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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** [$C_6H_4(CO)_2$N-X-CONHOH, X = amino acid residues of glycine, β-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 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.

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N-phthaloylamino acid hydroxamates of general formula $C_6H_4(CO)_2$N-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 phtalimide, 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-α), 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-α 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_4 hydrolase (LTA_4). Over-expression of MMPs has been implicated in a number of diseases, including arthritis, multiple sclerosis and various human cancers. Dimartino and coautors explained the anti-arthritic activity of hydroxamates by two parallel inhibitions, TNF-α and metalloproteinase (25). ACE enzyme plays a key role in the control of blood pressure and the LTA_4 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. Ureases 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 hydroxamato 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).

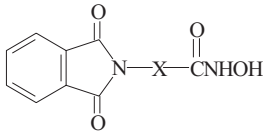
Two enzymes are involved in the process of post-translational protein modifications. The first, peptide deformilase (PDF), is believed to be an essential enzyme in both Gram-positive and Gram-negative bacteria and the second, methionine amino peptidase (MAP), is not essential. PDF enzyme catalyzes the 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 antimicrobic examinations in this work, *N*-phthaloyl-amino acid hydroxamates **1–3** (Fig. 1) were synthesized from the amino acids glycine, β-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^{-1}. In dissolution of compound **3**, a small amount of dimethylsulfoxyde was added (40 μL in 80 mL water). Glycine-hydroxamic acid used as standard was purchased from the Sigma company (USA).



1 X = CH_2
2 X = CH_2CH_2
3 X = CHC_6H_5

Fig. 1. The structure of *N*-phthaloyl-glycine hydroxamic acid (**1**), *N*-phthaloyl-β-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 10^4–10^5 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^{-1}) 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^{-1} for compounds **1** and **3** and starting from 2 to 0.004 mg mL^{-1} 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 nutritient 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^{-1}; *BC* (*MBC*) = 0.016 mg mL^{-1}], some strains of *Enterococcus* sp. (*MIC* = 0.008 mg mL^{-1}; *MBC* = 0.016 mg mL^{-1}) and *Sarcina lutea* (*MIC* = 0.016 mg mL^{-1}; *MBC* = 0.031 mg mL^{-1}) (Table II). Compound **1** showed the best activity against *Enterococcus sp.* (for strains ER1 and ER4 *MIC* = 0.016 mg mL^{-1}) and *Sarcina lutea* (*MIC* = 0.063 mg mL^{-1}) (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^{-1}; *MBC* = 0.125 mg mL^{-1}) (Table IV). *Enterococcus faecalis* showed with compound **2** the same *MIC* values, but the measured *MBC* concentration was very high (2 mg mL^{-1}).

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^{-1} 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)	
		1	**3**
Gram-positive	*Bacillus cereus* ATCC 11778	–	21
	Bacillus subtilis NCTC 8236	16	–
	Sarcina flava R28	20	30
	Sarcina lutea ATCC 9341	8	23
	Staphylococcus aureus ATCC 6538P	13	20
	Staphylococcus aureus SR2	12	19
	Staphylococcus aureus SR5	15	21
	Staphylococcus aureus SJ3	11	22
	Staphylococcus aureus SJ4	13	18
	Enterococcus sp. ER1	16	21
	Enterococcus sp. ER2	15	20
	Enterococcus sp. ER3	16	22
	Enterococcus sp. ER4	17	19
	Enterococcus sp. ER5	15	21
	Listeria monocytogenes	–	–
Gram-negative	*Klebsiella oxytoca* 1	12	32
	Salmonella enteritidis	17	–
	Proteus mirabilis 1	–	11
	Proteus mirabilis 1A	–	9
	Proteus mirabilis 1B	–	9
	Proteus rettgeri	–	10
	Proteus vulgaris	–	12
	Proteus sp.	–	13
	Escherichia coli R 16	–	30
	Escherichia coli R30	–	29
	Escherichia coli R19	–	30
	Escherichia coli 0157; A2	–	31
	Escherichia coli 0128; B12	–	30
	Pseudomonas aeruginosa	–	21
	Yersinia enterocolitica 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-β-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^{-1} for *Yersinia*. The results for *Pseudomonas aeruginosa* were as follows: 0.250 for compound **3** and 0.500 mg mL^{-1} 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^{-1})								
	1.000	0.500	0.250	0.125	0.063	0.031	0.016	0.008	0.004
Gram-positive bacteria									
Bacillus cereus ATCC11778	*BC*[a]	*BS*[b]	+	+	+	+	+	+	+
Sarcina flava R28	*BC*[a]	*BC*[a]	*BC*[a]	*BC*[a]	*BS*[b]	+	+	+	+
Sarcina lutea ATCC 9341	*BC*[a]	*BC*[a]	*BC*[a]	*BC*[a]	*BC*[a]	*BC*	*BS*[b]	+	+
Staphylococcus aureus ATCC6538P	*BC*[a]	*BC*[a]	*BS*[b]	+	+	+	+	+	+
Staphylococcus aureus SR2	*BC*[a]	*BC*[a]	*BC*[a]	*BS*[b]	+	+	+	+	+
Staphylococcus aureus SR5	*BC*[a]	*BC*[a]	*BC*[a]	*BS*[b]	+	+	+	+	+
Staphylococcus aureus SJ3	*BC*[a]	*BC*[a]	*BS*[b]	+	+	+	+	+	+
Staphylococcus aureus SJ4	*BC*[a]	*BC*[a]	*BS*[b]	+	+	+	+	+	+
Enterococcus sp. ER1	*BC*[a]	*BC*[a]	*BC*[a]	*BC*[a]	*BC*[a]	*BC*	*BS*[b]	+	+
Enterococcus sp. ER2	*BC*[a]	*BC*[a]	*BC*[a]	*BC*[a]	*BC*[a]	*BC*[a]	*BC*[a]	*BS*[b]	+
Enterococcus sp. ER3	*BC*[a]	*BC*[a]	*BC*[a]	*BC*[a]	*BC*[a]	*BC*	*BS*[b]	+	+
Enterococcus sp. ER4	*BC*[a]	*BC*[a]	*BC*[a]	*BC*[a]	*BC*[a]	*BC*	*BS*[b]	+	+
Enterococcus sp. ER5	*BC*[a]	*BC*[a]	*BC*[a]	*BC*[a]	*BC*[a]	*BC*[a]	*BC*[a]	*BS*[b]	+
Listeria monocytogenes	*BC*[a]	*BS*[b]	+	+	+	+	+	+	+
Gram-negative bacteria									
Klebsiella oxytoca	*BC*[a]	*BS*[b]	+	+	+	+	+	+	+
Proteus mirabilis 1	*BC*[a]	*BS*[b]	+	+	+	+	+	+	+
Proteus mirabilis 1A	*BC*[a]	*BS*[b]	+	+	+	+	+	+	+
Proteus rettgeri	*BC*[a]	*BC*[a]	*BS*[b]	+	+	+	+	+	+
Proteus vulgaris	*BC*[a]	*BC*[a]	*BS*[b]	+	+	+	+	+	+
Proteus sp.	*BC*[a]	*BS*[b]	+	+	+	+	+	+	+
Esherichia coli R16	*BC*[a]	*BC*[a]	*BS*[b]	+	+	+	+	+	+
Esherichia coli R30	*BC*[a]	*BC*[a]	*BC*[a]	*BS*[b]	+	+	+	+	+
Esherichia coli R 19	*BC*[a]	*BC*[a]	*BC*[a]	*BS*[b]	+	+	+	+	+
Esherichia coli 0157; A2	*BC*[a]	*BC*[a]	*BS*[b]	+	+	+	+	+	+
Esherichia coli 0128; B12	*BC*[a]	*BC*[a]	*BS*[b]	+	+	+	+	+	+
Yersinia enterocolitica O3	*BC*[a]	*BC*[a]	*BC*[a]	*BC*[a]	*BC*[a]	*BC*[a]	*BC*[a]	*BS*[b]	+

+ 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^{-1})							
	1.000	0.500	0.250	0.125	0.063	0.031	0.016	0.008
Gram-positive bacteria								
Bacillus subtilis NCTC8236	*BC*	*BS*	+	+	+	+	+	+
Sarcina flava R28	BC	*BC*	*BS*	+	+	+	+	+
Sarcina lutea ATCC 9341	BC	*BC*	*BC*	*BC*	*BS*	+	+	+
Staphylococcus aureus ATCC 6538P	*BC*	*BS*	*BS*	+	+	+	+	+
Staphylococcus aureus SR2	BC	*BC*	*BS*	+	+	+	+	+
Staphylococcus aureus SR5	BC	*BC*	*BS*	+	+	+	+	+
Staphylococcus aureus SR3	*BC*	*BS*	+	+	+	+	+	+
Staphylococcus aureus SR4	*BC*	*BS*	+	+	+	+	+	+
Enterococcus sp. ER1	*BC*	*BC*	*BC*	*BC*	*BC*	*BS*	+	+
Enterococcus sp. ER2	*BC*	*BC*	*BC*	*BC*	*BC*	*BC*	*BS*	+
Enterococcus sp. ER3	*BC*	*BC*	*BC*	*BC*	*BC*	*BS*	+	+
Enterococcus sp. ER4	*BC*	*BC*	*BC*	*BC*	*BC*	*BC*	*BS*	+
Enterococcus sp. ER5	*BC*	*BC*	*BC*	*BC*	*BC*	*BS*	+	+
Gram-negative bacteria								
Klebsiella oxytoca	*BC*	*BS*	+	+	+	+	+	+
Salmonella enteritidis	+	+	+	+	+	+	+	+
Yersinia enterocolitica O3	*BC*	*BS*	+	+	+	+	+	+

+ Growth of bacteria.

Structure-affinity relationships for aliphatic LpxC inhibitors with saturated C_6–C_{12} chain revealed that the more lipophilic inhibitor, with longer chain lengths of C_{10}, exhibited significant binding affinity for *Aquifex Aeolicus* Lpxc to chain C_6 (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*-β-*alanine hydroxamic acid (**2**)*

Bacteria	Concentration (mg mL^{-1})						
	2.000	1.000	0.500	0.250	0.125	0.063	0.031
Gram-positive bacteria							
Bacillus subtilis	*BS*	+	+	+	+	+	+
Bacillus cereus	*BS*	*BS*	+	+	+	+	+
Bacillus pumilus	*BS*	+	+	+	+	+	+
Sarcina lutea	*BC*	*BC*	*BC*	*BC*	*BC*	*BS*	+
Staphylococcus aureus	*BC*	*BC*	*BS*	+	+	+	+
Enterococcus sp.	*BS*	*BS*	*BS*	*BS*	+	+	+
Enterococcus faecalis	*BC*	*BS*	*BS*	*BS*	*BS*	*BS*	+
Listeria monocytogenes	*BC*	*BS*	*BS*	+	+	+	+
Gram-negative bacteria							
Esherichia coli	*BC*	*BS*	*BS*	*BS*	*BS*	+	+
Salmonella enteritidis	*BC*	*BC*	*BS*	+	+	+	+
Klebsiella oxytoca	*BC*	*BS*	*BS*	*BS*	+	+	+
Pseudomonas aeruginosa	*BC*	*BS*	*BS*	+	+	+	+
Proteus mirabilis	*BC*	*BS*	*BS*	+	+	+	+
Yersinia sp.	*BC*	*BC*	*BC*	*BC*	*BS*	+	+
Serratia 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 determinated by the volume of α-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 hydroxamato 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^{-1})					
		1.000	0.500	0.250	0.125	0.063	0.031
Candida tropicalis	**1**	*FC*[a]	*FC*[a]	*FS*[b]	+	+	+
	2	*FC*[a]	*FC*[a]	*FC*[a]	*FS*[b]	+	+
	3	*FC*[a]	*FS*[b]	*FS*[b]	+	+	+
Candida albicans	**1**	*FS*[b]	+	+	+	+	+
	2	*FC*[a]	*FC*[a]	*FS*[b]	*FS*[b]	+	+
	3	*FC*[a]	*FS*[b]	*FS*[b]	+	+	+
Candida glabrata	**1**	*FC*[a]	*FS*[b]	+	+	+	+
	2	*FC*[a]	*FS*[b]	+	+	+	+
	3	*FC*[a]	*FS*[b]	+	+	+	+
Candida kefyr	**1**	*FC*[a]	*FC*[a]	*FC*[a]	*FC*[a]	+	+
	2	*FS*[b]	*FS*[b]	+	+	+	+
	3	*FC*[a]	*FC*[a]	*FC*[a]	*FC*[a]	+	+
Candida parapsilosis	**1**	*FS*[b]	*FS*[b]	+	+	+	+
	2	*FC*[a]	*FS*[b]	+	+	+	+
	3	*FC*[a]	*FC*[a]	*FC*[a]	*FC*[a]	*FS*[b]	+
Criptococus neoformans	**1**	*FC*[a]	+	+	+	+	+
	2	*FC*[a]	*FS*[b]	+	+	+	+
	3	*FC*[a]	*FC*[a]	*FC*[a]	*FC*[a]	+	+
Candida krusei	**2**	*FC*[a]	*FS*[b]	+	+	+	+
Geotrichum sp.	**2**	*FS*[b]	*FS*[b]	+	+	+	+
Saccharomyces cerevisiae	**2**	*FS*[b]	+	+	+	+	+
Hansenula anomala	**2**	*FS*[b]	+	+	+	+	+

+ Growth of yeasts.
[a] Total inhibition of growth.
[b] 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^{-1} 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^{-1}) was obtained in the determination of compound **3** activity against *Candida parapsilosis*.

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REFERENCES

1. O. L. Hoffmann and A. E. Smith, A new group of plant growth regulators, *Science* **109** (1949) 588.
2. H. Koch, Phytopharmakologische Untersuchung von Thalidomid, seinen Metaboliten and einigen strukturverwandten Verbindungen, *Sci. Pharm.* **39** (1971) 209–247.
3. Lj. Butula, N. Kujundžić, M. Malnar and I. Vukušić, Mitodepressive effect of some *N*-substituted phthalimides on *Lepidium sativum* L., *Pharmazie* **30** (1975) 753.
4. T. Midtvet, The effect of thalidomide on the growth curve of a riboflavine-dependent microbe, *Acta Pathol. Microbiol. Scand.* **58** (1963) 355–362.
5. P. Kant and R. K. Saksena, Synthesis and antimicrobial activity of some new 2-phenyl-3-*p*-(2′-methyl-3′-aryl-4′-oxo-thiazolin-2′-yl) phenyl-quinazolin-4-ones and 2-phenyl-3-*p*-(1′-aryl-3-phthalimido-4′-methylazetidin-2′-one-2′-yl) phenyl-quinazolin-4-ones, *Indian J. Het. Chem.* **12** (2003) 315–318.
6. G. L. Kennedy, J. R. D. W. Arnold and M. L. Keplinger, Mutagenicity studies with captan, captofol, folpet and thalidomide, *Food Cosmet. Toxicol.* **13** (1975) 55–61.

7. E. Fernandez-Martinez, M. S. Morales-Rios, V. Perez-Alvarez and P. Muriel, Effects of thalidomide and 3-phthalimido-3-(3,4-dimethoxyphenyl)-propanamide on bile duct obstruction-induced cirrhosis in the rat, *Drug Dev. Res*. **54** (2001) 209–218.
8. E. R. Lepper, S. S. W. Ng, M. Gutschow, M. Weiss, S. Hauschildt, T. K. Hecker, F. A. Luzzio, K. Eger and W. D. Figg, Comparative molecular field analysis and comparative molecular similarity indices analysis of thalidomide analogues as angiogenesis inhibitors, *J. Med. Chem*. **47** (2004) 2219–2227.
9. X. X. Zhu, T. Giordano, Q. S. Yu, H. W. Holloway, T. A. Perry, D. K. Lahiri, A. Brossi and N. H. Greig, Thiothalidomides: Novel isosteric analogues of thalidomide with enhanced TNF-alpha inhibitory activity, *J. Med. Chem.* **46** (2003) 5222–5229.
10. M. E. Franks, G. R. Macpherson and W. D. Figg, Thalidomide, *Lancet* **363** (2004) 1802–1811.
11. M. Sayarlioglu, M. C. Kotan, N. Totcu, H. Arslanturk and A. Gul, Treatment of recurrent perforating intestinal ulcers with thalidomide in Behcet's disease, *Ann. Pharmacother.* **38** (2004) 808– 811.
12. R. Srinivasan and G. R. Lichtenstein, Recent developments in the pharmacological treatment of Crohn's disease, *Expert Opin. Investig. Drugs* **13** (2004) 373–391.
13. K. Q. Lu, S. Brennenman, R. Burns, A. Vink, E. Gaines, A. Haake and A. Gaspari, Thalidomide inhibits UVB-induced mouse keratinocyte apoptosis by both TNF-alpha-dependent and TNF--alpha-independent pathways, *Photoderm. Photoimm. Photomed.* **19** (2003) 272–280.
14. H. R. Gockel, A. Lugering, J. Heidemann, M. Schmidt, W. Domschke, T. Kucharzil and N. Lugering, Thalidomide induced apoptosis in human monocytes by using a cytochrome c-dependent patway, *J. Immunol.* **172** (2004) 5103–5109.
15. A. R. Kerr and J. A. Ship, Management strategies for HIV-associated aphthous stomatitis, *Am. J. Clin. Dermat*. **4** (2003) 669–680.
16. L. Tsenova, A. Bergtold, V. H. Freedman, R. A. Young and G. Kaplan, Tumor necrosis factor alpha is a determinant of pathogenesis and disease progression in mycobacterial infection in the central nervous system, *Proc. Natl. Acad. Sci. USA* **96 (**1999) 5657–5662.
17. Y. S. Kim, J. S. Kim, H. C. Jung and I. S. Song, The effects of thalidomide on the stimulation of NF-kappa B activity and TNF-alpha production by lipopolysaccharide in the human colonic epithelial cell line, *Mol. Cells* **17** (2004) 210–216.
18. A. Orzeszko, J. Vilpo, L. Vilpo and B. Kaminska, Synthesis and anticancer activity of 5′-phthaloyl-nucleosides, *Pharmazie* **58** (2003) 169–172.
19. P. Yogeeswari, D. Sriram, V. Saraswat, J. V. Ragavendran, M. M. Kumar, S. Murugesan, R. Thirumurugan and J. P. Stables, Synthesis and anticonvulsant and neurotoxicity evaluation of *N*-4--phthalimido phenyl (thio) semicarbazides, *Eur. J. Pharm. Sci***. 20** (2003) 341–346.
20. S. Lentzsch, M. S. Rogers, R. LeBlanc, A. E. Birsner, J. H. Shah, A. M. Treston, K. C. Anderson and R. J. D'Amato, *S*-3-amino-phthalimido-glutarimide inhibits angiogenesis and growth of B-cell neoplasias in mice, *Cancer Res.* **62** (2002) 2300–2305.
21. J. N. Neilands, Hydroxamic acids in nature, *Science* **156** (1967) 1443–1447.
22. Z. Eckstein and T. Urbanski, The biological active naturally occurring and synthetic hydroxamic acid derivatives, *Wiad. Chem.* **37** (1983) 348–388.
23. E. M. F. Muri, M. J. Nieto, R. D. Sindelar and J. S. Williamson, Hydroxamic acids as pharmacological agents, *Curr. Med. Chem*. **9** (2002) 1631–1653.
24. I. A. Butovich and C. C. Reddy, Inhibition of potato lipoxygenase by linoleyl hydroxamic acid kinetic and EPR spectral evidence for a two-sted reaction, *Biochem. J*. **365** (2002) 865–871.
25. M. Dimartino, C. Wolff, W. High, G. Stroup, S. Hoffman, J. Laydon, J. C. Lee, D. Bertolini, W. A. Galloway, M. J. Crimmin, M. Davis and S. Davis, Anti-arthritic activity of hydroxamic acid-based pseudopeptide inhibitors of matrix metalloproteinases and TNF-α processing, *Inflamm. Res.* **46** (1997) 211–215.

26. S. Odake, T. Morikawa, M. Tsuchiya, I. Imamura and K. Kobashi, Inhibition of *Helicobacter pylori* urease by hydroxamic acid derivatives, *Biol. Pharm. Bull.* **17** (1994) 1329–1332.
27. M. Arnold, D. A. Brown, O. Deeg, W. Errington, W. Haase, K. Herlihy, T. J. Kemp, H. Nimir and R. Werner, Hydroxamate-bridged dinuclear nickel complexes as models for urease inhibition, *Inorg. Chem.* **37** (1998) 2920–2925.
28. E. M. F. Muri, H. Mishra, S. M. Stein and J. S. Williamson, Molecular modeling, synthesis and biological evaluation of heterocyclic hydroxamic acids designed as *Helicobacter pylori* urease inhibitors, *Lett. Drug Des. Disc.* **1** (2004) 30–34.
29. E. Jabri, M. B. Carr, R. P. Hausinger and P. A. Karplus, The crystal structure of urease from *Klebsiella aerogenes*, *Science* **268** (1995) 998–1004.
30. M. C. Pirrung, L. N. Turney, A. L. McClerren and C. R. H. Raetz, High-throughput catch and release synthesis of oxazoline hydroxamates. Structure-activity relationships in novel inhibitors of *Escherichia coli* LpxC: In vitro enzyme inhibition and antibacterial properties, *J. Am. Chem. Soc.* **125** (2003) 1575–1586.
31. J. E. Jackman, C. R. H. Raetz and C. A. Fierke, Site-directed mutagenesis of the bacterial metalloamidase UDP-(3-*O*-acyl)-*N*-acetylglucosamine deacetylase (LpxC). Identification of the zinc binding site, *Biochemistry* **40** (2001) 514–523.
32. C. P. McClure, K. M. Rusche, K. Peariso, J. E. Jackman, C. A. Fierke and J. E. Penner-Hahn, EXAFS studies of the zinc sites of UDP-(3-*O*-acyl)-*N*-acetylglucosamine deacetylase (LpxC), *J. Inorg. Biochem.* **94** (2003) 78–85.
33. D. A. Whittington, K. M. Rusche, H. Shin, C. A. Fierke and D. W. Christianson, Crystal structure of LpxC, a zinc-dependent deacetylase essential for endoxin biosynthesis, *Proc. Nat. Acad. Sci.* **100** (2003) 8146–8150.
34. C. R. H. Raetz, Bacterial endotoxins: Extraordinary lipids that activate eucaryotic signal transduction, *J. Bacteriology* **175** (1993) 5745–5753.
35. F. Dardel, S. Ragusa, C. Lazennec, S. Blanquet and T. Meinnel, Solution structure of nickel-peptide deformylase, *J. Mol. Biol.* **280** (1998) 501–513.
36. A. Becker, I. Schlichting, W. Kabsch, D. Groche, S. Schultz and A. F. V. Wagner, Iron center, substrate recognition and mechanism of peptide deformylase, *Nat. Struct. Biol.* **5** (1998) 1053–1058.
37. M. K. Gupta, P. Mishra, P. Prathipati and A. K. Saxena, 2D-QSAR in hydroxamic acid derivatives as peptide deformylase inhibitors and antibacterial agents, *Bioorg. Med. Chem.* **10** (2002) 3713–3716.
38. J. Matijević-Sosa and Lj. Butula, Synthesis and mitodepressive activity of some phthalimidoalkanehydroxamic acids, *Acta Pharm.* **43** (1993) 185–194.
39. *European Pharmacopoeia*, 4th ed., Council of Europe, Strasbourg 2002, pp. 160–161.
40. L. M. Prescott, J. P. Harley and D. A. Klein, *Microbiology*, Wm. C. Brown Publishers, Dubuque 1993, pp. 328.
41. S. Nikolić, M. Medić-Šarić and J. Matijević-Sosa, QSPR and QSAR study of phthalimidohydroxamic acids, *Acta Pharm.* **45** (1995) 15–24.

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, β-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 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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