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Antimicrobial activity of some hydroxamic acids

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Several hydroxamic acids, *viz.*, *N*-benzyl-*N*′-hydroxysuccinamide (BHS) (**1**), poly[α,β-(*N*-hydroxy)-DL-aspartamide] (PHA) (**2**), poly[α,β-(*N*-hydroxy-*N*-methyl-DL-aspartamide)] (PMHA) (**3**) and poly [α,β-(*N*-hydroxy)-DL-aspartamide]/poly[α,β-(*N*-2-hydroxyethyl)-DL-aspartamide] (PHA-PHEA 1:1) (**4**) were prepared and screened for their antimicrobial activity. Ten Gram-positive and 7 Gram-negative species of bacteria, 5 *Candida* species, 4 dermatophyte species and 3 mould species were used in tests. Compound **1** showed no antimicrobial activity on any of the tested microorganisms. Other compounds (**2–4**) showed a narrow spectrum of antibacterial activity, but no antifungal activity.

Keywords: hydroxamic acid, polyhydroxamic acid, antimicrobial activity

The current interest in hydroxamic and polyhydroxamic acids is related to the variety of their biological activities, as well as to their role as iron chelators and microbial siderophores (1–3). Antibacterial, antifungal, antitumor and anti-inflammatory activities of hydroxamic acids are connected with their ability to inhibit various enzymes, *viz.*, matrix metalloproteinases (4, 5), 5-lipoxygenase (6, 7), urease (8) or ribonucleotid reductase (9). Some ion exchange resins are based on hydroxamic acids (10), and a number of hydroxamic acids are currently accepted therapeutic agents (desferrioxamine B, hydroxycarbamide, ibuproxam, oxametacin, bufexamac, adrafinil) (11). The promising therapeutic potential of this class of compounds prompted us to screen antimicrobial activity of several hydroxamic acids previously synthesized by our group (12). In this paper, we report the results of the undertaken antimicrobial evaluation.

EXPERIMENTAL

Melting points were determined on a Boëtius Microheating Stage (Franz Küstner Nachf. KG, Germany). IR spectra were recorded on a FTIR Perkin Elmer Paragon 500 spectrometer (Perkin Elmer, UK). For TLC, silica gel plates Kieselgel 60 F_{254} (Merck, Germany) and the following solvent mixtures were used: *i*-propanol/conc. ammonium hy-

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droxide/water (7:1:2) and chloroform/methanol (7:3). Spots were visualized by short-wave UV light and iodine vapour.

L-aspartic acid was purchased from Kemika (Croatia), while *N*-methylhydroxylamine hydrochloride and *N*-methylhydroxylamine hydrochloride were purchased from Aldrich (USA). All solvents were of analytical grade purity and were dried prior to use.

Syntheses

Hydroxamic and polyhydroxamic acids were prepared according to previously described procedures. Melting points, IR and TLC data were in agreement with the published data.

N-*benzyl*-N′-*hydroxysuccinamide (BHS) (**1**).* – The compound was synthesized by a slightly modified procedure published previously (13). To a solution of *N*-hydroxysuccinimide (4.59 g, 0.004 mol) in ethanol (40 mL), benzylamine (4.28 g, 0.004 mol) was added dropwise. The reaction mixture was stirred one day at room temperature, then refluxed 2.5 h and left overnight. The precipitated product was filtered off, washed with ethanol and dried in vacuum at 40 °C, under P_2O_5. Yield: 7.69 g (86%). m.p. 144–146 °C; m.p. (13) 149 °C.

Poly[α,β-(N-*hydroxy)-DL-aspartamide*] *(PHA) (**2**).* – Polyhydroxamic acid **2** was prepared by aminolysis of the succinimide units of poly-DL-(2,5-dioxo-1,3-pyrrolidinediyl) (PSI) by hydroxylamine, according to our procedure published previously (12). PSI was prepared by thermal polycondensation of L-aspartic acid (14, 15).

Poly[α,β-(N-*hydroxy*-N-*methyl-DL-aspartamide*)] *(PMHA) (**3**).* – Polymeric compound **3** was prepared by aminolysis of PSI by *N*-methylhydroxylamine (12).

Poly[α,β-(N-*hydroxy)-DL-aspartamide*]-*poly*[α,β-(N-2-*hydroxyethyl)-DL-aspartamide*] *(PHA-PHEA) (**4**).* – This copolymer was synthesized by partial aminolysis of PSI by hydroxylamine and further aminolysis by 2-aminoethanol (12). The ratio of PHA and PHEA units was 1:1. Structures of the tested compounds are shown in Fig. 1.

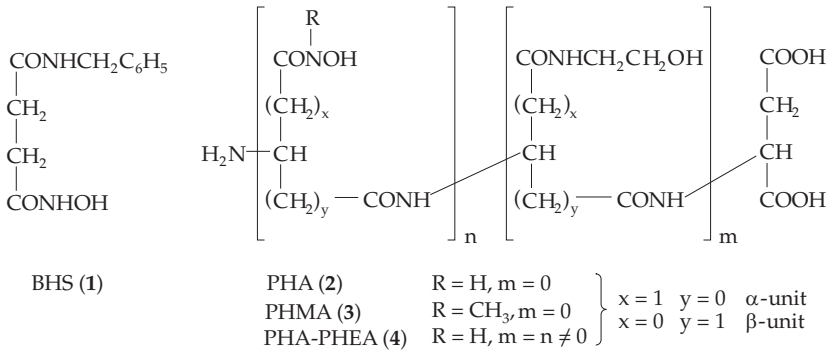

Fig. 1. Chemical structure of *N*-benzyl-*N*′-hydroxysuccinamide (BHS) (**1**), poly[α,β-(*N*-hydroxy)--DL-aspartamide] (PHA) (**2**), poly[α,β-(*N*-hydroxy-*N*-methyl-DL-aspartamide)] (PMHA) (**3**) and poly[α,β-(*N*-hydroxy)-DL-aspartamide]/poly[α,β-(*N*-2-hydroxyethyl)-DL-aspartamide] (PHA-PHEA 1:1) (**4**).

Microorganisms tested

Investigated microorganisms (17 species of Gram-positive and Gram-negative bacteria and 12 species of fungi including yeasts, dermatophytes and moulds) are listed in Table I. All tested species were from the collection of the Department of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia. Gram-positive and Gram-negative bacteria were cultivated on Müller-Hinton agar (Merck, Germany) (MHA) while *Streptococcus pyogenes* ATCC 110401 was cultivated on blood agar (BA). Yeasts and moulds were cultivated on Sabouraud 2%-glucose agar (SGA) (Biolife, Italy) supplemented with 50 mg L^{-1} chloramphenicol. Dermatophytes were also cultivated on

*Table I. Antimicrobial activity of compounds **1**–**4** determined by the difusion method*

Microorganism	Inhibition zone diameters (mm)			
	1	**2**	**3**	**4**
Gram-positive bacteria				
Bacillus subtilis NCTC 8236	–	32	–	32
Bacillus pumilus NCTC 8241	–	20	–	18
Bacillus cereus ATCC 11778	–	–	–	–
Bacillus stearothermophilus MFBF	–	26	–	16
Staphylococcus aureus ATCC 29213	14	14	18	14
Staphylococcus aureus ATCC 25923	–	–	–	–
Staphylococcus epidermidis MFBF	–	–	17	12
Sarcina lutea ATCC 9341	–	33	–	26
Enterococcus faecalis R-13	–	–	–	–
Enterococcus faecalis ATCC 29212	–	25	17	17
Streptococcus pyogenes ATCC 20478	–	–	–	–
Streptococcus pyogenes ATCC 110401	–	23[a]	–	23[a]
Listeria monocytogenes MFBF	–	17	–	20
Listeria monocytogenes R1	–	25	–	21
Listeria monocytogenes R15	–	26	–	23
Gram-negative bacteria				
Klebsiella oxytoca MFBF	–	–	–	–
Serratia sp. MFBF	–	–	–	–
Pseudomonas aeuruginosa 14	–	21	–	–
Pseudomonas aeuruginosa R-301	–	–	–	–
Pseudomonas aeuruginosa 91	15[b]	18	21	–
Pseudomonas aeuruginosa 8	–	–	–	–
Pseudomonas aeuruginosa 39R	–	–	–	–
Proteus mirabilis MFBF	–	–	16	–
Proteus mirabilis R-611	–	–	–	–
Proteus mirabilis R-624	–	–	–	–
Yersinia enterocolitica R1	–	–	23	17
Yersinia enterocolitica R15	–	–	20	13
Shigella sonnei MFBF	–	12	19	–
Acinetobacter sp. MFBF	–	–	–	–

Table I. continued

Microorganism	Inhibition zone diameters (mm)			
	1	**2**	**3**	**4**
Yeasts MFBF				
Candida albicans	–	–	–	–
Candida tropicalis	–	–	–	–
Candida kefyr	–	–	–	–
Candida krusei	–	–	–	–
Candida glabrata	–	–	14	13
Dermatophytes MFBF				
Microsporum gypseum	–	–	–	–
Microsporum canis	–	–	–	–
Trichophyton menthagrophytes	–	–	–	–
Trichophyton rubrum	–	–	–	–
Moulds MFBF				
Aspergillus flavus	–	–	–	–
Fusarium sp.	–	–	–	–
Penicillium sp.	–	–	–	–

[a] In a very short period compounds **1** and **4** caused hemolysis of the blood agar used for *Streptococcus pyogenes* ATCC 11435 culture. [b] The tested compound has bacteriostatic activity. – no antibacterial/antifungal activity.

SGA with addition of 50 mg L^{-1} cycloheximide to prevent growth of other microorganisms. All tested yeasts were cultivated for 72 h at 25 ± 2 °C and dermatophytes and moulds for 7 days for inoculums preparation.

Antimicrobial investigation

Prior to the analysis, substance **1** was dissolved in 80% ethanol to a final concentration of 0.025 g mL^{-1}. Substances **2–4** were dissolved in sterile distilled water to final concentrations of 0.157 g mL^{-1} (**2**), 0.123 g mL^{-1} (**3**) and 0.079 g mL^{-1}. Antimicrobial activity of compounds **1–4** was tested by the agar diffusion method described in *European Pharmacopoeia* (16).

For the agar diffusion method, 25 ± 2 mL of sterile and melted MHA and/or BA and SGA at 45–50 °C was inoculated with 1 mL of approximately 10^8 CFU mL^{-1} of bacterial cells and 1–5 × 10^6 CFU mL^{-1} of yeast cells or conidia and mycelial structures of dermatophytes and moulds in sterile saline in Petri dishes (9 cm diameter). Density of bacterial inoculum was measured with McFarland's standard solution of freshly prepared barium sulphate in sterile water (density of 0.1 mL of 1% $BaCl_2$ in 9.9 mL of 1% H_2SO_4 equals approximately 3 × 10^8 bacterial cells mL^{-1}).

After drying MHA, BA and SGA at room temperature for a maximum of 30 minutes, holes of 6 mm in diameter were made with stainless steel cylinders and filled with solutions of the tested compounds: 50 µL of each compound for *Bacillus subtilis*; 30 µL (**1**), 20 µL (**2**) 10 µL (**3** and **4**) for the other microorganisms tested. Plates were then incubated at 4 °C for 1 hour and at 25 ± 2 °C for 24 h for bacteria, 48 h for yeasts and 3–7 days for dermatophytes and moulds. After the incubation period, inhibition zones were measured and recorded.

Minimum microbicidal (*MMC*) and inhibitory (*MIC*) concentrations were determined using the serial broth dilution method (17). *MMC* was determined by the broth two--fold macro-dilution method in nutrition medium containing the test compounds (0.02–100%, *V/V*) and incubated at 37 °C for bacteria or 25 °C for *Candida* species. The highest dilution of the test compound preventing appearance of turbidity was considered to be the minimum inhibitory concentration. All samples showing no turbidity were subcultured into Müeller Hinton or Sabouraud agar. The highest compound dilution from which the microorganisms did not recover and grow when transferred to fresh medium was the *MMC*.

RESULTS AND DISCUSSION

One hydroxamic (BHS, **1**) and three polyhydroxamic acids, *viz.* PHA (**2**), PMHA (**3**) and PHA-PHEA (**4**), were tested for their antimicrobial activities. Antimicrobial activities of the tested substances obtained by the diffusion and dilution methods are presented in Tables I and II.

*Table II. Minimum microbicidal (*MMC*) and inhibitory concentrations (*MIC*) of compounds **1–4** determined by the broth dilution method*[a]

Microorganisms	**1**		**2**		**3**		**4**	
	MMC	*MIC*	*MMC*	*MIC*	*MMC*	*MIC*	*MMC*	*MIC*
Bacillus subtilis NCTC 8236	–	–	316.0	39.5	–	–	79.33	39.67
Bacillus pumilus NCTC 8241	–	–	–	[b]	–	–	–	[b]
Bacillus stearothermophilus MFBF	–	–	29.8	14.9	–	–	158.7	79.33
Staphylococcus aureus ATCC 29213	[b]	–	79.0	19.75	–	–	317.3	79.0
Staphylococcus epidermidis MFBF	–	–	–	–	184.13	5.76	237.98	39.67
Sarcina lutea ATCC 9341	–	–	39.5	4.94	–	–	158.66	39.67
Enterococcus faecalis ATCC 29212	–	–	631.0	316.0	245.73	7.68	386.7	158.66
Listeria monocytogenes MFBF	–	–	9.88	7.41	–	–	79.33	14.88
Listeria monocytogenes R1	–	–	3.84	1.92	–	–	4.94	2.49
Listeria monocytogenes R15	–	–	3.84	1.92	–	–	19.84	9.92
Pseudomonas aeuruginosa MFBF 14	–	–	[b]	–	–	–	–	–
Pseudomonas aeuruginosa MFBF 91	[b]	–	9.88	7.41	491.0	245.5	–	–
Proteus mirabilis MFBF	–	–	–	–	184.13	5.76	–	–
Yersinia enterocolitica R1	–	–	–	–	61.38	46.04	59.54	14.88
Yersinia enterocolitica R15	–	–	–	–	92.06	46.04	79.38	19.83
Shigella sonnei MFBF	–	–	[b]	–	[b]	–	–	–
Candida glabrata MFBF	–	–	–	–	[b]	–	[b]	–

[a] in mg mL^{-1}

[b] Microorganisms developed resistance to the tested compounds although antimicrobial activity was determined by the dilution method.

MFBF and R – Number of strains from the collection of microorganisms of the Department of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia; ATCC – American Type Culture Collection, Rockville, USA; NCTC – National Collection of Type Cultures, London, Great Britain.

Hydroxamic acid **1** showed no antimicrobial activity on most of the Gram-positive and Gram-negative bacteria. In fact, one strain of *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* 91 developed resistance to the tested compound, although antimicrobial activity was observed by the dilution method. Species of *Candida*, dermatophytes and moulds were resistant to compound **1**. Similar findings were obtained for *Bacillus pumilus*, *P. aeruginosa*, *Shigella sonnei* and *Candida glabrata*. These microorganisms developed resistance to substances **2**, **3** and **4**, respectively.

Compound **3** showed antimicrobial activity against *Staphylococcus epidermidis*, *Enterococcus faecalis*, *P. aeruginosa* and *Yersinia enterocolitica*, but at high concentrations (Table II). For example, *MMC* for *P. aeruginosa* was found to be 491.0 mg mL^{-1}, which is a significantly higher concentration compared to *MMC* of trimethoprim (0.1–0.125 mg mL^{-1}) (18) or galangin (0.23 mg mL^{-1}), an active compound in propolis extracts (19).

Gram-positive bacteria were more sensitive than Gram-negative bacteria to the activities of compounds **2** and **4**. In fact, only *P. aeruginosa* was sensitive to polyhydroxamic acid **2**, while *Y. enterocolitica* was sensitive to polyhydroxamic acid **4**. In addition, compounds **2** and **4** caused hemolysis of the blood agar and cannot be used for human treatment. These substances could be used only as disinfectants.

CONCLUSIONS

Hydroxamic and polyhydroxamic acids showed a narrow spectrum of antimicrobial activity. Minimal bactericidal concentrations of these substances are significantly higher than *MMC* of most antimicrobial drugs for human treatment. Also, polyhydroxamic acids (**2** and **4**) caused hemolysis of the blood agar and they cannot be used in antimicrobial therapy of humans. These compounds could be possibly used in disinfection but at high concentrations.

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SAŽETAK

Antimikrobni učinak nekih hidroksamskih kiselina

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U radu je ispitano antimikrobno djelovanje nekoliko hidroksamskih kiselina: *N*-benzil-*N'*-hidroksi-sukcinamida (BHS) (**1**), poli[α,β-(*N*-hidroksi)-DL-aspartamida] (PHA) (**2**), poli[α,β-(*N*-hidroksi-*N*-metil-DL-aspartamida)] (PMHA) (**3**) i poli[α,β-(*N*-hidroksi)-DL--aspartamid]/poli[α,β-(*N*-2-hidroksietil)-DL-aspartamid] kopolimera (PHA-PHEA) (**4**). Antimikrobno djelovanje ispitano je na 10 Gram-pozitivnih i 7 Gram-negativnih bakterija,

5 vrsta kandida, 4 vrste dermatofita i 3 vrste plijesni. Supstancija **1** ne pokazuje antimikrobno djelovanje na ispitivane vrsta bakterija i gljivica. Ostali spojevi (**2**–**4**) imaju relativno slabo antimikrobno djelovanje na Gram-pozitivne i Gram-negativne bakterije i ne pokazuju antifungalni učinak.

Ključne riječi: hidroksamska kiselina, polihidroksamska kiselina, antimikrobno djelovanje

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