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Synthetic *N*-(Hydroxy)urea-based Dipeptides as Potential Antibacterial Agents

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Background: A type of *N*-(hydroxyl)urea-based dipeptides was synthesized as a new class of antibacterial agents based on a naturally occurring hydroxamate-containing dipeptide 3-((1-((2-[Hydroxymethyl]-1-pyrrolidinyl)carbonyl)-2-methylpropyl)carbamoyl)octanohydroxamic acid (actinonin, **1a**). Actinonin has been assumed to inhibit bacterial peptide deformylase (PDF), which is a metalloprotease and required for final protein maturation for bacterial survival in all of the bacterial species. Methods: In preliminary screening, *N*-(hydroxyl)ureido-norleucyl-valyl-indolin-1-ylamide (**11c**) was two-fold more potent than actinonin against *S. aureus*. Both *N*-(hydroxyl)ureido-norleucyl-valyl-(4-benzyl)piperazin-1-ylamide (**11a**) and **11c** also showed moderate potency against *B. subtilis* and *E. coli* with MIC at 25 μg/ml. **Results:** In the bacteria-sensitive test, the potency of **11a** and **11c** were relatively equal to kanamycin with MIC of 25 μg/ml and 50 μg/ml, respectively. **Conclusion:** No cytotoxic effects on HEK 293 cells were observed at 10 μM levels for the tested compounds.

Key words: antibacterial agent, actinonin, peptide deformylase, *N*-(Hydroxy)ureas

INTRODUCTION

There has been a great deal of work and contributions on the discovery of antimicrobial agents from natural sources and through synthetic chemistry since the beginning of the twentieth century. Many broad-spectrum agents have really been magic bullets in the treatment of multiple diseases that have actually been fatal, showing high mortality rates. Unfortunately, the frequent clinical use of these drugs has elicited the progressive development of bacterial resistance in multiple species. Nevertheless, the number of new drugs developed for clinical use has markedly declined in the past two decades. Recently, the rising prevalence of multi-drug resistant bacteria has urgently raised the impetus for search of novel antimicrobials. Although efforts in traditionally cell-based screening or modification of routinely used antibiotics generate new agents, these approaches have always failed to surmount the emergence of resistance. The unraveling of genomes provides alternative strategies for identifying new targets. The gene encoding peptide deformylase (PDF) is present in all sequenced pathogenic bacterial genomes and PDF is required in the N-terminal methionine excision for the last step of protein synthesis in all organisms,[1] making it an attractive target for developing therapeutics against formidable pathogens.[2,3]

There have been mounting reports describing inhibitors of PDF, but few of these have displayed prominent antimicrobial activity. Actinonin (Figure 1), a naturally occurring antibiotic,[4] assumed to possess the transition-state structural feature of the PDF substrate-processing step, has been shown to be a potent inhibitor of PDF. By screening a variety of chemical libraries, certain potent antimicrobial compounds have been disclosed, for instance BB-3497 and VRC-3379 (Figure 1),[5,6] which, nevertheless, contain a metal-chelating bidentate moiety. This information suggests that moieties composed of non-bounding electrons of heteroatoms possess metal-binding abilities within the adopted conformation. In this study, we described a series of derivatives with *N*-(hydroxyl)ureido dipeptides and investigated their antimicrobial activities.

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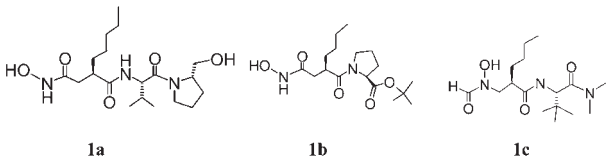
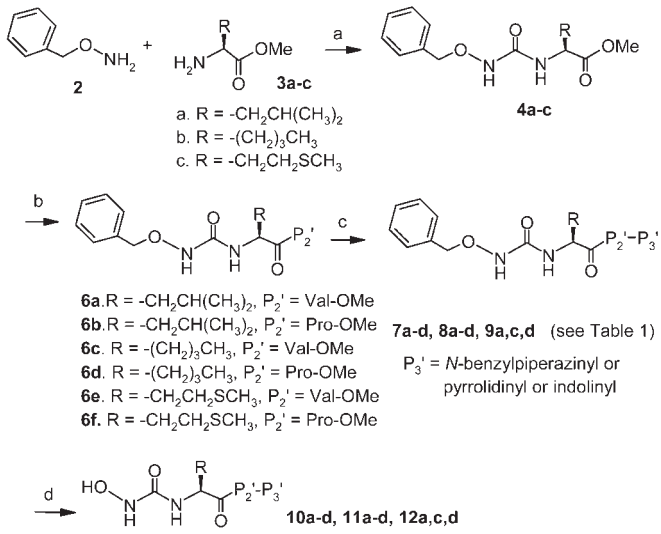


1a 1b 1c

Fig. 1 Chemcal structures of actinonin (1a), VRC3379 (1b), and BB-3497 (1c)

Scheme 1.[a]



[a] **Reagents and conditions:** a) Cl_3CO-CO-$OCCl_3$, r.t. b) i. LiOH, THF. ii. valine methyl ester (**5a**) or proline methyl ester (**5b**), BOP, DIEA. c) i. LiOH, THF. ii. *N*-benzylpiperazine (**6x**) or pyrrolidine (**6y**), or indoline (**6z**), EDCI, HOBt. d) $H_{2(g)}$, 10% Pd-C.

METHODS

Bacteria

Escherichia coli JM109, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 25923 were obtained from the Culture Collection and Research Center, Hsin-Chu, Taiwan, R.O.C. Clinical isolate of *Bacillus substilis* TSGH743 was obtained from the Culture Collection of Tri-Service General Hospital, Taipei, Taiwan. All bacteria were stored in Luria-Bertani broth (Difco Laboratories, Detroit, MI, USA) with 15% (v/v) glycerol at -70°C.

Synthesis of *N*-(hydroxy)urea-based dipeptides

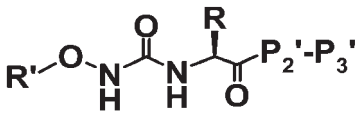
*Synthesis of N-(hydroxyl)ureido-norleucyl-valyl-indolin-1-ylamide (**11c**) as a typical example* (Scheme 1): a). Preparation of **4**: Triphosgene (0.37 mmol, 110 mg, 0.37 eq) was dissolved in CH2Cl2 (2 ml). A mixture of amino acid methyl ester hydrochloride (1 mmol, 1 eq) and diisopropylethylamine (DIEA, 2.2 mmol, 3.78 ml, 2.2 eq) in CH_2Cl_2 (3.5 ml) was slowly added to the stirred solution of triphosgene over a period of 30 min using a syringe pump. After 5 min of stirring, a solution of *O*-benzylhydroxyamine hydrochloride (1 mmol, 1 eq) and DIEA (2.2 mmol, 3.78 ml, 2.2 eq) in CH_2Cl_2 (2 ml) was added in one portion. The reaction mixture was stirred for 10 min at r.t, evaporated to dryness and diluted with EtOAc. The mixture was washed with 10% $KHSO_4$, 5% $NaHCO_3$ and brine, dried over $MgSO_4$ and evaporated to give the title compound after purification through chromatography (*n*-Hex : EtOAc= 1:1). b). *Synthesis of N-(hydroxyl)ureido-norleucyl-valyl-indolin-1-ylamide (**11c**)*: A solution of *N*-[(*O*-benzyl)-hydroxy)]ureido-norleucyl-valine methyl ester (**6c**) in 50 ml of THF/MeOH/H_2O (3:1:1) was treated with LiOH (2.5 eq) at room temperature for 12 h to give a solid, which was directly condensed with indoline (**6z**) in the presence of EDCI and HOBt to yield *N*-[(*O*-benzyl)-hydroxy)]ureido-norleucyl-valyl-indolin-1-ylamide (**8c**). 10% Pd-C was added to a solution of **8c** in 24 ml of EtOAc/MeOH and stirred under $H_{2(g)}$ to give the title compound **11c**, after purification from silica gel chromatography: $[\alpha]_D$ -54.4° (c 0.48, MeOH); FABMS: m/z 391 $[M+H]^+$; HR-FABMS exact mass calcd for $C_{20}H_{31}N_4O_4$ $[M+H]^+$ 391.2347, found 391.2349 ; ^{1}H NMR (300 MHz, CDCl3): δ 6.92-7.38 (m, 4H, Ar-Hs), 4.41-4.58 (m ,2H, 2α-CH), 3.15-3.33 (m, 2H, N-CH_2), 2.13-2.63 (m, 3H, Ar-CH_2, Val-β-CH), 1.12-1.95 (m, 7H, CH_2-CH_2-CH_2, OH), 0.76-1.01 (m, 9H, $C(CH_3)_2$, CH_3).

Determination of Antimicrobial Activity

To determine the antimicrobial activity of antibiotics, minimal inhibitiory concentrations (MICs) of antibiotics for Gram-negative (*E. coli and Pseudomonas aeruginosa*) and Gram-positive (*Staphylococcus aureus* and *Bacillus substilis*) bacterial species were determined by a broth dilution method in 2-ml volumes of Mueller-Hinton broth (Difco). To prepare the inoculum of the test organism, four well isolated colonies were selected from an LB Agar plate culture and transferred to a tube containing 5 ml of LB broth. After overnight incubation at 37°C, the cell concentration was adjusted to 10^8 CFU/ml and 20 μl of the cell suspension was used as inoculum for each tube containing 2-ml of broth plus antibiotics. All cultures were incubated at 37°C for approximately 18 h. MIC is defined as the lowest concentration (μg/ml) of antibiotic that can completely inhibit the growth of the test organism.

Table 1 The chemical structures of the synthetic *N*-(hydroxyl)urea-based dipeptides

Synthetic *N*-(hydroxyl)ureas



Compound	R	P2'	P3'	Yield (%)
7a (R'=Bzl), 10a (R'=H)	$CH_2CH(CH_3)_2$	Valyl	*N*-Benzylpiperazinyl	43-68
7b (R'=Bzl), 10b (R'=H)	$CH_2CH(CH_3)_2$	Valyl	Pyrrolidinyl	61-79
7c (R'=Bzl), 10c (R'=H)	$CH_2CH(CH_3)_2$	Valyl	Indolinyl	55-72
7d (R'=Bzl), 10d (R'=H)	$CH_2CH(CH_3)_2$	Prolyl	Indolinyl	45-89
8a (R'=Bzl), 11a (R'=H)	$CH_2CH_2CH_2CH_3$	Valyl	*N*-Benzylpiperazinyl	59-71
8b (R'=Bzl), 11b (R'=H)	$CH_2CH_2CH_2CH_3$	Valyl	Pyrrolidinyl	49-70
8c (R'=Bzl), 11c (R'=H)	$CH_2CH_2CH_2CH_3$	Valyl	Indolinyl	53-98
8d (R'=Bzl), 11d (R'=H)	$CH_2CH_2CH_2CH_3$	Prolyl	Indolinyl	55-74
9a (R'=Bzl), 12a (R'=H)	$CH_2CH_2SCH_3$	Valyl	*N*-Benzylpiperazinyl	63-73
9c (R'=Bzl), 12c (R'=H)	$CH_2CH_2SCH_3$	Valyl	Indolinyl	52-92
9d (R'=Bzl), 12d (R'=H)	$CH_2CH_2SCH_3$	Prolyl	Indolinyl	45-64

Cell Viability Test

The cytotoxicity of the test compounds were assessed by using human HEK293 cell lines and performed as previously described.[7] The human cell line was supplemented with DMEM medium in 5% CO_2 at 37°C. The tests were conducted with 96-well microtiter plates and each of the test compounds was serially diluted in 1% dimethyl sulfoxide. Ten microliters of each solution was added to wells with one well as a control, containing 1% dimethyl sulfoxide without the drug. The cells were suspended in appropriate assay medium and added to each well. The cells were exposed to the testing compounds for 72 h at 37°C in the presence of 5% CO_2. One day later, an MTT solution in phosphate-buffered saline (PBS) was added to each well, and the suspension was reincubated for 3 h under the same conditions. The formazan's product from the MTT cleavage was detected by recording the absorbance change at 550 nm; the percentage of cell viability compared with that of cells in the corresponding control well was calculated with the following equation:

$$\text{Cell viability (\%)} = [1 - At/Ao] \times 100$$

In the equation, At is the absorbance of the test compound and Ao is the absorbance of the control well.

Statistical Analysis

All values are expressed as the mean±s.d. Statistical analysis was performed using the Student's t-test.

RESULTS

Chemistry

At the first step, we established a *O*-benzyl protected form of *N*-(hydroxyl)ureido functionality from *in-situ* condensation of each of the amino acid esters **3a-c**, *O*-benzyl-hydroxylamine, and triphosgene. Thus, *N*-(*O*-benzyl-hydroxyl)ureido-leucine, -norleucine, and -methionine methyl esters **4a-c** were obtained in 77-93% yields from corresponding amino acid esters under mild conditions. Hydrolysis of these methyl ester intermediates under basic conditions gave the free acids followed by condensation with valine methyl ester (**5a**) or proline methyl ester (**5b**) using BOP reagent which was completed with good yield to afford the desired dipeptide intermediates **6a-f**. Subsequently, repeat saponification of **6a-f** followed by incorporation with a series of lipophilic amines **6x-z** as selected to optimize the relative activities and also to probe the diversity of the binding pockets. At the final step, the *O*-benzyl protected group of these resulting ureido dipeptides **7-9** were removed through catalytic hydrogenation with 10% palladium on carbon to yield *N*-(hydroxyl)ureido dipeptides **10-12**. The chemical structures of these synthetic *N*-(hydroxyl)ureido dipeptides were characterized by the fully satisfactory spectra (^{1}H-NMR, MS, and HR-FABMS).

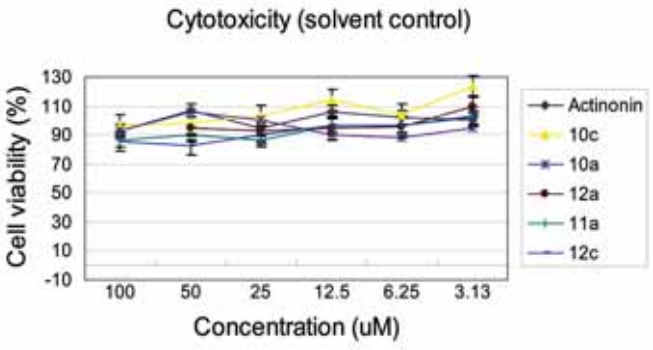
The biological activities of these synthetic compounds on the growth inhibition of certain infectious microorganisms were measured by using a bacteria-sensitivity test. First, the repeated studies to determine the efficacy of the antibacterial activities of the series of *N*-(hydroxy)urea-based dipeptides was carried out on these Gram-positive and Gram-negative bacterial species. The results are presented in Table 2 along with data for actinonin and kanamycin as comparison. Apparently, most *N*-(hydroxy)ureas showed moderate activities and selectivity, with IC_{50}s at the micromolar levels against *B. subtilis*. Among these compounds, *N*-(hydroxyl)ureido-norleucyl-prolyl-indolin-1-ylamide (**11d**) displayed the most promising antibacterial activities with improved potency, while *N*-(hydroxyl)ureido-norleucyl-valyl-indolin-1-ylamide (**11c**) still maintained potent activities, as compared to the lead actinonin. Moreover, **11d**, possessing a *n*-butyl side chain at the P_1' position like VRC3379, which is one-carbon short related to the *n*-pentyl group of actinonin, displayed an improved inhibitory potency. According

Table 2 The minimal inhibitory concentrations (MICs, microgram/ml) of the synthetic *N*-(hydroxy) urea-based dipeptides

Compound	*S. aureus*	*B. Subtilis*	*P. aeruginosa*	*E. coli*
10a	25	<1.56	>50	12.5
10b	>50	25	>50	>50
10c	25	<1.56	25	25
10d	>50	25	>50	50
11a	12.5	<1.56	25	12.5
11b	>50	>50	>50	>50
11c	3.125	<1.56	25	12.5
11d	3.125	<1.56	12.5	6.25
12a	25	<1.56	50	25
12c	6.25	<1.56	>50	12.5
12d	>50	12.5	50	>50
Actinonin	6.25	<1.56	>50	12.5
Kanamycin	1.56	<1.56	50	<1.56

to these results, keeping valyl residue or replacing it with proline at the P_2' position, both **11c** and **11d** still preserved the potent anti-G-(+) activities. These compounds showed broad potency against some G-(-) bacteria, such as *P. aeruginosa* and *E. coli*, which might be attributed to an additional hydrophobic indolinyl group at the *C*-terminus. Nevertheless, isosterically replacing norleucyl residue of compounds **11** with methionine at P_1' position, *N*-(hydroxyl)ureido-methionyl-valyl-indolin-1-ylamide (**12c**) still maintained its broad activity against *S. aureus*, *B. subtilis*, and *E. coli* as it possesses indolinyl residue, comparable to the structure of actinonin. On the other hand, either changing the valyl residue of the P_2' position or replacing the *C*-terminal indolinyl group only worsened the antibacterial activities as *N*-(hydroxyl)ureido-methionyl-valyl-(4-benzyl)piperazin-1-ylamide (**12a**) and *N*-(hydroxyl)ureido-methionyl-prolyl-indolin-1-ylamide (**12d**) were 2~4-fold less potent against *S. aureus* and *E. coli* than actinonin. Most of these antibacterial *N*-(hydroxy)ureido-based small peptides presented no significant cytotoxicity on the HEK293 cells, as shown in Figure 2. 90% of the cells survived with these compounds at the concentration of 10 μM levels.

These studies clearly show that keeping a lipophilic residue (e.g. norleucyl) at the P_1' position and a relatively stringent side-chain at the P_2' position are requisite for the antibacterial potency. Moreover, the antibacterial activities can be improved with isosteric replacement of the natural hydroxamate residue of actinonin with an *N*-(hydroxyl)ureido moiety and proper C-terminal manipulation.



Values are mean±s.d., n = 3.

Fig. 2 Cell viability ratio of certain *N*-(hydroxy)urea-based dipeptides

DISCUSSION

In summary, we have delicately explored the potential of certain *N*-(hydroxyl)urea-based small peptides to be potent antibacterial agents, assuming that the antibacterial effects of these small peptides are probably due to their inhibition of deformylase activity based on their structural similarity to that of actinonin. Some of the synthetic *N*-(hydroxyl)ureas showed a relatively high selective potency against certain G-(-) bacteria without diminishing their broad antibacterial potency.

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