mu\text{M}$, though they had different carbon numbers and steric structure in their acyl residues. The inhibitory potencies of these compounds are close to those of octano-¹¹⁾ and hippuro-hydroxamic acid⁵⁾ which are the most potent inhibitors hitherto reported. These observations suggest that the presence of the $-\text{CONHCH}_2-$ group between the alkyl residue and functional hydroxamic acid group might be significantly advantageous for binding with the active site of the enzyme, and so difference in hydrophobicity or steric structure of alkyl residues did not markedly affect inhibitory potencies. The similar effect of the $-\text{CONHCH}_2-$ group against inhibitory potencies was also found in a congener of substituted hippurohydroxamic acid.⁵⁾ The apparent I_{50} value of the acyl GHAs against the ureolytic activity of *P. mirabilis* in human urine was several times higher than that against the urease activity of sword bean in the phosphate buffer. This marked difference in inhibitory potency might be attributable to the competition between the hydroxamic acids and urea²⁾ existing originally at about 0.3 M in urine, or to the difference in enzymatic properties between plant and bacterial ureases, and/or to permeability of the hydroxamic acids across the cell membrane of the bacterium because urease is an intracellular enzyme, whereas the bacterium could not degrade the hydroxamic acids as verified experimentally.

Urinary excretion rates of *n*-aliphatic acyl GHAs in rats were markedly lower than those of branched aliphatic acyl GHAs, suggesting that metabolic degradation rates of the former in the liver are more rapid than those of the latter. 2,2-Dimethylpropionyl GHA of fatty acyl GHAs had the weakest inhibitory action and the highest urinary excretion rate, probably due to a steric hindrance for the binding with the active site of the enzyme and an interference for the metabolic degradation by the *tert*-butyl group. As men-

tioned above, about 90% of the unchanged 2,2-dimethylpropionyl GHAs was excreted in urine within 2 hr after the oral administration, and there was no significant difference in the urinary excretion rate between the oral and the intraperitoneal administrations. These observations indicate that this compound was completely absorbed from the upper-gastrointestinal tracts and rapidly excreted in the urine.

In our previous report,⁵⁾ the effective index which is a ratio of the urinary excretion rate (%) to the apparent I_{50} value (μM) of a hydroxamic acid was employed for the evaluation of the compounds. Among the acyl GHAs tested, the indexes of branched acyl GHAs were higher: those of DL 2-methyl-*n*-butyroyl, 2-ethyl-*n*-butyroyl- and 2,2-dimethylpropionyl GHA were calculated to be 0.85, 0.40 and 0.24, respectively, wherein the I_{50} values against the ureolytic activity of the bacterium were used. As described above, when 2,2-dimethylpropionyl GHA was orally administered with the dose of 20 mg/rat, its concentrations in 0–2 hr-, 2–4 hr- and 4–6 hr-urine were 6.070, 610 and 210 μM , respectively. On the other hand, its I_{50} value against the ureolytic activity of the bacterium was 45 μM , suggesting that its urinary concentration is enough for the inhibition of the ureolytic activity of the bacteria in the urinary tract infections. In fact, the treatment of these acyl GHAs has been demonstrated to be effective for the prevention of the experimental development of urinary phosphate stones in rats (unpublished).

Furthermore, most of the hydroxamic acids examined in the present report showed no mutagenicity in the Ames assay test system (TA 98, TA 100).⁶⁾ Therefore, these compounds are expected to be prospective therapeutic agents for the treatment of phosphate stones.

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