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Bacicyclin, a new antibacterial cyclic hexapeptide from *Bacillus* sp. strain BC028 isolated from *Mytilus edulis*

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ABSTRACT

A new cyclic hexapeptide, cyclo-(Gly-Leu-Val-IIe-Ala-Phe), named bacicyclin (**1**), was isolated from a marine *Bacillus* sp. strain associated with *Mytilus edulis*. The sequences of the amino acid building blocks of the cyclic peptide and its structure were determined by 1D- and 2D-NMR techniques. Marfey's analysis showed that the amino acid building blocks had L-configuration in all cases except for alanine and phenylalanine, which had D-configuration. Bacicyclin (**1**) exhibited antibacterial activity against the clinically relevant strains *Enterococcus faecalis* and *Staphylococcus aureus* with minimal inhibitory concentration values of 8 and 12 μM, respectively. These results demonstrate the potential of marine bacteria as a promising source for the discovery of new antibiotics.

Molluscans are wide spread filter feeders in aquatic environments and are known as producers of biological active compounds, such as polyproniates, alkaloids, peptides, defensins, and proteins playing an important role in humoral immunity of the producing organisms or in the defense against pathogens.[1–4] Antimicrobial peptides were also isolated from the blue mussel *Mytilus edulis*. Mytilin A, mytilin B, and mytimycin have been found in *Mytilus edulis* from the White Sea (North Karelia region, Russia).[5] Crude extracts of *M. edulis* from the Baltic Sea exhibited antifouling effects and prevented attachment of common fouling organisms such as the barnacle *Balanus amphitrite*, the bacterium *Cobetia marina*, and *Amphora coffeaeformis*, a benthic diatom.[6] *Mytilus* sp. also provides a habitat for a diverse array of bacteria. *Mytilus galloprovincialis* obtained from the Mediterranean Sea (Italy) was colonized mainly by members of the genus *Aeromonas*, though representatives of *Moraxella*, *Pseudomonas*, *Alcaligenes*, *Acinetobacter*, *Flavobacterium*, *Chromobacterium*, *Photobacterium*, *Flexibacter*, *Lucibacterium*, and *Vibrio* were also present.[7] *Mytilus trossulus* from Peter the Great Gulf (Russia, Sea of Japan) was shown to harbor representatives of six genera of Gram-negative bacteria including *Pseudoalteromonas*, *Pseudomonas*, *Vibrio*, *Photobacterium*, *Cytophaga*/*Flavobacterium*/*Bacteroides*, and *Moraxella*, as well as the Gram-positive *Streptomyces*.[8] The presence of the genus *Bacillus* in *Mytilus* sp. was not reported in the former studies.

In an effort to study biodiversity, metabolic profiles, and antimicrobial activities of bacteria associated with *M. edulis* from the Kiel Fjord (Baltic Sea, Germany), the *Bacillus* sp. strain BC028 was selected for detailed chemical analysis. The crude extract from the culture broth exhibited antibacterial activity against *Pseudomonas* sp. strain MB140 which had also been isolated from *M. edulis*. A new antibiotic cyclic hexapeptide composed of phenylalanine, alanine, glycine, leucine, isoleucine, and valine was identified in the present study.

Total number of 116 isolates was obtained from the blue mussel *Mytilus edulis* (Kiel Fjord, Germany). The isolates belong to the classes Proteobacteria (75%), Actinobacteria (15%), Firmicutes (9%), and Bacteroidetes (1%). Altogether 14 genera were found, including *Achromobacter*, *Aeromonas*, *Agromyces*, *Algoriphagus*, *Bacillus*, *Enterobacter*, *Listonella*, *Microbacterium*, *Pseudomonas*, *Rahnella*, *Rhodococcus*, *Shewanella*, *Stenotrophomonas*, and *Vibrio* (data not shown). Strain BC028, the producer of the peptide described in this study, was classified as member of the genus *Bacillus* (class Firmicutes) based on phylogenetic analysis of the nearly complete 16S rDNA gene sequence. The strains *Bacillus cereus* ATCC 14579^T (GenBank accession No. AE016877), *Bacillus thuringiensis* ATCC 10792^T (GenBank accession No. D16281), and

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Bacillus weihenstephanensis DSM 11821^T (GenBank accession No. AB021199) were the closest related validly described type strains with similarities of 99.8%, 99.7%, and 99.6%, respectively.

Compound **1** was isolated as white powder from the culture broth of *Bacillus* sp. strain BC028 associated with the blue mussel *Mytilus edulis*.[9] It has the molecular formula of $C_{31}H_{48}N_6O_6$ by HR-ESIMS (TOF) analysis (*m*/*z* 623.3546 for $[M+Na]^+$), with 11 degrees of unsaturation. The presence of several doublets and doublets of doublets at 4–5 ppm, attributed to α-protons of amino acids, and the characteristic chemical shifts for the amide carbonyls at 170–180 ppm suggest the peptidic nature of the molecule. In agreement with the possible occurrence of amide functionalities, the IR spectrum showed bands at 3347 and 1639 cm^{-1}. The compounds turned purple with ninhydrin reagent only upon hydrolysis with 6 M HCl, which is consistent with a cyclic structure.[10,11]

The ^{13}C NMR spectrum displayed 6 ester/amide-type carbonyls (δ 171.5, 173.2, 173.4, 173.9, 175.5, 179.1), five α-methine carbons (δ 49.9, 52.7, 56.8, 58.9, 60.6), α- methylene carbon (41.4), seven methyl groups, three methylene groups, aromatic signals for a mono-substituted phenyl ring (112.3, 119.4, 124.6, and 128.9). Analysis of COSY, HSQC, NOESY (mixing time 300 ms), and HMBC data assigned six partial structures (Table 1): one valine (Val), one alanine (Ala), one leucine (Leu), one isoleucine (Ile), one glycine (Gly), and one phenylalanine (Phe) residue. HMBC correlations (Fig. 1) from each α-proton to the carbonyl carbon of the neighboring amino acid and to its own carbonyl carbon were detected. Since only 10 of the calculated 11 degrees of unsaturation could be accounted for by the functionalities in the eight individual fragments, it became obvious that compound **1** had a cyclic structure.

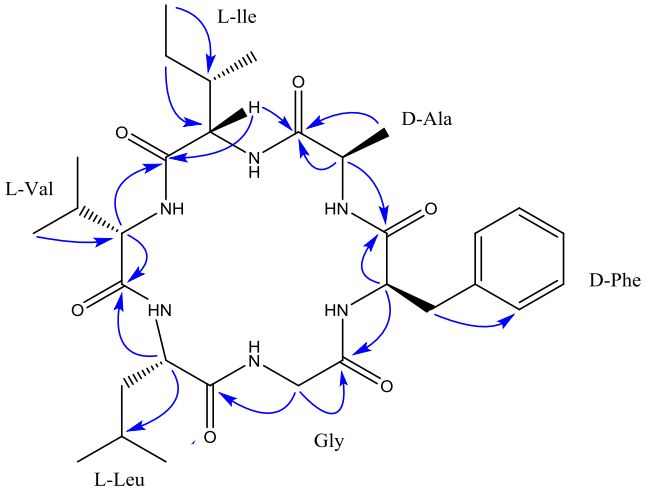

Fig. 1. Structure and selected HMBC correlations of bacicyclin (**1**).

Given the established constitution of the new cyclic peptide **1**, the absolute configurations of the constituting amino acids were determined by acid hydrolysis followed by chiral derivatization with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, FDAA).[12] HPLC analysis of the Marfey's derivatives in com-

Table 1
NMR-spectroscopic data of bacicyclin (**1**) in methanol d_4 (^1H: 600 MHz; ^{13}C: 150 MHz, δ in ppm, *J* in Hz).

Amino acid	δ_C	δ_H, mult	COSY	HMBC	NOESY
Gly					
CO	179.1				
α	41.4	3.36, t (8.4)		CO, Leu-CO	Val-β, Ala-α
Leu					
CO	171.5				
α	52.7	3.8, t (8.4)	β/β′	CO, γ, Val-CO	Val-α,Ile- α
β/β′	41.8	1.58, m	α,γ	CO, α	
γ	26.2	1.62, m	β/β′,δ,δ′	α	
δ	22.5	0.91, d (7.8)	γ	α, β/β′	
δ′	21.6	0.96, d (7.7)	γ	α, β/β′	
Val					
CO	175.5				
α	60.6	4.21, dd (10.8,6.2)	β	CO, Ile-CO	Leu-α
β	31.8	1.98, m	α, γ /γ′	CO	
γ	19.5	0.89, d (6.4)	β	α	
γ′	18.9	0.88, d (6.6)	β	α	
Ile					
CO	173.2				
α	58.9	4.19, d (8.5)	β	CO, γ, Ala-CO	Gly-α
β	36.1	2.03, m	α,γ	CO, δ	Leu-β/β′
γ	12.1	0.84	γ′		
γ′	27.5	1.28	γ,δ	α	
δ	14.6	0.86	γ′	β	
Ala					
CO	173.4				
α	49.9	4.3, q (9.0)	β	CO, β, Phe-CO	Phe-α, Leu-α
β	18.1	1.17, d (8.4)	α	CO, α	
Phe					
CO	173.9				
α	56.8	4.37, dd (11.4,4.2)	β	CO, Bz-I, Gly-CO	
β/β′	29.1	3.31/3.05, m	α	CO, Bz-o	Gly-α
Bz-I	128.9				
Bz-o	112.3	7.22, m	Bz-m	Bz-I, Bz-m, Bz-p	
Bz-m	119.4	6.98, m	Bz-o	Bz-I, Bz-o, Bz-p	
Bz-p	124.6	7.18, m			