

# Antimikrobna aktivnost N-ftaloil-aminokiselinskih hidroksamata

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## Antimicrobial activity of *N*-phthaloylamino acid hydroxamates

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Antibacterial and antifungal activity of *N*-phthaloylamino acid hydroxamates **1–3** [ $C_6H_4(CO)_2N-X-CONHOH$ , X = amino acid residues of glycine,  $\beta$ -alanine or *D*-phenylglycine], was examined against 44 strains of Gram-positive and Gram-negative bacteria, and 10 species of yeasts. The level of antimicrobial activity was established using the *in vitro* agar assay and the standard broth dilution susceptibility test. *N*-phthaloyl-*D*-phenylglycine-hydroxamic acid (**3**), the substance with the highest lipophilicity ( $\log P$ ), showed the best antibacterial activity, especially against Gram-negative bacteria. Minimum inhibitory concentration of **3** was  $0.008 \text{ mg mL}^{-1}$  in the activity against *Yersinia enterocolitica* O3, confirmed by a large inhibition zone (30 mm) by the diffusion test. Hydroxamates inhibit growth by chelation of the PDF enzyme metal in both Gram-positive and Gram-negative bacteria, and LpxC enzyme in Gram-negative enzyme. Phthalimides appear to contribute to inhibition by destabilizing m-RNA. Antifungal activity of substances **1–3** is not very expressed.

**Keywords:** hydroxamic acid, phthalimide, antibacterial activity, lipophilicity

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*N*-phthaloylamino acid hydroxamates of general formula  $C_6H_4(CO)_2N-X-CONHOH$  (X = amino acid residue) contain two biologically active groups in their structures, phthalimido and *N*-hydroxyamido.

Phthalimides have been known for a long time as plant growth regulators (1–3), bacteriostatics (4, 5) and fungicides (6). Thalidomide is the best-known phthalimide, a hypnotic/sedative drug with teratogenic effect. Nevertheless, thalidomide has never completely vanished as a therapeutic substance. The drug was found to have a powerful anti-inflammatory effect owing to its ability to inhibit the production of the cytokine tumour necrosis factor alpha (TNF- $\alpha$ ), a potent stimulator of inflammation, cellular necrosis and tissue damage in general (7). A recently discovered anticancer activity of thalidomide is based on inhibition of the growth of new vessels, the process of angiogenesis (8). Thalidomide is being increasingly used in the clinical management of a wide spectrum of im-

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munologically-mediated and infectious diseases and cancers such as erythema nodosum leprosum, multiple myeloma, renal and intestinal carcinoma, Behçet's, Crohn's and a number of dermatologic diseases, rheumatoid arthritis and wasting syndrome in AIDS (9–15). It is also effective for mycobacterial infection in the central nervous system such as tuberculous meningitis caused by *Mycobacterium bovis* or *Mycobacterium bacillus* (16). The way phthalimide reduces TNF- $\alpha$  production is associated with induction of the degradation process of TNF-alpha m-RNA (17–20).

The other active part in phthalimidohydroxamate structures is the *N*-hydroxyamido group (hydroxamic acid). Some natural hydroxamic acids, products of various microorganisms and fungi, act as growth factors or possess antitumour and antibacterial activity (21). In biomedical sciences, hydroxamic acid moieties are used in the design of therapeutics targeted at cancer, cardiovascular diseases, Alzheimer's disease, malaria, allergic and infective diseases, metal poisoning and other metal overload diseases, e.g. after transfusions in the genetic blood disease Cooley's anemia (22).

It seems that the powerful biological activity of structurally heterogeneous hydroxamic acids is related to their common ability to form very stable chelates with a variety of metal ions. By blocking the metal ion in the active centre of enzyme, they can inhibit a number of metalloenzymes. The process of enzymes inactivation, as supposed earlier, involves redox reactions, e.g. inhibition of lipoxygenase is a result of the reduction of Fe(III) to Fe(II) ions (23). Kinetic and EPR spectral evidence disputed such opinion (24). Some important Zn(II) containing enzymes easily inhibited by hydroxamates are matrix metalloproteinases (MMPs), angioconverting enzyme (ACE) and leukotriene A<sub>4</sub> hydrolase (LTA<sub>4</sub>). Over-expression of MMPs has been implicated in a number of diseases, including arthritis, multiple sclerosis and various human cancers. Dimartino and coauthors explained the anti-arthritic activity of hydroxamates by two parallel inhibitions, TNF- $\alpha$  and metalloproteinase (25). ACE enzyme plays a key role in the control of blood pressure and the LTA<sub>4</sub> enzyme is thought to be a pro-inflammatory mediator. Anti-inflammatory activity of hydroxamates includes inhibitions of 5-lipoxygenase (5-LO) and cyclooxygenase (COX), which are iron-containing metalloenzymes. The hydroxamate derivatives of indometacin and ibuprofen appear to be dual inhibitors, of 5-LO and COX (23).

Antimicrobial activity of hydroxamates includes inhibition of enzymes necessary for the growth of bacteria or yeasts as well as enzymes, the causes of pathogenicity. Urease, product of *Helicobacter pylori* (HP), is considered to be a major causative fact in peptic ulcer diseases and it is also central to the virulence of *Proteus mirabilis* and *Klebsiella aerogenes*. Ammonia produced by strong HP urease elevates the pH level in the stomach and breaks gastric mucus, while the ammonia itself inhibits the consumption of oxygen and reduces the production of ATP in gastric mucous cells or in the mitochondria. Ureasases are also implicated in the infection-induced urinary stones, pyelonephritis and hepatic encephalopathy. Urease is a nickel-dependent metalloenzyme very effectively inhibited by hydroxamates (26–29).

Studies of hydroxamate dependent inhibition of *Escherichia coli*, as well as *Pseudomonas aeruginosa* and *Aquifex aeolicus*, have established that the main inhibitor-sensitive enzyme in Gram-negative bacteria is LpxC [UDP-3-(*R*-3-hydroxymyristoyl)-*N*-acetylglucosamine-deacetylase]. It is essential for the removal of acetyl group from 2-*N* position of the lipid A precursor. Lipid A is part of lipopolysaccharide – a causative agent of pa-

thogenicity in the outer membrane of Gram-negative bacteria. Enzyme LpxC is Zn(II) amidase with zinc binding motifs not found in other zinc metalloenzymes (30–34).

Two enzymes are involved in the process of post-translational protein modifications. The first, peptide deformylase (PDF), is believed to be an essential enzyme in both Gram-positive and Gram-negative bacteria and the second, methionine amino peptidase (MAP), is not essential. PDF enzyme catalyzes the removal of the *N*-formyl group from *N*-formyl-methionine. It is an iron(II)-containing metalloenzyme, not present in eukaryotic protein synthesis (23). Although several different chelating groups for iron in the PDF enzyme have been described, hydroxamate remains the preferred group (35–37).

## EXPERIMENTAL

### Chemistry

For antimicrobial examinations in this work, *N*-phthaloyl-amino acid hydroxamates 1–3 (Fig. 1) were synthesized from the amino acids glycine,  $\beta$ -alanine and D-phenylglycine in three steps. Amino acids were phthaloylated by phthalanhydride to *N*-phthaloylamino acids, which were converted by thionyl chloride to *N*-phthaloyl-aminoacylchlorides and then translated into hydroxamic acids by hydroxylamine hydrochloride. Preparations were carried out according to our previously published method (38).

Solutions of samples for antimicrobial testing were made in water, in concentration range 2–4 mg mL<sup>-1</sup>. In dissolution of compound 3, a small amount of dimethylsulfoxide was added (40  $\mu$ L in 80 mL water). Glycine-hydroxamic acid used as standard was purchased from the Sigma company (USA).

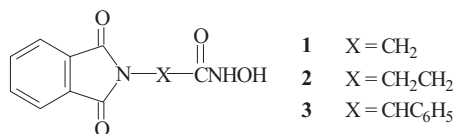


Fig. 1. The structure of *N*-phthaloyl-glycine hydroxamic acid (1), *N*-phthaloyl- $\beta$ -alanine hydroxamic acid (2) and *N*-phthaloyl-D-phenylglycine hydroxamic acid (3).

### Microbiological tests

Antimicrobial activity testing was performed with 44 strains of Gram-positive and Gram-negative bacteria and 10 species of yeasts from the collection of the Department of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb. The bacterial growth inhibition assays were carried out by the diffusion test and the dilution susceptibility test. The agar diffusion method was applied according to the European Pharmacopoeia (39). Testing inocula 10<sup>4</sup>–10<sup>5</sup> cells (0.5 mL portion) were swabbed onto solidified Mueller-Hinton agar for bacteria and Sabouraud dextrose agar for yeasts. Steel cylinders (8 x 10 mm) were placed on the agar and water solution of compound 1 or water solution with 0.05 % DMSO of compound 3 were applied in a volume of 0.25 mL. After 2 h

of diffusion at 4 °C, the agar plates were incubated for 18–24 h at either 37 °C for bacteria, or 48 h at 25 °C for yeasts. The diameters of clear inhibition zones around the cylinder were measured after incubation if the tested substance inhibited bacterial or yeast growth.

Inhibitory concentrations (in mg mL<sup>-1</sup>) were determined by the broth dilution susceptibility test. Test strains were grown in a nutrition medium containing progressively lower concentrations of the test substances. Dilutions of samples were made in the range from 1 to 0.004 mg mL<sup>-1</sup> for compounds **1** and **3** and starting from 2 to 0.004 mg mL<sup>-1</sup> for compound **2**. The prepared test dilutions were incubated at 37 °C for bacteria or 25 °C for yeasts. The last tube, as a positive growth control, was free of test compounds. In the case of solution containing DMSO, two growth controls were made, with and without DMSO. A sample was deemed free of viable germs if the nutritive solution appeared clear after incubation. All samples showing no turbidity were subcultured into Mueller-Hinton or Sabouraud agar. Concentrations at which the microorganisms did not grow in fresh medium were bactericidal (BC) or fungicidal (FC) concentrations, *i.e.*, the concentrations that irreversibly kill microorganisms. The lowest concentration among BC is the minimum bactericidal concentration (MBC) while the minimum fungicidal concentration (MFC) is the lowest among FC concentrations.

The subcultures from clear tubes that result in growth into fresh medium lacking test substance show a bacteriostatic (BS) or fungistatic (FS) concentration, which reversibly inhibit growth and do not kill the present microorganisms. The lowest sample concentration that prevents appearance of turbidity was established to be the minimum inhibitory concentration (MIC) and can be cidal or static (40).

## RESULTS AND DISCUSSION

It has been established by the diffusion antibacterial test that *N*-phthaloyl-D-phenylglycine hydroxamic acid (**3**) inhibits 27/30 strains of bacteria in contrast to *N*-phthaloyl-glycine hydroxamic acid (**1**) which inhibits 16/30 strains (Table I). The zones of inhibition were also bigger in compound **3** (average 21.3 mm) compared to those of compound **1** (13.9 mm). The dilution test showed the best activity of compound **3** against *Yersinia enterocolitica* 03 [BS (MIC) = 0.008 mg mL<sup>-1</sup>; BC (MBC) = 0.016 mg mL<sup>-1</sup>], some strains of *Enterococcus* sp. (MIC = 0.008 mg mL<sup>-1</sup>; MBC = 0.016 mg mL<sup>-1</sup>) and *Sarcina lutea* (MIC = 0.016 mg mL<sup>-1</sup>; MBC = 0.031 mg mL<sup>-1</sup>) (Table II). Compound **1** showed the best activity against *Enterococcus* sp. (for strains ER1 and ER4 MIC = 0.016 mg mL<sup>-1</sup>) and *Sarcina lutea* (MIC = 0.063 mg mL<sup>-1</sup>) (Table III). Dilution test of *N*-phthaloyl-β-alanine hydroxamic acid (**2**) showed moderate or low activity against 15/15 bacteria, the best of which were against *Sarcina lutea* (MIC = 0.063 mg mL<sup>-1</sup>; MBC = 0.125 mg mL<sup>-1</sup>) (Table IV). *Enterococcus faecalis* showed with compound **2** the same MIC values, but the measured MBC concentration was very high (2 mg mL<sup>-1</sup>).

The influence of compounds **1–3** on the growth of yeasts, especially *Candida*, is shown in Table V. Growth inhibitions were not prominent. The lowest inhibitory concentration was 0.063 mg mL<sup>-1</sup> determined for compound **3** against *Candida parapsilosis*. The growth control of the water solution with 0.05 vol. % DMSO (used for dissolution of compound **3**) was positive for bacteria and yeasts.

Table I. Antibacterial activity of compounds **1** and **3** (diffusion test)

Bacteria	Inhibition zone (mm)		
	<b>1</b>	<b>3</b>	
Gram-positive	<i>Bacillus cereus</i> ATCC 11778	–	21
	<i>Bacillus subtilis</i> NCTC 8236	16	–
	<i>Sarcina flava</i> R28	20	30
	<i>Sarcina lutea</i> ATCC 9341	8	23
	<i>Staphylococcus aureus</i> ATCC 6538P	13	20
	<i>Staphylococcus aureus</i> SR2	12	19
	<i>Staphylococcus aureus</i> SR5	15	21
	<i>Staphylococcus aureus</i> SJ3	11	22
	<i>Staphylococcus aureus</i> SJ4	13	18
	<i>Enterococcus</i> sp. ER1	16	21
	<i>Enterococcus</i> sp. ER2	15	20
	<i>Enterococcus</i> sp. ER3	16	22
	<i>Enterococcus</i> sp. ER4	17	19
	<i>Enterococcus</i> sp. ER5	15	21
	<i>Listeria monocytogenes</i>	–	–
Gram-negative	<i>Klebsiella oxytoca</i> 1	12	32
	<i>Salmonella enteritidis</i>	17	–
	<i>Proteus mirabilis</i> 1	–	11
	<i>Proteus mirabilis</i> 1A	–	9
	<i>Proteus mirabilis</i> 1B	–	9
	<i>Proteus rettgeri</i>	–	10
	<i>Proteus vulgaris</i>	–	12
	<i>Proteus</i> sp.	–	13
	<i>Escherichia coli</i> R 16	–	30
	<i>Escherichia coli</i> R30	–	29
	<i>Escherichia coli</i> R19	–	30
	<i>Escherichia coli</i> 0157; A2	–	31
	<i>Escherichia coli</i> 0128; B12	–	30
	<i>Pseudomonas aeruginosa</i>	–	21
	<i>Yersinia enterocolitica</i> O3	7	30

The differences in inhibition of investigated *N*-phthaloylamino acid hydroxamates can be explained by the level of lipophilicity ( $\log P$ , octanol/water partition coefficient) of structures **1**–**3**. In general, the most lipophilic compound, *N*-phthaloyl-D-phenylglycine hydroxamic acid (**3**,  $\log P = -6.776$ ), showed the highest inhibition and the least lipophilic compound *N*-phthaloyl-glycine hydroxamic acid (**1**,  $\log P = -8.367$ ) the weakest one. Compound **2** (*N*-phthaloyl- $\beta$ -alanine hydroxamic acid,  $\log P = -7.837$ ) gave the expected values between compounds **3** and **1**, closer to **1** (41). For example, *BS* (*MIC*) for compound **3** was 0.008, for **2** it was 0.125 and for compound **1**, 0.500 mg mL<sup>-1</sup> for *Yersinia*. The results for *Pseudomonas aeruginosa* were as follows: 0.250 for compound **3** and 0.500 mg mL<sup>-1</sup> for compound **2**, while hydroxamic acid **1** showed no inhibition. In some cases compound **2** was more active than **1**, but never compared to compound **3**.

Table II. Bactericidal (BC) and bacteriostatic (BS) concentrations of *N*-phthaloyl-*D*-phenylglycine hydroxamic acid (**3**)

Bacteria	Concentration (mg mL <sup>-1</sup> )								
	1.000	0.500	0.250	0.125	0.063	0.031	0.016	0.008	0.004
Gram-positive bacteria									
<i>Bacillus cereus</i> ATCC11778	BC <sup>a</sup>	BS <sup>b</sup>	+	+	+	+	+	+	+
<i>Sarcina flava</i> R28	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BS <sup>b</sup>	+	+	+	+
<i>Sarcina lutea</i> ATCC 9341	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC	BS <sup>b</sup>	+	+
<i>Staphylococcus aureus</i> ATCC6538P	BC <sup>a</sup>	BC <sup>a</sup>	BS <sup>b</sup>	+	+	+	+	+	+
<i>Staphylococcus aureus</i> SR2	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BS <sup>b</sup>	+	+	+	+	+
<i>Staphylococcus aureus</i> SR5	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BS <sup>b</sup>	+	+	+	+	+
<i>Staphylococcus aureus</i> SJ3	BC <sup>a</sup>	BC <sup>a</sup>	BS <sup>b</sup>	+	+	+	+	+	+
<i>Staphylococcus aureus</i> SJ4	BC <sup>a</sup>	BC <sup>a</sup>	BS <sup>b</sup>	+	+	+	+	+	+
<i>Enterococcus</i> sp. ER1	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC	BS <sup>b</sup>	+	+
<i>Enterococcus</i> sp. ER2	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BS <sup>b</sup>	+
<i>Enterococcus</i> sp. ER3	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC	BS <sup>b</sup>	+	+
<i>Enterococcus</i> sp. ER4	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC	BS <sup>b</sup>	+	+
<i>Enterococcus</i> sp. ER5	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BS <sup>b</sup>	+
<i>Listeria monocytogenes</i>	BC <sup>a</sup>	BS <sup>b</sup>	+	+	+	+	+	+	+
Gram-negative bacteria									
<i>Klebsiella oxytoca</i>	BC <sup>a</sup>	BS <sup>b</sup>	+	+	+	+	+	+	+
<i>Proteus mirabilis</i> 1	BC <sup>a</sup>	BS <sup>b</sup>	+	+	+	+	+	+	+
<i>Proteus mirabilis</i> 1A	BC <sup>a</sup>	BS <sup>b</sup>	+	+	+	+	+	+	+
<i>Proteus rettgeri</i>	BC <sup>a</sup>	BC <sup>a</sup>	BS <sup>b</sup>	+	+	+	+	+	+
<i>Proteus vulgaris</i>	BC <sup>a</sup>	BC <sup>a</sup>	BS <sup>b</sup>	+	+	+	+	+	+
<i>Proteus</i> sp.	BC <sup>a</sup>	BS <sup>b</sup>	+	+	+	+	+	+	+
<i>Esherichia coli</i> R16	BC <sup>a</sup>	BC <sup>a</sup>	BS <sup>b</sup>	+	+	+	+	+	+
<i>Esherichia coli</i> R30	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BS <sup>b</sup>	+	+	+	+	+
<i>Esherichia coli</i> R 19	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BS <sup>b</sup>	+	+	+	+	+
<i>Esherichia coli</i> 0157; A2	BC <sup>a</sup>	BC <sup>a</sup>	BS <sup>b</sup>	+	+	+	+	+	+
<i>Esherichia coli</i> 0128; B12	BC <sup>a</sup>	BC <sup>a</sup>	BS <sup>b</sup>	+	+	+	+	+	+
<i>Yersinia enterocolitica</i> O3	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BC <sup>a</sup>	BS <sup>b</sup>	+

+ Growth of bacteria.

a Total inhibition of growth.

b Partial inhibition of growth (here identical to MIC).

The big difference between the behaviour of the most lipophilic structure **3** and the least lipophilic **1** against Gram-negative bacteria is noteworthy. The fact that the more lipophilic substance showed better inhibition can be explained by its easier passing through the lipophilic membrane where it inhibits the LpxC enzyme. To be useful against the LpxC enzyme, an inhibitor should recognize Zn coordination and the surfaces in the enzyme active site – a hydrophobic tunnel occupied by appropriately positioned fatty acid substituents.

Table III. Bactericidal (BC) and bacteriostatic (BS) concentrations of *N*-phthaloyl-glycine hydroxamic acid (1)

Bacteria	Concentration (mg mL <sup>-1</sup> )							
	1.000	0.500	0.250	0.125	0.063	0.031	0.016	0.008
Gram-positive bacteria								
<i>Bacillus subtilis</i> NCTC8236	BC	BS	+	+	+	+	+	+
<i>Sarcina flava</i> R28	BC	BC	BS	+	+	+	+	+
<i>Sarcina lutea</i> ATCC 9341	BC	BC	BC	BC	BS	+	+	+
<i>Staphylococcus aureus</i> ATCC 6538P	BC	BS	BS	+	+	+	+	+
<i>Staphylococcus aureus</i> SR2	BC	BC	BS	+	+	+	+	+
<i>Staphylococcus aureus</i> SR5	BC	BC	BS	+	+	+	+	+
<i>Staphylococcus aureus</i> SR3	BC	BS	+	+	+	+	+	+
<i>Staphylococcus aureus</i> SR4	BC	BS	+	+	+	+	+	+
<i>Enterococcus</i> sp. ER1	BC	BC	BC	BC	BC	BS	+	+
<i>Enterococcus</i> sp. ER2	BC	BC	BC	BC	BC	BC	BS	+
<i>Enterococcus</i> sp. ER3	BC	BC	BC	BC	BC	BS	+	+
<i>Enterococcus</i> sp. ER4	BC	BC	BC	BC	BC	BC	BS	+
<i>Enterococcus</i> sp. ER5	BC	BC	BC	BC	BC	BS	+	+
Gram-negative bacteria								
<i>Klebsiella oxytoca</i>	BC	BS	+	+	+	+	+	+
<i>Salmonella enteritidis</i>	+	+	+	+	+	+	+	+
<i>Yersinia enterocolitica</i> O3	BC	BS	+	+	+	+	+	+

+ Growth of bacteria.

Structure-affinity relationships for aliphatic LpxC inhibitors with saturated C<sub>6</sub>–C<sub>12</sub> chain revealed that the more lipophilic inhibitor, with longer chain lengths of C<sub>10</sub>, exhibited significant binding affinity for *Aquifex Aeolicus* Lpxc to chain C<sub>6</sub> (33). Stronger LpxC inhibition does not automatically mean better antibacterial activity. Very hydrophobic groups often exhibit excellent enzyme inhibitory activity while showing little to no antibacterial activity. Such steric bulky groups may become trapped in the cell membrane, where they can associate with bacterial lipid bilayers, bind to bacterial proteins, interact with some enzymes or receptors, resulting in low inhibitor concentrations in the cytoplasm. Despite the fact that antibacterial and LpxC enzyme inhibitory properties are not strictly correlated, it is most important that there are no compounds that exhibit significant antibacterial activity but lack enzyme inhibitory activity (30).

Antibacterial activity of *N*-phthaloyl-amino acid hydroxamates 1–3 against both Gram-positive and Gram-negative bacteria can be explained by inhibition of the PDF enzyme. Comparison of the results obtained from isolated PDF and the whole cell system showed that inhibitions were similar. Thus, the whole cell antibacterial activity could be mainly attributed to the inhibition of PDF enzyme as the most important enzyme in protein



Table IV. Bactericidal (BC) and bacteriostatic (BS) concentrations of *N*-phthaloyl- $\beta$ -alanine hydroxamic acid (2)

Bacteria	Concentration (mg mL <sup>-1</sup> )						
	2.000	1.000	0.500	0.250	0.125	0.063	0.031
Gram-positive bacteria							
<i>Bacillus subtilis</i>	BS	+	+	+	+	+	+
<i>Bacillus cereus</i>	BS	BS	+	+	+	+	+
<i>Bacillus pumilus</i>	BS	+	+	+	+	+	+
<i>Sarcina lutea</i>	BC	BC	BC	BC	BC	BS	+
<i>Staphylococcus aureus</i>	BC	BC	BS	+	+	+	+
<i>Enterococcus</i> sp.	BS	BS	BS	BS	+	+	+
<i>Enterococcus faecalis</i>	BC	BS	BS	BS	BS	BS	+
<i>Listeria monocytogenes</i>	BC	BS	BS	+	+	+	+
Gram-negative bacteria							
<i>Escherichia coli</i>	BC	BS	BS	BS	BS	+	+
<i>Salmonella enteritidis</i>	BC	BC	BS	+	+	+	+
<i>Klebsiella oxytoca</i>	BC	BS	BS	BS	+	+	+
<i>Pseudomonas aeruginosa</i>	BC	BS	BS	+	+	+	+
<i>Proteus mirabilis</i>	BC	BS	BS	+	+	+	+
<i>Yersinia</i> sp.	BC	BC	BC	BC	BS	+	+
<i>Serratia</i> sp.	BC	BS	BS	BS	+	+	+

+ Growth of bacteria.

synthesis of bacteria. Quantitative structure relationship (QSAR) studies demonstrated the importance of small lipophilic substituents for increasing inhibitory activity, but not of bulky lipophilic ones (23, 37). The barriers determined by the volume of  $\alpha$ -aminoacyl hydroxamates were established also by studying HP urease inhibition (23, 36).

From the mentioned results on structure-inhibition relationships from the references, it can be seen that inhibition, in general, increases with lipophilicity but is sterically limited. With regard to the fact that antibacterial activity and inhibition of the PDF enzyme are almost identical and, what is more, this enzyme participates in all bacteria, it can be concluded that the inhibitions of bacteria with *N*-phthaloyl-amino acid hydroxamates 1–3, presented in this work, are caused first by inhibition of PDF enzyme. The presented results further indicate participation of LpxC enzyme inhibition because of the great difference in the behavior of Gram-negative bacteria. The process of inhibition also includes other metalloenzymes, certainly some specific urease in some Gram-negative bacteria.

The presented results of inhibition dependence on the hydrophobicity of hydroxamate structures 1–3 are in agreement with the published ones.

In general, hydroxamic acids are capable of inhibiting bacterial enzymes due to their high chelating affinity towards metal ion in the active centre of the enzyme (28, 32,

Table V. Fungicidal (FC) and fungistatic (FS) activity against yeasts of *N*-phthaloyl-amino acid hydroxamates 1–3

Yeasts	Compd. No.	Concentration (mg mL <sup>-1</sup> )					
		1.000	0.500	0.250	0.125	0.063	0.031
<i>Candida tropicalis</i>	1	FC <sup>a</sup>	FC <sup>a</sup>	FS <sup>b</sup>	+	+	+
	2	FC <sup>a</sup>	FC <sup>a</sup>	FC <sup>a</sup>	FS <sup>b</sup>	+	+
	3	FC <sup>a</sup>	FS <sup>b</sup>	FS <sup>b</sup>	+	+	+
<i>Candida albicans</i>	1	FS <sup>b</sup>	+	+	+	+	+
	2	FC <sup>a</sup>	FC <sup>a</sup>	FS <sup>b</sup>	FS <sup>b</sup>	+	+
	3	FC <sup>a</sup>	FS <sup>b</sup>	FS <sup>b</sup>	+	+	+
<i>Candida glabrata</i>	1	FC <sup>a</sup>	FS <sup>b</sup>	+	+	+	+
	2	FC <sup>a</sup>	FS <sup>b</sup>	+	+	+	+
	3	FC <sup>a</sup>	FS <sup>b</sup>	+	+	+	+
<i>Candida kefyr</i>	1	FC <sup>a</sup>	FC <sup>a</sup>	FC <sup>a</sup>	FC <sup>a</sup>	+	+
	2	FS <sup>b</sup>	FS <sup>b</sup>	+	+	+	+
	3	FC <sup>a</sup>	FC <sup>a</sup>	FC <sup>a</sup>	FC <sup>a</sup>	+	+
<i>Candida parapsilosis</i>	1	FS <sup>b</sup>	FS <sup>b</sup>	+	+	+	+
	2	FC <sup>a</sup>	FS <sup>b</sup>	+	+	+	+
	3	FC <sup>a</sup>	FC <sup>a</sup>	FC <sup>a</sup>	FC <sup>a</sup>	FS <sup>b</sup>	+
<i>Cryptococcus neoformans</i>	1	FC <sup>a</sup>	+	+	+	+	+
	2	FC <sup>a</sup>	FS <sup>b</sup>	+	+	+	+
	3	FC <sup>a</sup>	FC <sup>a</sup>	FC <sup>a</sup>	FC <sup>a</sup>	+	+
<i>Candida krusei</i>	2	FC <sup>a</sup>	FS <sup>b</sup>	+	+	+	+
<i>Geotrichum</i> sp.	2	FS <sup>b</sup>	FS <sup>b</sup>	+	+	+	+
<i>Saccharomyces cerevisiae</i>	2	FS <sup>b</sup>	+	+	+	+	+
<i>Hansenula anomala</i>	2	FS <sup>b</sup>	+	+	+	+	+

+ Growth of yeasts.

<sup>a</sup> Total inhibition of growth.<sup>b</sup> Partial inhibition of growth (identical to MIC).

37). Phthalimides contribute to bacterial growth inhibition by mechanisms that are not quite clear, probably by destabilizing m-RNA of bacterial enzymes (10, 17, 18).

To find if phthalimido or hydroxamato groups contribute to better inhibition, the influence of *N*-phthaloyl-glycine and glycine hydroxamic acid on the growth of bacteria was also examined. As expected, the inhibition of most bacteria was stronger with *N*-phthaloyl-glycine hydroxamic acid (1) than with *N*-phthaloyl-glycine or with glycine hydroxamic acid. A similar result was obtained for the growth inhibition of *Lepidium sativum* L. (38). According to the differences in activity between hydroxamato and phthalimido moieties, phthalimido structure was more effective against some bacteria, hydroxamato structure against the other.

Further, no regularity among activities against yeasts was found for compounds 1–3.

## CONCLUSIONS

The examined *N*-phthaloylamino acid hydroxamates 1–3 showed a broad antibacterial activity, especially the activity of the most lipophilic compound, *N*-phthaloyl-*D*-phenylglycine hydroxamic acid (3). MIC was 0.008 mg mL<sup>-1</sup> for compound 3 against *Yersinia enterocolitica* O3, confirmed by a large inhibition zone (30 mm) in the diffusion test.

The inhibition of both Gram-positive and Gram-negative bacteria by the examined phthalimido-hydroxamates can be contributed to inhibition of probably the most important enzyme for the growth of bacteria, the PDF enzyme. Owing to the fact that this enzyme is not involved in the eukariotic cytoplasmatic protein synthesis and therefore PDF inhibited bacteria can not develop resistance against antimicrobial agents, PDF is a potentially attractive target for antibacterial drug design.

The inhibition of Gram-negative bacteria by the examined substances includes also inactivation of the LpxC enzyme, the key of endotoxin, as well as some specific enzymes e.g. urease, one of exotoxins. Owing to its inhibitory activity depending on lipophilicity, *N*-phthaloyl-*D*-phenylglycine hydroxamic acid (3) was established to possess the best antimicrobial activity against Gram-negative bacteria.

The results of the antibacterial investigations described in this work are in agreement with some earlier published data and provide another contribution to the structure-antibacterial activity relationship.

The inhibitory activity of tested substances against yeasts was rather weak. The best MIC value (0.063 mg mL<sup>-1</sup>) was obtained in the determination of compound 3 activity against *Candida parapsilosis*.

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S A Ž E T A K

**Antimikrobna aktivnost *N*-ftaloil-aminokiselinskih hidroksamata**

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Ispitano je djelovanje *N*-ftaloil-aminokiselinskih hidroksamata 1–3 opće formule  $C_6H_4(CO)_2N-X-CONHOH$  ( $X$  = aminokiselinski ostatak glicina,  $\beta$ -alanina ili *D*-fenilglicina) na 44 soja Gram-pozitivnih i Gram-negativnih bakterija i 10 vrsta kvasaca. Antibakterijski i antifungalni učinak testiran je postupkom difuzije na hranjivom agaru i standardnom metodom dilucije. Najbolja inhibicija rasta, osobito izražena prema Gram-negativnim bakterijama, utvrđena je za *N*-ftaloil-*D*-fenilglicin hidroksamsku kiselinu (3) s najvećom lipofilnošću. Za soj *Yersinia enterocolitica* O3 minimalna inhibitorna koncentracija ( $0,008 \text{ mg mL}^{-1}$ ) potvrđena je inhibicijskom zonom od 30 mm pomoću difuzijskog testa. Hidroksamati inhibiraju rast keliranjem metala PDF enzima kod Gram-pozitivnih i Gram-negativnih bakterija, te LpxC enzima kod Gram-negativnih bakterija. Ftalimido struktura pridonosi inhibiciji, pretpostavlja se, destabilizacijom *m*-RNA. Antifungalna aktivnost spojeva 1–3 nije osobito izražena.

*Ključne riječi:* hidroksamska kiselina, ftalimid, antibakterijsko djelovanje, lipofilnost

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