

Inhibitors of *N*^ε-acetyl-L-ornithine deacetylase: synthesis, characterization and analysis of their inhibitory potency

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Received: 30 January 2009 / Accepted: 10 July 2009 / Published online: 2 August 2009
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Abstract A series of *N*^ε-acyl (alkyl)- and *N*^ε-alkoxycarbonyl-derivatives of L- and D-ornithine were prepared, characterized, and analyzed for their potency toward the bacterial enzyme *N*^ε-acetyl-L-ornithine deacetylase (ArgE). ArgE catalyzes the conversion of *N*^ε-acetyl-L-ornithine to L-ornithine in the fifth step of the biosynthetic pathway for arginine, a necessary step for bacterial growth. Most of the compounds tested provided IC₅₀ values in the μM range toward ArgE, indicating that they are moderately strong inhibitors. *N*^ε-chloroacetyl-L-ornithine (**1g**) was the best inhibitor tested toward ArgE providing an IC₅₀ value of 85 μM while *N*^ε-trifluoroacetyl-L-ornithine (**1f**), *N*^ε-ethoxycarbonyl-L-ornithine (**2b**), and *N*^ε-acetyl-D-ornithine (**1a**) weakly inhibited ArgE activity providing IC₅₀ values between 200 and 410 μM. Weak inhibitory potency toward *Bacillus subtilis*-168 for *N*^ε-acetyl-D-ornithine (**1a**) and *N*^ε-fluoro- (**1f**), *N*^ε-chloro- (**1g**), *N*^ε-dichloro- (**1h**), and *N*^ε-trichloroacetyl-ornithine (**1i**) was also observed. These data correlate well with the IC₅₀ values determined for

ArgE, suggesting that these compounds might be capable of getting across the cell membrane and that ArgE is likely the bacterial enzymatic target.

Keywords ArgE inhibitors · Acetylornithine derivatives · Synthesis · Inhibitory and antibacterial activity

Abbreviations

AA	Amino acid analysis
ACN	Acetonitrile
ArgE	<i>N</i> ^ε -acetyl-L-ornithine deacetylase
Boc	<i>tert</i> -Butoxycarbonyl
(Boc) ₂ O	Di-(<i>tert</i> -butylcarbonate) anhydride
DIC	<i>NN</i> -Diisopropylcarbodiimide
DCM	Dichloromethane
DIEA	<i>NN</i> -Diisopropylethylamine
DMAP	4-(Dimethylamino)pyridine
DMF	<i>NN</i> -Dimethylformamide
ESI MS	Electro spray ionization mass spectrometry
Et	Ethyl
Fmoc	[(Fluoren-1-yl-methoxy)carbonyl; 9-fluorenyl methoxycarbonyl]
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HOBt	1-Hydroxybenzotriazole
IPTG	Isopropyl-β-D-thiogalactopyranoside
Me	Methyl
NAO	<i>N</i> -Acetyl-L-ornithine
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TFA	Trifluoroacetic acid
TIS	Tri-isopropylsilane
Tricine	<i>N</i> -tris[hydroxymethyl]methylglycine
Z	Benzyloxycarbonyl

The nomenclature and symbols of amino acids follow the Recommendations of IUPAC/IUB Joint Commission on Biochemical Nomenclature. Eur J Biochem (1984) 138: 9–37.

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Introduction

The increasing resistance of microorganisms to antimicrobial agents represents a serious public health problem (Hancock 1997). Today 1,500 people die each hour from an infectious disease; half of these are children under 5 years of age (Levy 1998). The Institute of Medicine, a part of the National Academy of Sciences, has estimated that the annual cost of treating antibiotic resistant infections in the US alone may be as high as \$30 billion (CDC 1995). These findings have stimulated a sustained search for new potent antimicrobial agents against drug resistant bacterial strains as well as new enzymatic targets (Teuber 1999). Microbial enzymes, especially those catalyzing metabolic processes exclusive to bacteria, are potential targets for potent and selective antibiotics. Since many of the broad-spectrum antibiotics contain β -lactam functional units that target enzymes involved in bacterial cell wall synthesis or pathways involved in cell replication (Levy 1998; Nemecek 1997), new enzymatic targets must be located so novel inhibitors can be synthesized, providing new classes of antibiotics. For this reason, several bacterial metallohydrolases containing dinuclear active sites have become the subject of intense efforts in inhibitor design (Holz et al. 2003; Bradshaw and Yi 2002; Daiyasua et al. 2001; Lipscomb and Sträter 1996; Wilcox 1996; Dismukes 1996).

The *argE*-encoded N^α -acetyl-L-ornithine deacetylase (EC 3.5.1.16; ArgE) is a bacterial metallohydrolase that contains a dinuclear active site and is a member of the arginine biosynthetic pathway in bacteria (McGregor et al. 2005). Prokaryotes synthesize arginine through a series of eight enzymatically catalyzed reactions that differ from those of eukaryotes by two key steps: (i) acetylation of glutamate and (ii) the subsequent deacetylation of the arginine precursor N^α -acetyl-L-ornithine (NAO) by ArgE (Cunin et al. 1986; Davis 1986; Ledwidge and Blanchard 1999). The arginine biosynthetic pathway is found in all Gram-negative and most Gram-positive bacteria including *Enterobacteriaceae* (Vogel and MacLellan 1970), *Mycobacterium* (Harris and Singer 1998), *Vibrionaceae* (Xu et al. 2000), and the thermophilic archaeon *Sulfolobus* (Van de Castele et al. 1990). Because ornithine is required, not only for the synthesis of arginine in bacteria, but also for polyamines involved in DNA replication and cell division, NAO deacetylation is critical for bacterial proliferation (Girodeau et al. 1986). Indeed, when an arginine auxotrophic bacterial strain void of NAO deacetylase activity was transformed with a plasmid containing *argE*, an Arg⁺ phenotype resulted (Meinzel et al. 1992). However, when the start codon (ATG) of *argE* in the same plasmid was changed to the Amber codon (TAG), the resultant plasmid

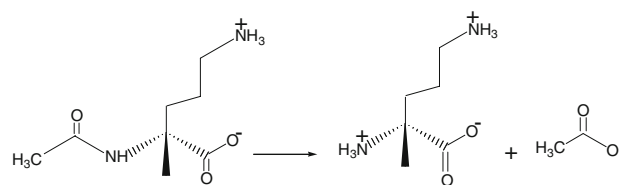


Fig. 1 Conversion of N^α -acetyl-L-ornithine to L-ornithine, catalyzed by ArgE

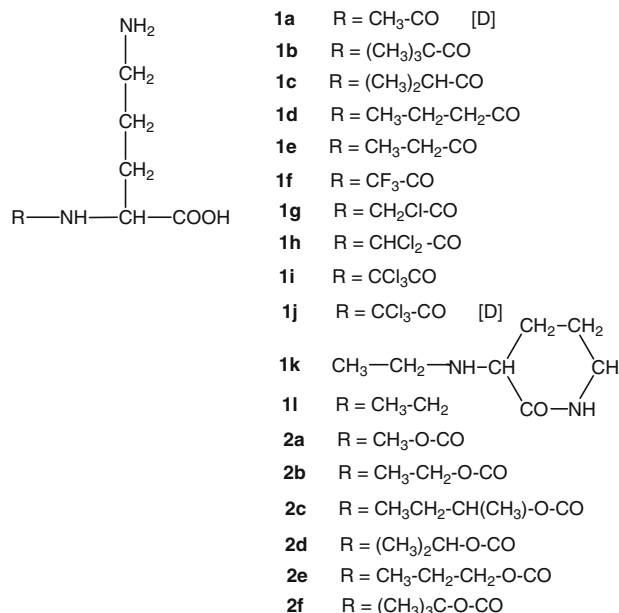


Fig. 2 General formula and structures of the ornithine **1a–1l** and **2a–2f** derivatives

was unable to relieve arginine auxotrophy in the same cell strain, therefore, ArgE is required for cell viability.

ArgE catalyzes the conversion of N^α -acetyl-L-ornithine to L-ornithine (Velasco et al. 2002) (Fig. 1). The substrate specificity of ArgE is broad in that several N^α -acylamino acids can be hydrolyzed including N^α -acetyl- or N^α -formylmethionine and N^α -acetylornithine (Javid-Majd and Blanchard 2000). Few inhibitors have been reported for ArgE (McGregor et al. 2007) to date, and fluoride ions were shown to be uncompetitive inhibitors exhibiting a modest K_i of 3.4 mM. Due to the crucial role ArgE plays in prokaryotic cell growth and proliferation, the development of specific and potent inhibitors of ArgE is of key importance. For that reason, we have designed a series of ornithine derivatives (Fig. 2) that were hypothesized to function as inhibitors of ArgE.

Compounds **1a–1j** (Fig. 3), **1k**, **1l** (Fig. 4), and **2a–2f** (Fig. 5) were synthesized and characterized, where the natural N^α -acetyl substituent was replaced. This general modification renders the corresponding amide bond resistant to hydrolytic cleavage by ArgE. Therefore, replacement of the

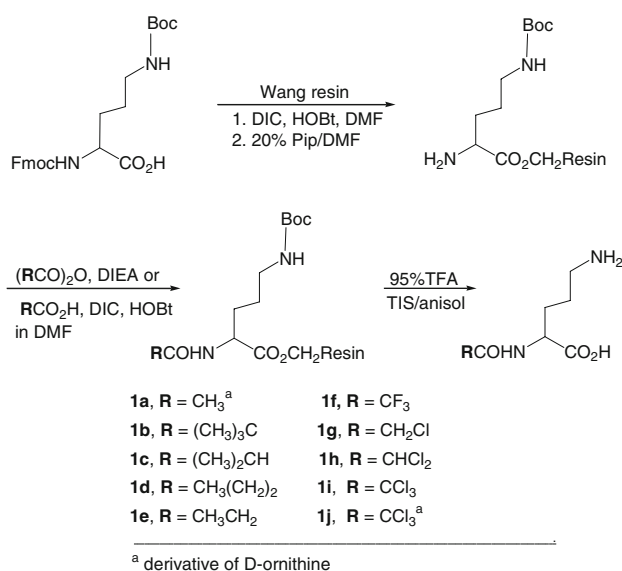


Fig. 3 Synthetic scheme for the preparation of the *N*^α-acyl-ornithine derivatives **1a–1j**

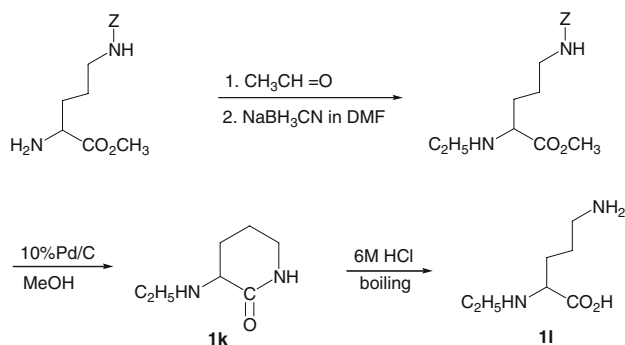


Fig. 4 Synthetic scheme for the preparation of the *N*^α-alkyl-ornithine derivatives **1k–1l**

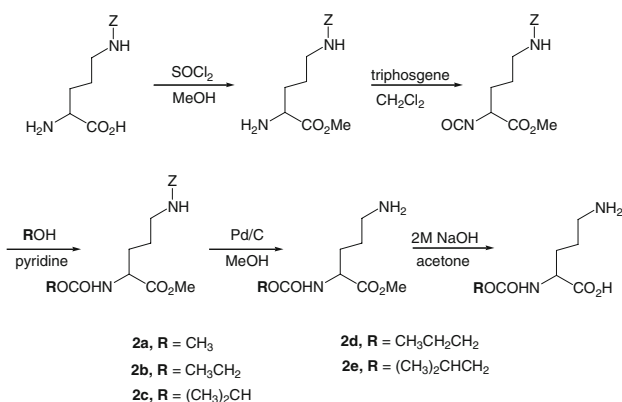


Fig. 5 Synthetic scheme for the preparation of the *N*^α-alkyloxycarbonyl-ornithine derivatives **2a–2f**

N^α-acetyl substituent was hypothesized to provide small molecule inhibitors of ArgE, which in turn may cause an interruption in the arginine biosynthetic pathway of bacteria,

inhibiting their growth. In an effort to gain insight into structure–activity relationships of this series of ornithine (Orn) derivatives, we determined their inhibitory potency toward the ArgE from *Escherichia coli* (Table 1). Most of the compounds tested provided IC₅₀ values in the μM range, indicating that they are moderately strong inhibitors. Weak inhibitory potency for a few of these Orn derivative toward *Bacillus subtilis*-168 was observed indicating that these derivatives are capable of getting across the cell membrane and that ArgE is the likely bacterial enzymatic target.

Materials and methods

General methodologies

H-*N*^δ-Z-Orn-OH was purchased from NovaBiochem, Switzerland and *N*^α-Fmoc-*N*^δ-Boc-Orn-OH, Wang resin (substitution 1.1 mmol/g) and triphosgene from Iris Comp, Germany. DIC, HOBt, DIEA, and reagents for *N*^α-acylation were purchased from Sigma-Aldrich, Czech Republic. Products were dried in a vacuum dry box (Salvis AG, Emmenbrücke-Luzern, Switzerland) at room temperature for 16 h. Tetracycline, LB broth, and LB agar were purchased from Sigma-Aldrich. The test organisms used were: *B. subtilis* 168, kindly provided by Prof. Yoshikawa (Princeton University, NJ) and *E. coli* B from the Czech Collection of Microorganisms (Brno, Czech Republic). Electrophoresis was carried out in a modified moist chamber apparatus (Durrum 1950) on Whatman No. 3 MM paper at 20 V/cm using 6% aqueous acetic acid (pH 2.5) and pyridine–acetate buffer (pH 5.7) as the electrolytes for 45 min. Electrophoretic mobilities *E*_{2,4}^{Gly}, *E*_{2,4}^{His}, and *E*_{5,7}^{His}, respectively, were expressed as the distance ratios of the compound and the reference amino acids Gly and His from the start line. Dried papers were stained with a 1% solution of ninhydrine in ethanol. Molecular weights of the compounds prepared were determined by mass spectroscopy using an ESI technique (Agilent 5975B MSD) from Agilent Technologies, USA. For HPLC, an SP 8800 pump and a TSP Chrom Jet SP4290 integrator and Spectra 100 UV detector were used. The ornithine derivatives were purified by semi-preparative HPLC on a Vydac RP-18, 25 × 1 cm, 10 μm column (Separation Groups, Hesperia, USA), flow rate 3 ml/min, detection at 220 nm, using an isocratic 0.05% aqueous TFA mobile phase. Analytical HPLC was carried out on a Supelco RP-18 Discovery 15 × 0.4 cm column, 5 μm (Supelco-Sigma-Aldrich, Czech Republic), with a flow rate of 1 ml/min at 220 nm, using a 0–100% gradient of ACN in 0.05% aqueous TFA over 40 min. Elemental analyses were performed at the Analytical Laboratory of the Institute of Organic Chemistry and Biochemistry, CAS. ¹H NMR and ¹³C NMR spectra were

Table 1 Analytical data and inhibitory activities of Orn **1a–1l** and **2a–2f** derivatives

Compound	Formula ^a Calc./found (m/z)	HPLC RT (min) ^{b–d}	ArgE IC50 value in mM	<i>B. subtilis</i> MIC (μM) ^e
1a^g , R=CH ₃	C ₇ H ₁₄ N ₂ O ₃ 174.2/175.1	2.85	0.41 ± 0.1	26.0 250
1b , R=CH ₃) ₃ C	C ₁₀ H ₂₀ N ₂ O ₃ 216.3/217.1	11.02	~ 5.4	19.5 h
1c , R=(CH ₃) ₂ CH	C ₉ H ₁₈ N ₂ O ₃ 202.3/203.1	11.22	1.21 ± 0.2	26.2 h
1d , R=CH ₃ (CH ₂) ₂	C ₉ H ₁₈ N ₂ O ₃ 202.3/203.1	11.12	0.42 ± 0.08	26.1 i
1e , R=CH ₃ CH ₂	C ₈ H ₁₆ N ₂ O ₃ 188.2/189.1	7.05	0.76 ± 0.04	26.0 h
1f , R=CF ₃	C ₇ H ₁₁ N ₂ O ₃ F ₃ 228.2/229.1	4.34	0.20 ± 0.04	23.9 250
1g , R=CH ₂ Cl	C ₇ H ₁₃ N ₂ O ₃ Cl 208.6/209.2	7.32	0.085 ± 0.007	21.7 250
1h , R=CHCl ₂	C ₇ H ₁₂ N ₂ O ₃ Cl ₂ 243.1/243.1	7.41	0.34 ± 0.03	41.2 500
1i , R=CCl ₃	C ₇ H ₁₁ N ₂ O ₃ Cl ₃ 277.5/276.9	7.68	0.45 ± 0.03	28.2 500
1j^g , R=CCl ₃	C ₇ H ₁₁ N ₂ O ₃ Cl ₃ 277.5/276.9	7.72	0.32 ± 0.02	28.2 i
1 k , C ₂ H ₅ –Orn(lactam)	C ₇ H ₁₄ N ₂ O 142.2/143.2	5.02	2.5 ± 0.2	22.1 h
1l , C ₂ H ₅ –Orn–OH	C ₇ H ₁₆ N ₂ O ₂ 160.21/161.1	4.21	2.7 ± 0.2	22.3 h
2a , R=CH ₃ O	C ₇ H ₁₄ N ₂ O ₄ 190.1/191.0	4.12	1.01 ± 0.11	26.1 i
2b , R=C ₂ H ₅ O	C ₈ H ₁₆ N ₂ O ₄ 204.2/205.1	5.22	0.25 ± 0.07	28.1 i
2c , R=(CH ₃) ₂ CHCH ₂ O	C ₁₀ H ₂₀ N ₂ O ₄ 232.3/233.3	9.51	1.16 ± 0.14	32.0 h
2d , R=(CH ₃) ₂ CHO	C ₉ H ₁₈ N ₂ O ₄ 218.2/219.2	7.56	1.40 ± 0.09	26.0 h
2e , R=CH ₃ (CH ₂) ₂ O	C ₉ H ₁₈ N ₂ O ₄ 218.2/218.2	7.58	0.71 ± 0.05	26.2 i
2f , R=(CH ₃) ₃ CO	C ₁₀ H ₂₀ N ₂ O ₄ 232.3/233.3	9.17	0.54 ± 0.05	29.0 i
Tetracycline	C ₂₂ H ₂₄ N ₂ O ₈ 444.4/445.3			45.2 12.5

^a Determined with ESI MS technique^b For HPLC a TSP instrument with an SP 8800 pump, an SP 4290 integrator, TSP Spectra 100 UV detector and 5 μm Supelco 15 × 0.4 cm column, 20 min gradient 0–50% ACN in 0.05% TFA and for compounds **1a**, **1k**, **1l**, **2a–2d** an isocratic analysis with 0.05% TFA were used^c Electrophoretic mobilities of derivatives **1a–1e** were in the range 0.92–1.14 (Gly) and 0.49–0.63 (His), those of the **2a–2f** were in the range 0.85–1.05 (Gly) and 0.41–0.52 (His), in 6% AcOH of the pH 2.4^d All compounds of series **1** and **2** gave correct elemental analyses of C, H, and N atoms in the range of 0.3%^e Maximal quantity of compound applied in agar plate^f MIC tested in concentrations 0.5–500 μM, tetracycline standard in the concentration range 0.5–500 μM^g Compound with D-Orn^h No inhibitionⁱ Very weak inhibitor

recorded on a spectrometer AVANCE-600 (¹H at 600.13 MHz; ¹³C at 150/90 MHz) from Bruker BioSpin Corporation (Billerica, MA, USA).

Synthesis of Orn derivatives

N^δ-Boc-Orn-O-Wang resin (Fig. 3)

To the Wang resin (5 g, 5.5 mmols of OH groups), swollen in DMF (3 × 20 ml), a solution of *N*^α-Fmoc-*N*^δ-Boc-Orn-OH (6 eq.; 15.0 g; 33.0 mmol), HOBt (6.6 eq.; 4.86 g;

36.3 mmol), DIC (6.6 eq.; 7.78 ml; 36.3 mmol), and DMAP (0.6 eq.; 0.4 g; 3.3 mmol) in DMF (30 ml) was added. This reaction mixture was stirred for 3 days at room temperature and then the resin was filtered and washed with 3 × 30 ml of DMF, 2-propanol, and DMF. After *N*^α-Fmoc group removal by 20% piperidine in DMF (20 ml) for 10 and 30 min, the H-*N*^δ-Boc-Orn-O-Wang resin was filtered, successively washed with 3 × 30 ml of DMF, 2-propanol, and MeOH and was dried in a desiccator over calcium chloride. The amount of Orn loaded onto the resin was found to be 0.45 mmol/g based on amino acid analysis (AA).

Table 2 Proton NMR data of ornithine derivatives in DMSO

Compound	HN	H _α	H _β	H _γ	H _δ	NH ₂	R
1a	8.21 d (<i>J</i> = 7.8)	4.18 m	1.75 m 1.57 m	1.57 m	2.78 m	7.83 b	CH ₃ CO: 1.85 s
1b	7.11 d (<i>J</i> = 5.5)	3.71 dt	1.67 m	1.53 m 1.47 m	2.74 m 2.70 m	7.76 b	(CH ₃) ₃ C: 1.10 s
1c	8.05 d (<i>J</i> = 8.2)	4.18 dt	1.77 m 1.59 m	1.56 m	2.77 m	7.74 b	(CH ₃) ₂ : 0.99 d, <i>J</i> = 6.8; 1.00 d, <i>J</i> = 6.8; CH: 2.44 h, <i>J</i> = 6.8
1d	8.12 d (<i>J</i> = 8.0)	4.19 dt	1.77 m 1.59 m	1.56 m	2.78 m	7.73 m	CH ₃ : 0.85 t, <i>J</i> = 7.4; CH ₂ : 1.51 m; CH ₂ CO: 2.09 t, <i>J</i> = 7.3
1e	8.10 d (<i>J</i> = 8.0)	4.19 dt	1.76 m 1.58 m	1.56 m	2.78 m	7.74 b	CH ₃ : 0.99 t, <i>J</i> = 7.6; CH ₂ CO: 2.13 q, <i>J</i> = 7.6
1f	9.77 b	4.27 um	1.90 m 1.74 m	1.58 m	2.79 m	7.76 b	–
1g	8.57 d (<i>J</i> = 7.8)	4.23 ddd	1.81 m 1.64 m	1.56 m	2.78 bt	7.75 b	CH ₂ Cl: 4.12 s
1h	8.95 d (<i>J</i> = 7.7)	4.22 dt	1.84 m 1.68 m	1.56 m	2.79 bt	7.79 b	CHCl ₂ : 6.55 s
1i	9.19 bd	4.18 m	1.91 m 1.80 m	1.58 m	2.78 m	7.82 b	–
1j	9.18 bd	4.20 m	1.93 m 1.83 m	1.59 m	2.79 m	7.81 b	–
1k	8.80 b	4.12 ddd	2.20 m 1.65 m	1.90 m 1.77 m	3.14 m 3.17 m	8.21 b	CH ₃ : 1.19 t, CH ₂ NH: 2.98 m
1l	~ 3.50 b	3.94 m	1.99 m 1.90 m	1.76 m 1.62 m	2.77 m	8.25 b	CH ₃ : 1.23 t, <i>J</i> = 7.3, CH ₂ : 2.95 m
2a	6.40 d	3.66 q	1.64 m	1.50 m	2.71 m	7.81 b	CH ₃ O: 3.50 s
2b	7.12 d	3.84 m	1.73 m 1.59 m	1.57 m	2.76 m	7.74 b	CH ₃ : 1.15 t; CH ₂ O: 3.97 m
2c*	7.31 d (<i>J</i> = 8.0)	3.89 m	1.74 m 1.58 m	1.58 m	2.77 m	7.78 b	CH ₃ : 0.85 t + 0.845 t, <i>J</i> = 7.4; 1.13 d + 1.135 d, <i>J</i> = 6.3; CH ₂ : 1.49 m; CH-O: 4.58 m
2d	7.21 d (<i>J</i> = 7.8)	3.87 m	1.73 m 1.58 m	1.58 m	2.76 m	7.74 b	CH ₃ : 1.165 d and 1.160 d, <i>J</i> = 6.3; CH-O: 4.73 h, <i>J</i> = 6.3
2e	6.20 bd (<i>J</i> ~ 6.0)	3.59 q	1.64 m	1.46 m	2.68 m	7.73 b	CH ₃ : 0.87 t, <i>J</i> = 7.4; CH ₂ : 1.53 m; CH ₂ -O: 3.85 m
2f	5.955 d (<i>J</i> = 5.5)	3.52 bq	1.64 m	1.54 m 1.47 m	2.70 m	7.77 b	(CH ₃) ₃ C: 1.365 s

*Mixture of diastereoisomers 1:1 (some signals are doubled)

N^α-Acyl derivatives of ornithine **1a–1j** (Fig. 3; Table 1)

A solution of carboxylic acid (10 eq.; 3.9 mmol), HOBT (11 eq.; 0.58 g; 4.3 mmol), and DIC (11 eq.; 0.92 ml; 4.3 mmol) or an acetanhydride/DIEA mixture 2:3 (5 ml) in DMF (10 ml) was added to H-*N*^δ-Boc-L-Orn-O-Wang resin or H-*N*^δ-Boc-D-Orn-O-Wang resin (0.9 g; 0.39 mmol of Orn, loaded at 0.43 mmol/g). The reaction mixture was stirred for 1 day at room temperature, filtered, and washed with DMF, 2-propanol, and MeOH (3 × 10 ml). A ninhydrine test (Kaiser et al. 1970) was used to check the level of completion of the *N*-acylation reaction. When a slight blue color persisted, re-coupling was again carried out using 50% of the reagent amounts listed above reacted for 12 h. Detachment of the corresponding ornithine derivative from the resin and deprotection of the side-chain (removal of *N*^δ-Boc) were performed simultaneously by treating with a 50% TFA–5% anisole–5% TIS mixture in DCM (10 ml). Finally, the solution was evaporated in vacuo to dryness at 30°C and the residue was triturated with *tert*-butyl-methyl ether and anhydrous diethyl ether. The corresponding *N*^α-acyl-Orn-OH derivative was purified using preparative HPLC under

conditions described in the “General Methodologies” section and characterized by analytical HPLC, ESI MS spectroscopy, elemental analysis, and electrophoresis (Table 1) and ¹H NMR (Table 2) and ¹³C NMR (Table 3) spectroscopy.

HCl·H-N^δ-Z-Orn-OMe

Methylester was prepared according to the literature (Brenner and Huber 1953): anhydrous MeOH (30 ml) was added to SOCl₂ (5 ml; 33 mmol) dropwise at –5°C with stirring. H-*N*^δ-Z-Orn-OH (7.99 g; 30 mmol) was added and stirring was continued at room temperature until the solid dissolved. After 2 h the reaction mixture was evaporated to dryness, the resulting residue was dissolved in MeOH and the evaporation repeated. Electrophoresis *E*_{2.4}^{Gly} 1.15; *E*_{2.4}^{His} 0.73; *E*_{5.7}^{His} 0.86 indicated ~10% of the starting acid in the final product. Therefore, the esterification procedure was repeated using the same conditions. The second esterification afforded product (7.7 g; 24.4 mmol; 81%), which was free of the starting acid and was used directly for the synthesis of compounds **1k**, **1l**, and the corresponding isocyanate in the synthesis of compounds **2a–2e**.

Table 3 Carbon-13 NMR data for each ornithine derivative in DMSO

Compound	C α	C β	C γ	C δ	COOH	R
1a	51.55	28.31	24.04	38.72	173.69	CH ₃ : 22.60; CONH: 169.68
1b	53.43	29.40	23.41	38.96	176.14	<i>t</i> -Bu: 27.63; >C<: 80.50; CONH: 174.01
1c	51.32	28.29	24.13	38.78	173.77	(CH ₃) ₂ : 19.64, 19.83; >CH–: 33.92; CONH: 176.65
1d	51.43	28.26	24.11	38.75	173.74	CH ₃ : 13.85; CH ₂ : 18.94; CH ₂ : 37.24; CONH: 172.76
1e	51.42	28.32	24.07	38.75	173.74	CH ₃ : 10.10; CH ₂ : 28.44; CONH: 173.41
1f	52.40	27.12	24.14	38.58	171.86	CF ₃ : 116.07 q, <i>J</i> (C,F) = 288.1; CONH: 156.78 q, <i>J</i> (C,F) = 36.5
1g	51.98	28.21	23.92	38.70	173.04	CH ₂ Cl: 42.61; CONH: 166.32
1h	52.50	28.19	23.88	38.86	172.52	CHCl ₂ : 66.70; CONH: 163.84
1i	53.74	27.21	24.34	38.82	172.18	CCl ₃ : 92.81; CONH: 161.66
1j	53.84	27.11	24.24	38.84	172.13	CCl ₃ : 92.78; CONH: 161.64
1k	54.30	22.72	20.29	40.61	166.92	CH ₃ : 11.16, CH ₂ NH: 39.76
1l	58.01	26.04	22.87	38.26	170.23	CH ₃ : 11.24; CH ₂ : 41.19
2a	54.96	29.47	23.35	38.86	174.20	CH ₃ O: 51.45; OCONH: 156.00
2b	53.86	28.43	24.01	38.75	174.01	CH ₃ : 14.37; CH ₂ O: 60.03; OCONH: 156.31
2c*	53.55	28.01	24.19	38.73	173.94	CH ₃ : 9.83 and 19.82; CH ₂ : 28.81 + 28.88; CH–O: 71.68 + 71.70; OCONH: 156.38
2d	53.62	28.15	24.14	38.73	173.98	CH ₃ : 22.28 and 22.30; CH–O: 67.23; OCONH: 156.08
2e	54.89	29.77	23.98	39.19	173.69	CH ₃ : 10.54; CH ₂ : 22.27; CH ₂ –O: 65.27; OCONH: 155.54
2f**	58.14 (59.61)	31.54	26.12 (26.32)	41.78	182.04	(CH ₃) ₃ : 30.40 (30.29); >C<: 83.85; OCONH: 160.37

*A mixture of diastereoisomers 1:1 was observed (some signals are doubled)

**D₂O was used as this molecule is insufficiently soluble in DMSO; some carbon signals are doubled (hindered rotation around C–N bond)

N^α -Ethyl-Orn-OH **1l** and corresponding lactam **1k**
(Fig. 4; Table 1)

Chilled acetaldehyde (0.63 ml; 3.84 mmol; 3 eq.) was added to a stirred DMF (15 ml) solution of $\text{HCl}\cdot\text{H}\cdot\text{N}^\delta\text{-Z-Orn-OMe}$ (0.38 g; 1.28 mmol) in the presence of DIEA (0.25 ml; 1.5 mmol) at 0°C . NaBH_3CN (0.45 g; 3.84 mmol; 3 eq.) in DMF (12 ml) was added to the reaction mixture within 2 min at 0°C . After elevation to room temperature, the mixture was stirred for another 3 h. The solvent was evaporated and the above reaction repeated with acetaldehyde (0.21 ml; 1.28 mmol) and NaBH_3CN (0.15 g; 1.28 mmol) in DMF (12 ml) and stirred for another 5 h. The DMF was again evaporated and the residue dissolved in ethyl acetate. This solution was washed with water and dried with anhydrous Na_2SO_4 . ESI mass for $\text{C}_2\text{H}_5\text{-Orn(Z)-OCH}_3$ ($\text{C}_{16}\text{H}_{24}\text{N}_2\text{O}_4$, 308.2) was found to be m/z : 309.1 ($\text{M} + \text{H}^+$). This compound (0.28 g; 0.91 mmol) was hydrogenated with 10% Pd on charcoal (50 mg) in MeOH (10 ml) for 3 h at room temperature. After filtration of the catalyst and evaporation of the solvent, the semi-oily residue (0.16 g; 0.89 mmol) was found to be the lactam **1k** with the formula $\text{C}_2\text{H}_5\text{-NH-CH}(\text{CH}_2\text{CH}_2\text{CH}_2\text{NHCO})$. ESI mass was determined for $\text{C}_7\text{H}_{14}\text{N}_2\text{O}$ (142.2), m/z : 143.1 ($\text{M} + \text{H}^+$); ^1H NMR and ^{13}C NMR results are provided in Tables 2 and 3. Treatment with boiling 6 M HCl (10 ml) for 2 h, opened the lactam. The solvent was evaporated resulting in compound **1l**, which was characterized by ESI MS spectroscopy, elemental analysis, electrophoresis (Table 1), and ^1H NMR and ^{13}C NMR spectroscopy (Tables 2, 3).

Ornithine derivatives **2a–2e** (Fig. 5; Table 1)

A solution of $\text{HCl}\cdot\text{H}\cdot\text{N}^\delta\text{-Z-Orn-OMe}$ (6.3 g; 20 mmol) and triphosgene (1.97 g; 6.59 mmol) in DCM (78.3 ml) was stirred vigorously in the presence of saturated aqueous NaHCO_3 (78.3 ml) on ice for 30 min. The reaction mixture was poured into a separatory funnel. The organic layer was collected and the aqueous layer was washed three times with DCM (13 ml). The combined organic layers were dried with MgSO_4 , filtered, and evaporated at reduced pressure to give a colorless oil that turned, upon refrigeration, into a white solid, that was triturated with a light petroleum to yield 5.73 g (18.9 mmol; 94.5%) of $\text{N}^\delta\text{-Z-Orn-OMe}$ isocyanate (m.p. 89°C). For $\text{C}_{15}\text{H}_{18}\text{N}_2\text{O}_5$ (306.3) calculated: 58.82% C, 5.88% H, 9.15% N; found: 58.61% C, 5.93% H, 8.98% N. This isocyanate (1.21 g, 3.9 mmol) was refluxed with an excess of the corresponding alcohol (20 ml) in the presence of pyridine (0.7 ml) for 2 h. The reaction mixture was evaporated and the resulting residue was dried in a desiccator after which it

was triturated with anhydrous diethyl ether to obtain the product, which was used without further purification.

The corresponding $\text{N}^\alpha\text{-R-O-CO-N}^\delta\text{-Z-Orn-OMe}$ (4 mmol) in MeOH (15 ml) solution was hydrogenated on 10% Pd/C (100 mg) until CO_2 was detected as solid BaCO_3 upon reaction with $\text{Ba}(\text{OH})_2$. The catalyst was removed by filtration through charcoal and the MeOH evaporated. The residue, dissolved in acetone (10 ml), was further stirred with 2 M NaOH (2 ml) at room temperature for 2 h. After saponification the pH was adjusted to 3 using AcOH after which the acetone was evaporated. The acidic solution was applied to a Dowex column 50×2 (10 ml) and washed with water (100 ml). The corresponding $\text{N}^\alpha\text{-R-O-CO-Orn-OH}$ was released from the ion exchange resin by aqueous ammonia (100 ml). Evaporation of ammonia and lyophilization yielded derivatives **2a–2e** that were purified by preparative HPLC and characterized by analytical HPLC, ESI MS spectroscopy, elemental analysis, and electrophoresis (Table 1) and ^1H NMR and ^{13}C NMR spectroscopy (Tables 2, 3).

$\text{N}^\alpha\text{-Boc-Orn-OH}$, **2f** (Table 1)

The $\text{H}\cdot\text{N}^\delta\text{-Z-Orn-OMe}$ (0.29 g; 1.1 mmol) in a dioxane–water mixture (10 ml) was treated with $(\text{Boc})_2\text{O}$ (0.24 g, 1.1 mmol) adjusted to pH 8 by NaHCO_3 , for 2 h at room temperature. The dioxane was evaporated, and the aqueous solution was acidified using 10% citric acid. The resulting oil was taken-up in ethyl acetate (3×100 ml), dried using anhydrous Na_2SO_4 after which the solution was evaporated to dryness, yielding the fully protected ornithine derivative. Deprotection was achieved following the procedures described above to yield compound **2f**, which was purified and characterized similarly to compounds **2a–2e**.

NMR structure determination

The NMR spectrum of each ornithine derivative (**1a–1l** and **2a–2f**) was measured on a Bruker AVANCE-600 (^1H at 600.13 MHz; ^{13}C at 150.90 MHz) spectrometer in $\text{d}^6\text{-DMSO}$ at 27°C and referenced to the solvent: $\delta_{\text{H}}(\text{DMSO}) = 2.50$ and $\delta_{\text{C}}(\text{DMSO}) = 39.70$. Chemical shifts were determined from 1D ^1H and ^{13}C NMR spectra. Correlated 2D-homonuclear spectra (2D- $^1\text{H}, ^1\text{H}$ -COSY) and 2D-heteronuclear spectra (2D- $^1\text{H}, ^{13}\text{C}$ -HSQC and 2D- $^1\text{H}, ^{13}\text{C}$ -HMBC) were used for structural assignment of hydrogen and carbon signals. All of the NMR data are summarized in Tables 2 and 3.

Measurement of IC_{50} values

ArgE from *E. coli* was purified as previously described (McGregor et al. 2005). The purified enzyme exhibited a

single band on 12% SDS-PAGE, corresponding to its calculated M_r of 42,350 by comparison with molecular weight standards purchased from Sigma. It was subsequently concentrated to >1 mM and stored at 4°C. Protein concentrations were determined using the theoretical value of $\epsilon_{280} = 41,250 \text{ M}^{-1} \text{ cm}^{-1}$ (McGregor et al. 2007).

IC_{50} values for each ornithine derivative were determined for ArgE (Table 1) using a spectrophotometric assay with NAO as the substrate, as previously described (McGregor et al. 2005). All kinetic experiments were performed in 50 mM Chelex-100 treated sodium phosphate buffer at pH 7.5 and 25°C. The rate of NAO deacetylation was monitored as a decrease in absorbance at 214 nm. Catalytic activities were determined within $\pm 10\%$. Initial rates were determined at a minimum of five inhibitor concentrations providing a dose-response curve indicating the concentration required for 50% inhibition (IC_{50}) (Table 1).

Antimicrobial activity determination

A quick qualitative estimate of the antimicrobial properties of ornithine derivatives was determined utilizing the double-layer technique originally developed by microbial geneticists for bacteriophage titration. Briefly, petri dishes (90 mm in diameter) with 20 ml of LB agar were prepared. Two ml of melted “soft” agar made from LB broth and 0.5% agar, containing bacteria (about 10^7 colony forming units, CFU) were poured over the surface. Fresh bacterial cultures were always prepared in LB broth and added when the melted soft agar cooled to $\sim 45^\circ\text{C}$. The ornithine derivatives ($0.001\text{--}10 \text{ mg ml}^{-1}$) in water (2 μl) were dropped onto the surface of the solidified upper layer, and the plates were incubated at 37°C. Clear zones of inhibition appeared within a few hours and remained clear for days. The potency was estimated by the diameter and clarity of the zones formed.

Quantitatively, the minimal inhibitory concentration (MIC) was established by observing the growth of bacteria growth in multi-well plates (Mendes et al. 2004; Oren and Shai 1997; Lequin et al. 2006). Bacteria in mid-exponential phase were added to individual wells containing test compound solutions of different concentrations in LB broth (0.2 ml volume and compound concentration in the range $0.5\text{--}500 \mu\text{M}$) and incubated at 37°C for 20 h. The plates were shaken continuously in a Bioscreen C instrument (Helsinki, Finland), and absorbance measurement were recorded at 540 nm every 15 min. Routinely, $1\text{--}5$ million CFUs were used to determine activity (corresponding to an absorption of $0.015\text{--}0.020$ units at 540 nm). Orn derivatives were tested in duplicate in at least three independent experiments. Tetracycline was tested as a standard in the concentration range $0.5\text{--}500 \mu\text{M}$.

Results and discussion

Previously, the synthesis of some N^α -acyl-derivatives of ornithine, in which the corresponding acylchlorides were used for acylation of $\text{H-}N^\delta\text{-Z-Orn-OH}$, was described (Hlaváček et al. 2007). However, given the large number of purification steps for intermediates and the limited number of compounds that could be obtained, an alternative route for the preparation of a larger number of ornithine derivatives (**1a–1j**) that eliminates purification steps was designed. These compounds were synthesized by acylation of $N^\delta\text{-Boc-L- or D-Orn}$ bound to a Wang polystyrene carrier, which served as the carboxyterminal protection for this amino acid (Fig. 3). Coupling of the commercially available $N^\alpha\text{-Fmoc-}N^\delta\text{-Boc-L- or D-Orn-OH}$ to this resin was carried out by DIC with HOBt as the coupling reagents in the presence of DMAP in DMF. The Fmoc protecting group was removed by the addition of 20% piperidine in DMF followed by the addition of a series of N^α -acyl groups, using acetaldehyde with DIEA (**1a**) or the corresponding carboxylic acids (**1b–1j**) with DIC and HOBt as the coupling reagents in DMF. Detachment of **1a–1j** from the resin and removal of the $N^\delta\text{-Boc}$ protecting groups were performed simultaneously in the last step of the syntheses by the addition of 95% TFA with TIS and anisole as scavengers.

Utilization of Wang resin for carboxyterminal protection resulted in a significantly simplified method for the synthesis of N^α -acyl-derivatives of ornithine. This method should be widely applicable for the solid phase synthesis of any amino acid derivative. One limitation involves the reductive alkylation of $N^\delta\text{-Boc-ornithine}$ bound to the Wang carrier (within the preparation of derivative **1i**) likely due to the close proximity of the α -amino group to the solid support, which blocked reaction with acetaldehyde and NaBH_3CN . Therefore, the successful synthesis of **1i** was carried out in solution starting with $\text{H-}N^\delta\text{-Z-Orn-OMe}$ using acetaldehyde and NaCNBH_3 in DMF, yielding $\text{C}_2\text{H}_5\text{-Orn(Z)-OCH}_3$ (Meyer et al. 1995). It should be noted that after hydrogenolytic side-chain deprotection, catalyzed by Pd on charcoal, the lactam **1k** was found due to the intramolecular reaction between the methyl ester and the free δ -amino groups in the N^α -ethyl-derivative (Fig. 4). While **1k** was examined for its inhibitory potency toward ArgE, it was also used as a starting material to synthesize the linear ornithine derivative **1l** by acidic hydrolysis in boiling 6 M HCl.

Synthesis of urethane groups containing N^α -alkyloxy-carbonyl derivatives of ornithine was accomplished via the corresponding isocyanate using triphosgene in DCM (Fig. 5). Using this general synthetic method compounds **2a–2e** were prepared starting from $N^\delta\text{-Z-Orn-OMe}$, which was reacted with triphosgene under mild alkaline

conditions to yield the corresponding isocyanate (Tsai et al. 2002). After refluxing in pyridine with a series of alcohols (Hlaváček et al. 1976), the corresponding Orn derivatives of general formulae N^α -R-O-CO- N^δ -Z-Orn-OMe were obtained. Hydrogenation on Pd/C, followed by saponification with NaOH yielded **2a–2e**, which was desalted using ion exchange chromatography on Dowex 50 and purified by HPLC (Table 1). Triphosgene in DCM was found to exhibit excellent reactivity by modifying only the α -amino group under mild alkaline conditions. Interestingly, no cyclization or lactam formation was observed until after N^δ -Z deprotection by hydrogenolysis of the corresponding N^α -R-O-CO- N^δ -Z-Orn-OMe derivative. Finally, $(\text{CH}_3)_3\text{C-O-CO-Orn-OH}$ (**2f**) was prepared by direct reaction of $\text{H-}N^\delta$ -Z-Orn-OMe with $(\text{Boc})_2\text{O}$ in a dioxane–water mixture at pH 8 adjusted with NaHCO_3 (Moroder et al. 1976), followed by deprotection of the δ -amino and carboxyl groups, desalting and purification as previously described.

All of the Orn derivatives synthesized were examined for their ability to inhibit the catalytic activity of ArgE. Of the 18 compounds synthesized, nearly all inhibited ArgE activity as evidenced by IC_{50} values in the μM range. These data indicate that all of the ornithine derivatives prepared in this study are moderately strong inhibitors of ArgE. The Orn derivative that provided the best inhibitory response was N^α -chloroacetyl-L-ornithine (**1g**), which exhibited an IC_{50} value of 85 μM . Similarly, the trifluoroacetyl-(**1f**) and ethoxycarbonyl-(**2b**) ornithine derivatives provided IC_{50} values of 200 and 250 μM , respectively. It should be noted that the N^α -dichloroacetyl-L-ornithine (**1h**), the N^α -trichloroacetyl-D-ornithine (**1j**), the N^α -butyryl-L-ornithine (**1d**), and the N^α -acetyl-D-ornithine (**1a**) derivatives all exhibited moderate inhibition of ArgE providing IC_{50} values in the range 320–450 μM (Table 1). Inspection of these inhibitory data provides some clues into the active site of ArgE enzymes. The best inhibitors have small R groups with the relative inhibitory order $\text{CH}_2\text{Cl} \gg \text{CF}_3 > \text{CHCl}_2 > \text{CH}_3$ with the D and L derivatives of CHCl_2 inhibiting ArgE equally. This trend suggests that this R group must recognize specific binding pocket near to the dinuclear Zn(II) active site of ArgE.

In order to determine if Orn derivatives are potential antibiotic leads their antimicrobial activity was examined against *B. subtilis*-168 and *E. coli* B (Table 1). A majority of these ArgE inhibitors proved to be weak or very weak antimicrobial agents as evidenced by the drop diffusion tests performed with *B. subtilis*-168. However, no activity was detected on *E. coli*, even at the highest concentration used ($\text{MIC} \gg 250 \mu\text{M}$ and no clear zone for 10 mg/ml concentration on the agar plate). The effective derivatives (**1a**, **1d**, **1f–1j**, **2a**, **2b**, **2e**, **2f**) as determined from the drop diffusion test were also assayed for their MIC

determination. Only five Orn derivatives (**1a**, **1f–1i**) were weak inhibitors of *B. subtilis*-168 in the range 250–500 μM for CFUs amounting to millions of bacteria. As in the drop diffusion test, no MIC activity of Orn derivatives toward *E. coli* was found.

Probably, the weak activity of Orn derivatives toward *B. subtilis* might correspond to a decrease or elimination of the hydrolytic activity of ArgE via inhibition of the amide bond cleavage in N^α -acetylornithine. Above data correlate well with the IC_{50} values determined for ArgE, suggesting an indirect evidence that Orn derivatives are capable of getting across the cell membrane and that ArgE is the likely bacterial enzymatic target.

In conclusion, the new Orn based compounds described herein represent a new class of inhibitors for ArgE enzymes. Interestingly, several of these compounds exhibit weak antibacterial activity toward *B. subtilis*. Therefore, ornithine provides a realistic structural platform for the design of new inhibitors for ArgE enzymes that may possess antimicrobial activity. In general, small molecules that are non-degradable substrate analogues should provide effective inhibitors of bacterial enzymes and represent a reasonable approach toward the development of a previously undescribed antimicrobial agent. Ultimately, refining the structural aspects of these substrate analog inhibitors in order to match inhibitor structural features to those of the enzyme, including metal binding interactions, will be crucial to the discovery of a clinically relevant antimicrobial agent.

Acknowledgments This research was supported by the Research Project Z4 055 0506, the Grant Agency of the Czech Academy of Sciences (No. IAA400550614) and the National Science Foundation (CHE-0652981, RCH).

References

- Bradshaw R, Yi E (2002) Methionine aminopeptidases and angiogenesis. *Essays Biochem* 38:65–78
- Brenner M, Huber W (1953) Herstellung von α -Aminosäureestern durch Alkoholyse der Methylester. *Helv Chim Acta* 36:1109–1115
- CDC (1995) Hospital infection control practices advisory committee's recommendations for preventing the spread of vancomycin resistance. *MMWR Morb Mortal Wkly Rep* 44:1–13
- Cunin R, Glansdorff N, Pierard A, Stalon V (1986) Biosynthesis and metabolism of arginine in bacteria. *Microbiol Rev* 50:314–352
- Daiyasua H, Osakaa K, Ishinob Y, Toha H (2001) Expansion of the zinc metallo-hydrolase family of the L-lactamase fold. *FEBS Lett* 503:1–6
- Davis RH (1986) Compartmental and regulatory mechanisms in the arginine pathways of *Neurospora Crassa* and *Saccharomyces Cerevisiae*. *Microbiol Rev* 50:280–313
- Dismukes GC (1996) Manganese enzymes with binuclear active sites. *Chem Rev* 96:2909–2926
- Durum EL (1950) A microelectrophoretic and microionophoretic technique. *J Am Chem Soc* 72:2943–2948

- Girodeau J-M, Agouridas C, Masson MRP, LeGoffic F (1986) The lysine pathway as a target for a new genera of synthetic antibacterial antibiotics. *J Med Chem* 29:1023–1030
- Hancock REW (1997) Peptide antibiotics. *Lancet* 349:418–422
- Harris BZ, Singer M (1998) Identification and characterization of the *Myxococcus xanthus* *ArgE* gene. *J Bacteriol* 180:6412–6414
- Hlaváček J, Poduška K, Šorm F, Sláma K (1976) Peptidic analogues of insect juvenile hormone containing urethane type protecting groups. *Collect Czech Chem Commun* 41:317–324
- Hlaváček J, Zyka D, Pícha J, Jiráček J, Čerovský V, Slaninová J, Fučík V, Holz RC (2007) Synthesis and characterization of potential inhibitors of *dapE* and *argE* enzymes as new antimicrobial agents. In: Wilce J (Ed) Proceedings of 4th international peptide symposium in conjunction with 7th Australian peptide conference and 2nd Asia-Pacific international peptide symposium, Cairns Queensland Australia 2007, www.peptideoz.org, N_317
- Holz RC, Bzymek K, Swierczek SI (2003) Co-catalytic metallopeptidases as pharmaceutical targets. *Curr Opin Chem Biol* 7:197–206
- Javid-Majd F, Blanchard JS (2000) Mechanistic analysis of the *argE*-encoded *N*-acetylornithine deacetylase. *Biochemistry* 39:1285–1293
- Kaiser E, Colescott RL, Bossinger CD, Cook PI (1970) Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal Biochem* 34:595–598
- Ledwidge R, Blanchard JS (1999) The dual biosynthetic capability of *N*-acetylornithine aminotransferase in arginine and lysine biosynthesis. *Biochemistry* 38:3019–3024
- Lequin O, Ladram A, Chabbert L, Bruston F, Convert O, Vanhoye D, Chassaing G, Nicolas P, Amiche M (2006) Dermaseptin S9, an α -helical antimicrobial peptide with a hydrophobic core and cationic termini. *Biochemistry* 45:468–480
- Levy SB (1998) The challenge of antibiotic resistance. *Sci Am* 278:46–53
- Lipscomb WN, Sträter N (1996) Recent advances in zinc enzymology. *Chem Rev* 96:2375–2433
- McGregor W, Swierczek SI, Bennett B, Holz RC (2005) The *argE*-encoded *N*-Acetyl-L-ornithine deacetylase from *Escherichia coli* contains a dinuclear metallo-active site. *J Am Chem Soc* 127:14100–14107
- McGregor W, Bennett B, Holz RC (2007) Kinetic and spectroscopic characterization of the manganese(II)-loaded *argE*-encoded *N*-acetyl-L-ornithine deacetylase from *Escherichia coli*. *J Biol Inorg Chem* 12:603–613
- Meinzel T, Schmitt E, Mechulam Y, Blanquet S (1992) Structural and biochemical characterization of the *Escherichia coli* *argE* gene product. *J Bacteriol* 174:2323–2331
- Mendes MA, De Sousa BM, Marques MR, Palma MS (2004) Structural and biochemical characterization of the novel peptides from the venom of the neotropical social vamp *Agelaia palpes*. *Toxicon* 44:67–74
- Meyer J-P, Davis P, Lee KB, Porreca F, Yamamura HI, Hruby VJ (1995) Synthesis using a Fmoc-based strategy and biological activities of some reduced peptide bond pseudopeptide analogues of dynorphin A. *J Med Chem* 38:3462–3468
- Moroder L, Hallett A, Wunsch E, Keller O, Wersing G (1976) Di-tert-butylidylcarbonat- ein vorteilhaftes Reagenz zur Einführung der tert-Butoxycarbonyl-Schutzgruppe. *Hoppe-Seyler's Z Physiol Chem* 357:1651–1653
- Nemecek S (1997) Beating Bacteria. New ways to fend off antibiotic-resistant pathogens. *Sci Am* 276:38–39
- Oren Z, Shai Y (1997) Selective lysis of bacteria but not mammalian cells by dia-stereoisomers of mellitin: structure function study. *Biochemistry* 36:1826–1835
- Teuber M (1999) Spread of antibiotic resistance with food-borne pathogens. *Cell Mol Life Sci* 56:755–763
- Tsai JH, Takaoka LR, Powell NA, Nowick JS (2002) Synthesis of amino acid ester isocyanates: methyl (S)-2-isocyanato-3-phenylpropanoate. *Organic Syntheses* 78:220–221; Coll 10:544–555 (2004)
- Van de Casteele M, Demarez M, Legrain C, Glansdorff N, Pierard A (1990) Pathways of arginine biosynthesis in extreme thermophilic archae- and eubacteria. *J Gen Microbiol* 136:1177–1183
- Velasco AM, Leguina JJ, Lazcano A (2002) Molecular evolution of the lysine biosynthetic pathways. *J Mol Evol* 55:445–459
- Vogel HJ, MacLellan WL (1970) Acetylornithinase (*E. coli*). *Methods Enzymol* 17A:265–269
- Wilcox DE (1996) Binuclear metallohydrolases. *Chem Rev* 96:2435–2458
- Xu Y, Liang Z, Legrain C, Ruger HL, Glansdorff N (2000) Evolution of arginine biosynthesis in the bacterial domain: novel gene-enzyme relationships from psychrophilic *Moritella* strains (*Vibrionaceae*) and evolutionary significance of *N*-alpha-acetylornithinase. *J Bacteriol* 182:1609–1615