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RESEARCH ARTICLE

Antifungal dipeptides incorporating an inhibitor of homoserine dehydrogenase

Andrzej S. Skwarecki[1] | Marta Schielmann[2] | Dorota Martynow[2] | Marcin Kawczyński[1] | Aleksandra Wiśniewska[1] | Maria J. Milewska[1] | Sławomir Milewski[2]

[1] Department of Organic Chemistry, Gdańsk University of Technology, 11/12 Narutowicza St, 80-233 Gdańsk, Poland

[2] Department of Pharmaceutical Technology and Biochemistry, Gdańsk University of Technology, 11/12 Narutowicza St, 80-233 Gdańsk, Poland

Correspondence
Sławomir Milewski, Department of Pharmaceutical Technology and Biochemistry, Gdańsk University of Technology, 11/12 Narutowicza Str., 80-233 Gdańsk, Poland.
Email: slamilew@pg.gda.pl

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The antifungal activity of 5-hydroxy-4-oxo-L-norvaline (HONV), exhibited under conditions mimicking human serum, may be improved upon incorporation of this amino acid into a dipeptide structure. Several HONV-containing dipeptides inhibited growth of human pathogenic yeasts of the Candida genus in the RPMI-1640 medium, with minimal inhibitory concentration values in the 32 to 64 μg mL^{-1} range. This activity was not affected by multidrug resistance that is caused by overexpression of genes encoding drug efflux proteins. The mechanism of antifungal action of HONV dipeptides involved uptake by the oligopeptide transport system, subsequent intracellular cleavage by cytosolic peptidases, and inhibition of homoserine dehydrogenase by the released HONV. The relative transport rates determined the anticandidal activity of HONV dipeptides.

KEYWORDS

antifungal agents, dipeptides, homoserine dehydrogenase, oligopeptide uptake

1 | INTRODUCTION

Several inhibitors of enzymes catalyzing particular steps of amino acid biosynthesis pathways exhibit antifungal properties.[1] One of them is an antibiotic RI-331 (5-hydroxy-4-oxo-L-norvaline/2-amino-5-hydroxy-4-oxopentanoic acid, HONV), produced by *Streptomyces* spp.[2] This compound is active *in vitro* against several human pathogenic yeasts and the plant pathogen *Cladosporium fulvus* but has no effect against *Aspergillus* spp.[3-5] Furthermore, RI-331 was shown to be effective in the treatment of systemic murine candidiasis, being well tolerated in mice.[3,4] The molecular target of HONV is homoserine dehydrogenase, the key enzyme in the fungi-specific biosynthetic pathway of L-methionine, L-isoleucine, and L-threonine biosynthesis.[4-6] HONV acts as an enzyme-assisted suicide inhibitor of this enzyme.[7] Interestingly, this compound also modulates a glutathione-related drug resistance of tumor cells.[8]

Because homoserine dehydrogenase is a cytosolic enzyme, HONV must be internalized, ie, has to cross the cytoplasmic membrane to reach its target. The hydrophilic nature of its structure precludes fast passive diffusion, so the only possibility is an active transport by one of the amino acid permeases. These transporters, however, demonstrate strict substrate specificity, usually limited to proteinogenic amino acids. Therefore, the inefficient transport through the cytoplasmic membrane limits an antifungal activity of HONV. One of the possible approaches to solve this problem could be the application of the so-called Trojan horse strategy, ie, conjugation of an enzyme inhibitor with a molecular nanocarrier.[9] In the case of inhibitors of amino acid structure, facilitated transport is possible after their incorporation into oligopeptides, which are effectively taken up by oligopeptide permeases, demonstrating broad substrate specificity. Once internalized, such oligopeptides are cleaved by intracellular peptidases to release the inhibitor. Several examples of the successful application of this strategy for the construction of antimicrobials have been reported in the literature.[10-13] Herein, we present results of our studies on synthesis and biological properties of dipeptides incorporating HONV.

2 | RESULTS AND DISCUSSION

2.1 | Rationale for the construction of HONV-containing dipeptides

The amino acid inhibitor of homoserine dehydrogenase (HONV; Figure 1) was used for the construction of dipeptides as potential antifungal agents. The rationale for the design of HONV-containing oligopeptides was based on the previous findings concerning the optimization of structures of such compounds aimed at the maximization of uptake by oligopeptide transport systems, namely, (1) a preference

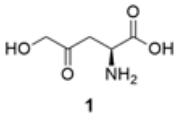


FIGURE 1 Structure of HONV

for dipeptides over the longer oligopeptides, (2) location of the "warhead" as the C-terminal amino acid, (3) aliphatic or aromatic amino acids as *N*-terminal components of a dipeptide.[14-16] Following these indications, 6 dipeptides with L-alanine, L-valine, L-norvaline (Nva), L-leucine, L-isoleucine, and L-phenylalanine as the *N*-terminal residues were obtained. Additionally, Gly-HONV and D-Leu-HONV were synthesized and evaluated for comparative purposes.

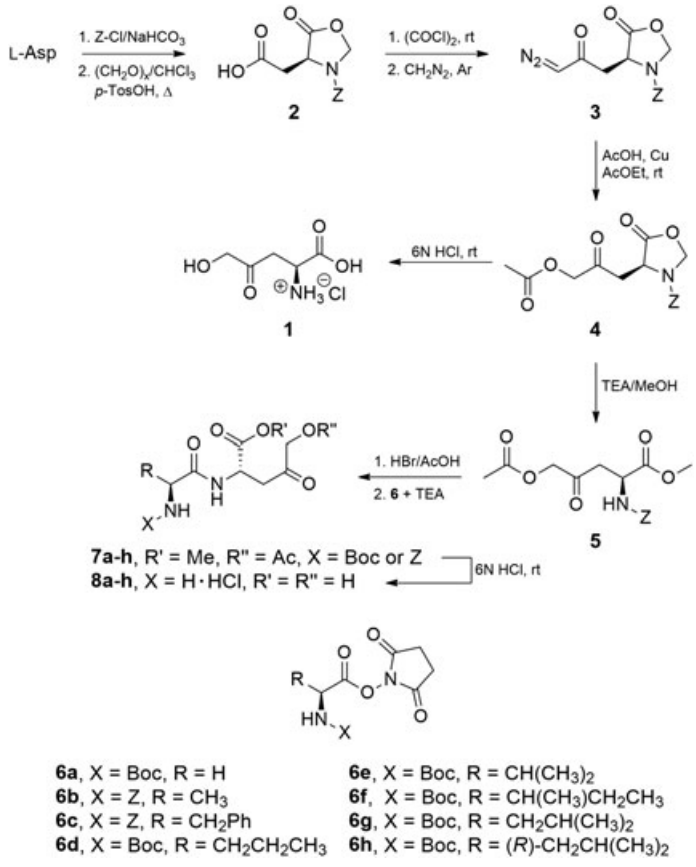
2.2 | Chemical synthesis

The HONV-containing dipeptides were synthesized by a multistep procedure involving 2 main stages: synthesis of an appropriately protected HONV and its conjugation with one of the *N*-protected amino acid hydroxysuccinimide esters, as shown in Scheme 1. Synthesis of *N*-benzyloxycarbonyl *O*-acetyl HONV methyl ester **5** was performed from L-aspartate as a starting substrate by the modified procedure of Chang et al.[17] Briefly, L-aspartate after protection of its α-amino group with Z was converted into (*S*)-3-benzyloxycarbonyl-4-methylcarboxy-1,3-oxazolidin-5-one **2** upon treatment with paraformaldehyde in the presence of *p*-toluenesulfonic acid. Subsequent treatment of **2** with oxalyl chloride, followed by the reaction with diazomethane, afforded diazoketene **3**. The diazo functionality was exchanged for the *O*-acetyl group derived from AcOH in the presence of powdered copper to give compound **4**. Opening of the 1,3-oxazolidin-5-one ring in **4** with TEA afforded *N*-benzyloxycarbonyl *O*-acetyl HONV methyl ester **5**, whereas treatment of **4** with HBr in acetic acid gave HONV hydrobromide **1**. Compound **5**, after deprotection of its amino functionality, was conjugated with one of the *N*-protected amino acid hydroxysuccinimide esters **6a-h**, to give fully protected dipeptides **7a-h**. The final deprotection with 6M HCl afforded hydrochlorides of dipeptides **8a-h**.

2.3 | Antifungal *in vitro* activity of HONV dipeptides

Antifungal *in vitro* activity of HONV and 8 dipeptides containing this compound as the *C*-terminal amino acid was determined against *Candida albicans* ATCC 10 231 cells in 3 growth media: the minimal YNB medium containing ammonium sulfate as a nitrogen source, the rich YPD medium, and the RPMI-1640 medium recommended by Central Laboratory Standards Institute (CLSI), the composition of which mimics that of the low molecular weight compounds pool of human serum. The determined minimal inhibitory concentration (MIC) values are summarized in Table 1. Activity of HONV strongly depended on growth medium composition. The lowest MIC value was noted in YNB minimal medium, the 8-fold higher one in rich YPD and the highest one in RPMI-1640. Dipeptides containing HONV were inactive in YPD and demonstrated lower than RI-331 activity in YNB, but 5 of them (**8b**, **8d-g**) were more active than RI-331 in RPMI-1640. Dipeptide D-Leu-HONV (**8h**) was inactive in all 3 growth media.

The range of activity of HONV dipeptides in RPMI-1640 medium was appreciable (MICs in the 64 to >1024 μg mL^{-1} range), and the



SCHEME 1 Synthesis of HONV and HONV-containing dipeptides

TABLE 1 Antifungal *in vitro* activity of HONV and its dipeptides

	MIC, μg mL^{-1}/mM		
	YNB-AS	RPMI-1640	YPD
HONV **1**	8/0.05	256/1.74	64/0.44
Gly-HONV **8a**	256/1.25	>1024	>1024
Ala-HONV **8b**	64/0.29	128/0.58	>1024
Phe-HONV **8c**	>1024	512/1.74	>1024
Nva-HONV **8d**	64/0.26	64/0.26	1024/4.16
Val-HONV **8e**	32/0.13	64/0.26	>1024
Ile-HONV **8f**	128/0.5	128/0.5	>1024
Leu-HONV **8g**	32/0.12	64/0.25	>1024
D-Leu-HONV **8h**	>1024	>1024	>1024

Minimal inhibitory concentration (MIC) values were determined against the model *Candida albicans* ATCC 10 231 strain by a serial dilution method in 3 growth media. The values given correspond to 3 consistent results of independent determinations.

highest was found for 3 dipeptides containing Nva, Val, or Leu as the *N*-terminal amino acid residue (**8d**, **8e**, and **8g**; MIC = 64 μg mL^{-1}).

A spectrum of anticandidal *in vitro* activity was determined for dipeptides Nva-HONV **8d** and Leu-HONV **8g** and compared to that of HONV **1**. The set of yeasts of the Candida genus used in this study comprised 6 *C. albicans* and 5 non-albicans strains. The former included *C. albicans* SC 5314, a mutant *C. albicans* PTR-OPT strain lacking all genes coding for di-tripeptide and oligopeptide permeases of the PTR and OPT families obtained from 5314 and 4 clinical strains: B3, B4, Gu4, and Gu5. The Gu5 and B4 isolates are resistant to the well-known antifungal drug fluconazole (FLU), because of the FLU-induced overexpression of genes encoding multidrug efflux pumps, *CDR1* and/or *CDR2* in the former and *MDR1* in the latter.[18] Their FLU-sensitive counterparts, Gu4 and B3, respectively, exhibit a basal expression of these resistance genes.

The MIC values determined in RPMI-1640 medium are shown in Table 2. Activity of HONV was generally low, with MICs = 256 or 512 μg mL^{-1}. Presence of multidrug efflux pumps did not lower the activity of either HONV or its dipeptides, thus suggesting that these compounds are not substrates for the drug exporters. A slightly higher activity of Nva-HONV and Leu-HONV against Gu5 cells than against Gu4 cells is additional evidence for the previously reported phenomenon of enhanced susceptibility of yeasts overexpressing the ABC-type drug transporters to oligopeptidic antifungals.[19] The *C. albicans* mutant lacking oligopeptide permeases was resistant to HONV-containing dipeptides but not to HONV itself. The non-albicans Candida strains were generally more sensitive to dipeptides than to HONV, with *Candida parapsilosis* being the least sensitive.

TABLE 2 Anticandidal spectrum of HONV and its 2 dipeptides

	MIC, μg mL^{-1}/mM		
	HONV 1	Nva-HONV 8d	Leu-HONV 8g
Candida albicans SC 5314	256/1.74	64/0.26	64/0.25
Candida albicans PTR-OPT	256/1.74	>1024	>1024
Candida albicans Gu4	256/1.74	64/0.26	64/0.25
Candida albicans Gu5	256/1.74	32/0.13	32/0.12
Candida albicans B3	256/1.74	64/0.26	64/0.25
Candida albicans B4	256/1.74	64/0.26	64/0.25
Candida glabrata	256/1.74	64/0.26	32/0.12
Candida krusei	256/1.74	32/0.13	64/0.25
Candida parapsilosis	512/3.48	128/0.52	128/0.5
Candida tropicalis	512/3.48	32/0.13	64/0.25
Coriaria arborea	256/1.74	64/0.26	64/0.25

Minimal inhibitory concentration (MIC) values were determined by a serial dilution method in RPMI-1640 medium. The values given correspond to 3 consistent results of independent determinations.

2.4 | Factors determining the activity of HONV-containing dipeptides

The previous studies on mechanism of antifungal action of HONV revealed that this amino acid is a strong inhibitor of homoserine dehydrogenase, an enzyme catalyzing the key step in the fungi-specific biosynthetic pathway of amino acids of the aspartate family, namely, L-Met, L-Ile, and L-Thr.[5] It is not surprising therefore that presence of these amino acids in the growth medium prevents the growth inhibitory effect of HONV, as shown in Table 3. The maximal protection was provided by the mixture of 3 amino acids, but each of them also exhibited some protective effect; the strongest one was noted for L-Met. The same phenomenon was observed for HONV-containing dipeptides, thus confirming that their antifungal activity is a consequence of homoserine dehydrogenase inhibition by the active component, ie, HONV. It is worth mentioning, therefore, that the RPMI-1640 medium contains the mixture of proteinogenic amino acids, including L-Met, L-Ile, and L-Thr at 0.1 to 0.33 mM concentration level. This is probably too low to supplement deficiency of these amino acids of biosynthetic origin in cells treated with HONV or its dipeptides but enough to effectively compete with HONV for respective amino acid permeases and thus prevent or strongly limit its accumulation in these cells.

The lack of activity of HONV-containing dipeptides against the *C. albicans opt1-opt5*Δ *ptr2*Δ *ptr22*Δ mutant (Table 2) clearly indicates that these compounds are transported to *C. albicans* cells by the oligopeptide transport system, most probably by the di-tripeptide permeases Ptr2p and Ptr22p. Such an assumption is additionally supported by the lack of activity of HONV-containing dipeptides in YPD medium. This medium contains an enzymatic digest of animal protein (peptone), ie, a mixture of oligopeptides that effectively compete with HONV dipeptides for the permease-mediated transport, thus preventing their intracellular accumulation, which is a prerequisite for the possible interaction of HONV with its target.

The effectiveness of transport was assessed by determination of the initial uptake rates of HONV or one of its dipeptides by *C. albicans* ATCC 10 231 cells from buffered solutions. The pHs of buffers (5.0 and 7.0) corresponded to those of the YNB-AS and RPMI-1640 medium, respectively. The uptake rates of HONV and the dipeptides tested were constant for at least 15 to 20 minutes and then gradually decreased. These properties allowed the determination of initial uptake velocities. The rates were corrected for passive binding of the compounds tested by measuring uptake in the presence of NaN_3, accounting for less than 3% of the initial rate of oligopeptide/amino acid uptake.

TABLE 3 Influence of amino acids on anticandidal *in vitro* activity of HONV and its 2 representative dipeptides

	MIC, μg mL^{-1}				
	YNB-AS	YNB-AS + Met	YNB-AS + Thr	YNB-AS + Ile	YNB-AS + (Met, Thr, Ile)
HONV **1**	8	256	64	128	>1024
Ala-HONV **8b**	64	512	128	128	>1024
Nva-HONV **8d**	64	512	128	128	>1024

Minimal inhibitory concentration (MIC) values were determined against *Candida albicans* ATCC 10 231 by a serial dilution method in YNB-AS medium. The values given correspond to 3 consistent results of independent determinations.

TABLE 4 Uptake and cleavage rates of HONV dipeptides.

	Uptake rate ± SD, nmol min^{-1} (mg dry weight)$^{-1}$		Cleavage rate ± SD, nmol min^{-1} (mg protein)$^{-1}$
	pH 5.0	pH 7.0	
HONV **1**	2.86 ± 0.42	1.32 ± 0.14	NA
Gly-HONV **8a**	3.24 ± 0.18	2.41 ± 0.22	14.5 ± 0.56
Ala-HONV **8b**	5.87 ± 0.66	4.66 ± 0.37	12.3 ± 0.82
Phe-HONV **8c**	3.79 ± 0.36	2.55 ± 0.24	13.3 ± 0.87
Nva-HONV **8d**	6.58 ± 0.46	5.84 ± 0.27	16.4 ± 1.44
Val-HONV **8e**	6.12 ± 0.75	4.99 ± 0.18	18.9 ± 1.83
Ile-HONV **8f**	6.57 ± 0.39	5.12 ± 0.33	15.1 ± 0.44
Leu-HONV **8g**	5.92 ± 0.43	4.47 ± 0.44	17.6 ± 1.33
D-Leu-HONV **8h**	0.27 ± 0.08	0.18 ± 0.06	0.5 ± 0.04

Abbreviation: NA, not applicable.

Each value is the mean of 3 independent determinations ± standard deviation.

The results of the determination of the initial uptake rates are presented in Table 4. The initial uptake velocities were generally higher at pH 5.0 than at pH 7.0. This was expected, as amino acid and oligopeptide permeases of *C. albicans* are ligand/H^+ symporters,[20,21] so their higher efficiency in acidic media is not surprising. All the dipeptides, except for D-Leu-HONV **8h**, were taken up faster than HONV, and generally, higher uptake rates were noted for dipeptides exhibiting higher anticandidal *in vitro* activity (lower MIC values in Table 1).

The velocity of dipeptide cleavage by peptidases present in cytosolic extracts prepared from *C. albicans* ATCC 10 231 cells was also determined, and the data are presented in Table 4. Results of this assay confirmed that all dipeptides but D-Leu-HONV **8h** were rapidly cleaved, with the rates in the 12.3 to 18.9 nmol min^{-1} (mg protein)$^{-1}$ range. It is clear, therefore, that the cleavage rate is not a factor limiting an anticandidal activity of HONV-containing dipeptides. Conversely, the uptake efficiency seems more important and confirms the previous observations concerning other oligopeptide antifungals constructed according to the "Trojan horse" strategy.[16]

3 | CONCLUSIONS

Incorporation of HONV into a dipeptide structure afforded compounds demonstrating slightly higher than HONV itself anticandidal *in vitro* activity in RPMI-1640 medium, the composition of which mimics that of the low molecular weight compounds pool of human serum. The HONV-containing dipeptides were transported to *C. albicans* cells by oligopeptide permeases, possibly of the PTR subfamily, and cleaved intracellularly by peptidases to release HONV, targeting the homoserine dehydrogenase. Relative transport rates determined the anticandidal activity of HONV dipeptides. It seems that further optimization of the oligopeptide carrier for HONV might be possible to get compounds exhibiting higher anticandidal activity that the most active HONV dipeptides described in this work.

4 | EXPERIMENTAL

4.1 | General

All solvents and reagents were used as obtained from commercial sources. ^{1}H NMR and ^{13}C NMR spectra were obtained at 500 and 125 MHz Varian Unity Plus spectrometers, and the deuterated solvents were used as internal locks. Melting points were determined on a melting point apparatus equipped with a thermometer and were uncorrected. Column chromatography was performed with silica gel (0.040-0.063 mm) by using the indicated solvent systems. Optical rotation was measured on an AUTOPOL V (Na D line) by using a microcell of 10-cm path length. High-resolution mass spectra were obtained using the Aqilent Technologies 6540 UHD Accurate – Mass Q-TOF LC/MS mass spectrometer.

4.2 | Synthesis

N-Benzyloxycarbonyl-L-amino acids were prepared from L-amino acids according to the method of Bergmann and Zervas.[22] *N-tert*-Butoxycarbonyl-L-amino acids were prepared from L-amino acids as described.[23,24] *N*-Benzyloxycarbonyl- or *N-tert*-butoxycarbonyl-L-amino

acid hydroxysuccinimide esters **6** were prepared from Z- or Boc-L-amino acids following the methodology of Anderson et al.[25]

4.3 | (*S*)-3-Benzyloxycarbonyl-4-methylcarboxy-1,3-oxazolidin-5-one (2)

The mixture of *N*-benzyloxycarbonyl-L-aspartic acid (0.1 mol, 26.7 g), paraformaldehyde (0.2 mol; 6 g), and *p*-toluenesulfonic acid (10 mmol, 1.9 g) in 200 mL of chloroform was heated at reflux for 2 hours, with removal of water with a Dean-Stark trap. After then, the solvent was removed by evaporation, and the oily residue was dissolved in 100 mL of ethyl acetate, washed with saturated $NaHCO_3$ solution (2 × 10 mL) and water (2 × 10 mL), and dried over anhydrous $MgSO_4$.. The solvent was evaporated to give a colorless syrup, which crystallized on standing overnight at 0°C. The final product was crystallized from ethyl acetate to afford 24 g (90%) of **2**; mp 82°C to 5°C, $[a]^{20}_D$ + 120 (*c*3, MeOH). (lit. $[a]^{20}_D$ + 125.7 (*c*3, MeOH); mp 82°C to 87°C.[26]

^{1}H NMR ($CDCl_3$, δ ppm): 3.05 to 3.10 (m, 1H), 3.30 to 3.40 (m, 1H), 4.36 to 4.40 (m, 1H), 5.10 to 5.25 (m, 2H), 5.30 to 5.35 (m, 1H), 5.40 to 5.45 (m, 1H), 7.35 to 7.45 (m, 5H); ^{13}C NMR ($CDCl_3$, δ ppm): 178.2, 175.1, 171.3, 152.8, 134.9, 128.6, 128.3, 78.2, 68.0, 51.4, 34.3.

4.4 | (*S*)-3-Benzyloxycarbonyl-4-(3-diazo-2-oxopropyl)-1,3-oxazolidin-5-one (3)

The mixture of (*S*)-*N*-benzyloxycarbonylo-4-carboxymethyl-1,3-oxazolidin-5-one **2** (18 mmol, 5 g) and oxalyl chloride (10 mL) was stirred for 15 hours at room temperature. An excess of chloride was evaporated, hexane (10 mL) was added, and the mixture was evaporated to dryness. This workup was repeated twice to remove the traces of oxalyl chloride. The product, 5.4 g (100% yield), obtained as a colorless oil, was dissolved in THF (20 mL, freshly distilled over $LiAlH_4$) and added dropwise to the ethereal solution of diazomethane, prepared from *N*-nitrozo-*N*-methylurea (0.15 mol, 16 g), maintaining the temperature of the reaction mixture below −5°C. The mixture was then stirred at 0°C for 10 hours under an atmosphere of argon. The resulting mixture was evaporated under reduced pressure, and the yellow solid residue obtained was purified by flash chromatography, with $CHCl_3$ as an eluent. After being concentrated under vacuum, the residue was crystallized from ethyl acetate to give the diazoketene **3** as yellow crystals (4.6 g, 85% yield); mp 94°C to 6°C; $[\alpha]_D$ + 115.5 (*c*2, $CHCl_3$).

^{1}H NMR ($CDCl_3$) δ:3.0 to 3.1 5(m, 1H), 3.30 to 3.40 (m, 1H), 4.33 to 4.35 (m, 1H), 5.05 to 5.20 (m, 3H), 5.25 to 5.35 (m, 1H), 5.40 to 5.55 (m, 2H), 7.35 to 7.40 (m, 5H).

4.5 | (*S*)-3-Benzyloxycarbonyl-4-(*O*-acetyl-3-hydroxy-2-oxopropyl)-1,3-oxazolidin-5-one (4)

The diazoketene **3** (15 mmol, 4.5 g) was dissolved in ethyl acetate (175 mL), and acetic acid (17.5 mL) and powdered copper (14 mmol, 0.9 g) were added. The reaction mixture was stirred for 42 hours at room temperature. The resulting suspension was filtered to remove copper, and the remaining solution was evaporated to give the crude product **4**. The residue was dissolved in ethyl acetate (100 mL), and the resulting solution was washed with saturated $NaHCO_3$ solution and water. After being concentrated under vacuum, the yellow residue was purified by flash chromatography, with hexane/ethyl acetate 7:2 *v*/v mixture as an eluent, to give **4** as a yellow oil (2.5 g, 50% yield).

^{1}H NMR ($CDCl_3$) δ:2.17 (s, 3H), 3.0 to 3.1 5(m, 1H), 3.30 to 3.40 (m, 1H), 4.35 to 4.38 (m, 1H), 4.60 to 4.65 (m, 2H), 5.10 to 5.25 (m, 2H), 5.4 (s, 1H), 5.45 to 5.60 (m, 1H), 7.35 to 7.40 (m, 5H).

MS-ESI: *m*/*z* $[M + H]^+$ found 335.1081. $C_{16}H_{17}NO_7$ requires 335.1005.

4.6 | Methyl (*S*)-2-benzyloxycarbonylamino-5-acetoxy-4-oxopentanoate (5)

Oxazolidinone **4** (7.5 mmol, 2.5 g) was added to the solution of TEA (25 mmol, 3.5 mL) in methanol (35 mL), at 0°C with stirring, under an atmosphere of argon. After 1 hour, the mixture was warmed to room temperature, and the stirring was continued for 12 hours. The solvent and an excess of TEA were evaporated, to give the crude product **5**. The residue was dissolved in ethyl acetate (100 mL), and the resulting solution was washed with 10% $KHSO_4$ solution and water. After being concentrated under vacuum, the yellow residue was purified by flash chromatography, with hexane/ethyl acetate 2:3, *v*/v mixture as an eluent, to give **5** as yellow crystals mp 65°C to 7°C; (1.5 g, 60% yield).

^{1}H NMR ($CDCl_3$) δ: 2.04 (s, 3H), 2.99 to 3.04 (dd, *J* = 4.4 and 18 Hz, 1H), 3.14 to 3.19 (dd, *J* = 4.4 and 18 Hz, 1H), 3.74 (s, 3H), 4.64 (s + m, 3H), 5.11 (s, 2H), 5.75 (d, 1H), 7.33 to 7.36 (m, 5H). MS-ESI: *m*/*z* $[M + H]^+$ found 338.1197. $C_{16}H_{17}NO_7$ requires 337.1162.

4.7 | Methyl (*S*)-2-*N*-[*N*-alkoxycarbonyl-2-aminoacyl] amino-5-acetoxy-4-oxopentanoate (7a-h)

Compound **5** (500 mg, 1.5 mmol) was dissolved in 5 mL of HBr in AcOH (45%), and the resulting solution was stirred for 1.5 hours at room temperature. Removal of acetic acid by evaporation under reduced pressure afforded a crude product as a bromide salt, which was used in the next reaction without purification. The salt was dissolved in 10 mL of THF, and 1 mL (7 mmol) of trimethylamine was added. After 15 minutes of stirring at room temperature, an active ester of one of the *N*-protected amino acids **6a-h** (1.5 mmol) was added as a solid, directly to the prepared solution. The mixture was stirred at room temperature for 60 hours. After this time, the solvent was removed under reduced pressure, and the residue was dissolved in 10 mL of $CHCl_3$ and washed twice with 10 mL of 10% aq. $NaHSO_4$ and water. The organic layer was separated and dried under anhydrous $MgSO_4$. Subsequent filtration and evaporation of organic solvents afforded the crude oily products, which were purified by column chromatography (silica gel, hexane/ethyl acetate, 3:2 *v*/v).

4.7.1 | Methyl (*S*)-2-*N*-[*N*-*tert*-butoxycarbonyl-glycyl] amino-5-acetoxy-4-oxopentanoate (7a)

Oil, 23% yield.

^{1}H NMR ($CDCl_3$) δ: 1.44 (s, 9H), 2.14 (s, 3H), 3.04 to 3.08 (dd, *J* = 4.4 and 18 Hz, 1H), 3.12 to 3.15 (dd, *J* = 4.4 and 18 Hz, 1H), 3.73 (s, 3H), 4.43 (m, 1H), 3.81 (s, 2H), 4.62 (s, 2H), 4.81 to 4.84 (m, 1H), 5.21 (br m, 1H), 7.03 (d, *J* = 7.8 Hz, 1H).

4.7.2 | Methyl (*S*)-2-*N*-[*N*-benzyloxycarbonyl-(*S*)-alanyl] amino-5-acetoxy-4-oxopentanoate (7b)

White crystals mp 115°C to 8°C ($CHCl_3$/hexane), 23% yield.

^{1}H NMR ($CDCl_3$) δ: 1.39 (d, *J* = 7.4 Hz, 3H), 2.14 (s, 3H), 3.04-3.14 (AB, 2H), 3.73 (s, 3H), 4.24 (m, 1H), 4.62 (AB, 2H), 4.8 (br m, 1H), 5.11 (s, 2H), 5.4 (br m, 1H), 6.93 (d, *J* = 6.3 Hz, 1H), 7.29 to 7.36 (m, 5H).

4.7.3 | Methyl (*S*)-2-*N*-[*N*-benzyloxycarbonyl-(*S*)-phenylalanyl]amino-5-acetoxy-4-oxopentanoate (7c)

White crystals mp 153°C to 5°C ($CHCl_3$/hexane), 50% yield.

^{1}H NMR ($CDCl_3$) δ: 2.14 (s, 3H), 3.05 to 3.09 5 (m, 3H), 3.11 to 3.16 (dd, *J* = 6.3 and 16.2 Hz, 1H), 3.71 (s, 3H), 4.43 (m, 1H), 4.57 (s, 2H), 4.76 (m, 1H), 5.08 (s, 2H), 5.27 (d, *J* = 6.3 Hz, 1H), 6.77 (d, *J* = 6.8 Hz, 1H), 7.17 to 7.36 (m, 10H).

4.7.4 | Methyl (*S*)-2-*N*-[*N*-*tert*-butoxycarbonyl-(*S*)-norvalyl] amino-5-acetoxy-4-oxopentanoate (7d)

Oil, 28% yield.

^{1}H NMR ($CDCl_3$) δ: 0.92 to 0.97 (dd, *J* = 7.3 and 14 Hz, 3H), 1.44 (s, 9H), 1.53 to 1.6 (m, 2H), 1.78 to 1.84 (m, 12), 2.16 (s, 3H), 3.04 to 3.08 (dd, *J* = 4.4 and 18 Hz, 1H), 3.12 to 3.16 (dd, *J* = 4.4 and 18 Hz, 1H), 3.74 (s, 3H), 4.07 (m, 1H), 4.64 (s, 2H), 4.80 to 4.83 (m, 1H), 5.06 (bs, 1H), 6.92 (d, *J* = 7.8 Hz, 1H).

4.7.5 | Methyl (*S*)-2-*N*-[*N*-*tert*-butoxycarbonyl-(*S*)-valyl] amino-5-acetoxy-4-oxopentanoate (7e)

Oil, 23% yield.

^{1}H NMR ($CDCl_3$) δ: 0.90 (d, *J* = 6.8 Hz, 3H), 0.97 (d, *J* = 6.8 Hz, 3H), 1.26 to 1.33 (m, 1H), 1.45 (s, 9H), 2.16 (s, 3H), 3.01 to 3.06 (dd, *J* = 4.4 and 18 Hz, 1H), 3.15 to 3.19 (dd, *J* = 4.4 and 18 Hz, 1H), 3.74 (s, 3H), 3.94 to 3.97 (m, 1H), 4.64 (s, 2H), 4.83 to 4.86 (m, 1H), 5.5 (d, *J* = 7.8 Hz, 1H), 6.81 (d, *J* = 6.8 Hz, 1H).

4.7.6 | Methyl (*S*)-2-*N*-[*N*-*tert*-butoxycarbonyl-(*S*)-isoleucyl]amino-5-acetoxy-4-oxopentanoate (7f)

White solid; mp 95°C to 7°C ($CHCl_3$/hexane), 25% yield.

^{1}H NMR ($CDCl_3$) δ: 0.9 to 0.96 (m, 6H), 1.12 to 1.32 (m, 2H), 1.44 (s, 9H), 1.88 (m, 1H), 2.16 (s, 3H), 3.01 to 3.05 (dd, *J* = 3.9 and 18 Hz, 1H), 3.14 to 3.19 (dd, *J* = 3.9 and 18 Hz, 1H), 3.74 (s, 3H), 3.99 (m, 1H), 4.64 (s, 2H), 4.83 to 4.85 (m, 1H), 5.03 (br m, 1H), 6.8 (d, *J* = 6.5 Hz, 1H).

4.7.7 | Methyl (*S*)-2-*N*-[*N*-*tert*-butoxycarbonyl-(*S*)-leucyl] amino-5-acetoxy-4-oxopentanoate (7g)

Oil, 25% yield.

^{1}H NMR ($CDCl_3$) δ: 0.93 to 0.96 (m, 6H), 1.42 to 1.49 (m, 1H), 1.44 (s, 9H), 1.62 to 1.69 (m, 2H), 2.15 (s, 3H), 3.04 to 3.14 (AB, 2H), 3.73 (s, 3H), 4.09 to 4.12 (m, 1H), 4.63 (s, 2H), 4.78 to 4.81 (m, 1H), 4.90 (d, *J* = 6.3 Hz, 1H), 6.97 (d, *J* = 7.8 Hz, 1H).

4.7.8 | Methyl (*S*)-2-*N*-[*N*-*tert*-butoxycarbonyl-(*R*)-leucyl] amino-5-acetoxy-4-oxopentanoate (7h)

Oil, 23% yield.

^{1}H NMR ($CDCl_3$) δ: 0.93 to 0.95 (m, 6H), 1.44 to 1.49 (m, 1H), 1.44 (s, 9H), 1.65 to 1.67 (m, 2H), 2.15 (s, 3H), 3.01 to 3.05 (dd, *J* = 4.4 and 18 Hz, 1H), 3.12 to 3.16 (dd, *J* = 4 and 18 Hz, 1H), 3.74 (s, 3H), 4.10 (m, 1H), 4.57 to 4.67 (AB, *J* = 17 Hz, 2H), 4.79 to 4.83 (m, 2H), 7.05 (d, *J* = 6.9 Hz, 1H).

4.8 | (*S*)-2-*N*-[2-aminoacyl]amino-5-hydroxy-4-oxopentanoic acid (8a-h)

The protected dipeptide **7a-h** was dissolved in 10 mL of 6M $HCl_{(aq)}$, and the solution was stirred at room temperature for 192 hours. After this time, the mixture was concentrated under reduced pressure, and the residue was purified by high-performance liquid chromatography.

4.8.1 | (*S*)-2-*N*-glycylamino-5-hydroxy-4-oxopentanoic acid (8a)

^{1}H NMR (D_2O) δ: 2.6 to 2.8 (m, 1H), 2.9 to 3 (m, 1H), 3.2 to 3.3 (m, 2H), 4.1 (t, *J* = 7 Hz, 1H), 4.25(m, 2H), 4.65 (m, 1H); ^{13}C NMR (D_2O) δ: 208, 175, 169, 69, 51, 42, 40. MS-ESI: *m/z* $[M + H]^+$ found 205.0657, $C_7H_{12}N_2O_5$ requires 204.0746.

4.8.2 | (*S*)-2-*N*-[(*S*)-alanyl]amino-5-hydroxy-4-oxopentanoic acid (8b)

^{1}H NMR (D_2O) δ: 1.4(d, *J* = 7 Hz, 3H), 2.6 to 2.8 (m, 1H), 2.9 to 3 (m, 1H), 3.9 (t, *J* = 6 Hz 1H), 4.18 (bs, 2H), 4.65 (m, 1H); ^{13}C NMR (D_2O) δ: 207, 174, 173, 69, 52, 51.3, 43, 19.5. MS-ESI: *m/z* $[M + H]^+$ found 219.1012, $C_8H_{14}N_2O_5$ requires 218.0903.

4.8.3 | (*S*)-2-*N*-[(*S*)-phenylalanyl]amino-5-hydroxy-4-oxopentanoic acid (8c)

^{1}H NMR (D_2O) δ: 2.76 to 2.88 5 (m, 1H), 2.96 to 2.99 (m, 1H), 3.05 (AB, *J* = 7.3 Hz, 2H), 4.07 (t, *J* = 7.3 Hz, 1H), 4.14 (m, 2H), 4.54 (t, *J* = 5.6 Hz, 1H), 7.11 to 7.25 (m, 5H); ^{13}C NMR (D_2O) δ: 206, 175, 171, 137, 130, 128, 68, 55, 50.5, 44.5, 40. MS-ESI: *m/z* $[M + H]^+$ found 295.1195 $C_{14}H_{18}N_2O_5$ requires 294.1216.

4.8.4 | (*S*)-2-*N*-[(*S*)-norvalyl]amino-5-hydroxy-4-oxopentanoic acid (8d)

^{1}H NMR (D_2O) δ: 0.76 (m, 3H), 1.24 (m, 2H), 1.70 (m, 2H), 2.91 (bs, 2H), 3.89 (t, *J* = 6 Hz 1H), 4.18 (bs, 2H), 4.65 (m, 1H); ^{13}C NMR (D_2O) δ: 208, 173.9, 170.1, 67.2, 53.0, 48.3, 38.9, 32.9, 17.6, 12.9. MS-ESI: *m/z* $[M + H]^+$ found 247.1245 $C_{10}H_{18}N_2O_5$ requires 246.1216.

4.8.5 | (*S*)-2-*N*-[(*S*)-valyl]amino-5-hydroxy-4-oxopentanoic acid (8e)

^{1}H NMR (D_2O) δ: 0.85 (d, *J* = 6.4 Hz, 3H), 0.86 (d, *J* = 6.4 Hz, 3H), 2.05 (octet, *J* = 6.4 Hz, 1H), 2.93 (bs, 2H), 3.63 (d, *J* = 5.4, 1H), 4.18 (bs, 2H), 4.68 (m, 1H); ^{13}C NMR (D_2O) δ: 208, 169.3, 67.2, 58.4, 48.3, 38.9, 30.1, 17.6, 16.9. MS-ESI: *m/z* $[M + H]^+$ found 247.2011; [M + Na] 269.2012. $C_{10}H_{18}N_2O_5$ requires 246.1216.

4.8.6 | (*S*)-2-*N*-[(*S*)-isoleucyl]amino-5-hydroxy-4-oxopentanoic acid (8f)

^{1}H NMR (D_2O) δ: 0.8 (t, *J* = 7 Hz, 3H), 1.1 (d, *J* = 6.5 Hz, 3H), 1.25 (q, *J* = 7 Hz, 2H), 1.6 to 17 (m, 1H), 2.91 (bs, 2H), 3.65 (t, *J* = 6.7 Hz, 1H), 4.2 (bs, 2H), 4.8 (m, 1H); ^{13}C NMR (D_2O) δ: 206, 174, 170, 68,

58, 50, 36, 25, 15, 11.5. MS-ESI: *m/z* $[M + H]^+$ found 261.1448. $C_{11}H_{20}N_2O_5$ requires 260.1372.

4.8.7 | (*S*)-2-*N*-[(*S*)-leucyl]amino-5-hydroxy-4-oxopentanoic acid (8g)

^{1}H NMR (D_2O) δ: 0.76 (d, *J* = 6.3 Hz, 3H), 0.77 (d, *J* = 6.3 Hz, 3H), 1.49 to 1.63 (m, 3H), 2.91 (bs, 2H), 3.82 (t, *J* = 6.7 Hz, 1H), 4.18 (bs, 2H), 4.62 (m, 1H); ^{13}C NMR (D_2O) δ: 206, 175, 174, 68, 53.5, 5044.542, 25, 23. MS-ESI: *m/z* $[M + H]^+$ found 261.1291 $C_{11}H_{20}N_2O_5$ requires 260.1372.

4.8.8 | (*S*)-2-*N*-[(*R*)-leucyl]amino-5-hydroxy-4-oxopentanoic acid (8h)

^{1}H NMR (D_2O) δ: 0.75 (d, *J* = 6.2 Hz, 3H), 0.79 (d, *J* = 6.2 Hz, 3H), 1.49 to 1.63 (m, 3H), 2.90 (bs, 2H), 3.84 (t, *J* = 6.7 Hz, 1H), 4.18 (bs, 2H), 4.62 (m, 1H). MS-ESI: *m/z* $[M + H]^+$ found 261.1611 $C_{11}H_{20}N_2O_5$ requires 260.1372.

4.9 | (*S*)-2-amino-5-hydroxy-4-oxopentanoic acid (1)

Oxazolidinone **4** was dissolved in 10 mL of 6M $HCl_{(aq)}$, and the solution was stirred at room temperature for 192 hours. After this time, the mixture was concentrated under reduced pressure, and the residue was purified by high-performance liquid chromatography.

^{1}H NMR (D_2O) δ: 2.98 to 3.03 (dd, *J* = 6.5 and 18 Hz, 1H), 3.05 to 3.09 (dd, *J* = 4.4 and 18 Hz, 1H), 4.14 to 4.16 (dd, *J* = 4.4 and 6.3 Hz, 1H), 4.20 (s, 2H); ^{13}C NMR (D_2O) δ: 208.7, 169.8, 66.9, 53.9, 37.7. MS-ESI: *m/z* $[M + H]^+$ found 148.0481 $C_5H_9NO_4$ requires 147.0532.

5 | BIOLOGICAL ASSAYS

5.1 | Strains and culture conditions

Reference and nonreference *C. albicans* strains used in this study are listed in a table provided as the Table S1. Cells were grown at 30°C in YPD medium (1% yeast extract, 2% peptone, and 2% glucose) and stored on YPD plates containing 2% agar. Susceptibility testing was performed in YPD and other 2 media: (1) RPMI-1640 without sodium bicarbonate, with L-glutamine + 2% glucose + 3.45% MOPS, pH adjusted to 7.0; and (2) YNB-AS (Yeast Nitrogen Base without amino acids, contains ammonium sulfate 5 g L^{-1}) + 2% glucose.

5.2 | Susceptibility testing methods

The *in vitro* growth inhibitory activity of antifungals was quantified by determination of MIC values by the serial 2-fold dilution method, using the 96-well microtiter plates in 3 media: buffered RPMI-1640, YNB-AS, and YPD. Conditions of the RPMI-1640-based assay were the same as outlined in the CLSI recommendations,[27] except for the end point readout that was done by spectrophotometric determination of cell density at 531 nm. Turbidity in individual wells was measured with a microplate reader (Victor^3; Perkin Elmer). MIC was defined as the lowest drug concentration that gave at least an 80% decrease in turbidity, relative to that of the drug-free growth control.

The 96-well microtiter plates were also used for determination of *in vitro* growth inhibitory activity in YPD and YNB-based media. Individual wells were inoculated with 5 × 10^3 cfu (colony forming units) mL^{-1} of *C. albicans* cells from the overnight culture in YPD medium. The inoculated plates were incubated 37°C for 24 hours, and then turbidity was measured with a microplate reader at 531 nm, as described above for the RPMI-1640-based assay.

5.3 | Determination of peptide uptake rates

Candida albicans ATCC 10 231 cells grown exponentially in the YNB-AS medium were harvested by centrifugation (3000*g*, 5 min), washed with 50mM potassium phosphate buffer (pH 5.0 or 6.5), and suspended in the same buffer containing 1% glucose, to a final cell density corresponding to $A_{660} \cong 1.0$. The cell suspension was incubated at 30°C. After 10 minutes, an HONV or dipeptide solution was added, to give a final concentration of 100 μM. At that moment and at 5-minute intervals thereafter, 2-mL samples of the cell suspension were withdrawn, immediately filtered through the Whatman GF/A filters, and the filtrates were used to determine the residual amino acid/peptide concentration. Then, the 1-mL portions of the filtrates were taken and combined with 1.25-mL aliquots of a solution containing 4% $Na_2B_4O_7 \times 10\ H_2O$ and 0.8 mg mL^{-1} of 2,4,6-trinitrobenzenesulfonate. The reaction was performed at 37°C for 30 minutes. The A_{420} values were measured, and the amino acid/peptide concentration was read from the appropriate standard curve. Data were plotted as nanomoles of an amino acid or oligopeptide, taken up by 1 mg (dry weight) of cells versus time. The initial uptake velocities were determined from the slopes of the linear part of the curves, in the 0- to 10-minute region.

5.4 | Preparation of cell-free extract

Candida albicans ATCC 10 231 cells from the overnight culture in YPD were harvested by centrifugation and washed with cold 25mM potassium phosphate buffer (pH 6.5). Cells were then suspended in a minimal amount of the buffer and disrupted with the French press. The resulting suspension was centrifuged (35 000*g*, 4°C, 45 min), and the supernatant was used as a cell-free extract for the determination of peptide cleavage rates.

5.5 | Determination of peptide cleavage rates

The incubation mixtures, consisting of 10 mL of a 200-μM peptide solution in 50mM potassium phosphate buffer (pH 6.5) and 2 mL of an appropriately diluted crude extract (final protein concentration, 0.1 to 0.5 mg mL^{-1}), were incubated at 30°C. At 5-minute intervals, 2-mL aliquots were withdrawn and heated at 100°C for 3 minutes. The resulting suspensions were centrifuged to remove the protein precipitates, and the concentration of free amino acids in the supernatant was determined by the Cd-ninhydrin procedure.[28]

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ORCID

Sławomir Milewski http://orcid.org/0000-0003-2616-4495

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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