Bioorganic & Medicinal Chemistry 20 (2012) 335–339

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Persipeptides A and B, two cyclic peptides from *Streptomyces* sp. UTMC 1154

Fatemeh Mohammadipanah [a,b], Josphat Matasyoh [a], Javad Hamedi [b], Hans-Peter Klenk [c], Hartmut Laatsch [a,*]

[a] Department of Organic Chemistry, University of Göttingen, Tammanstrasse 2, D-37077 Göttingen, Germany
[b] Microbial Biotechnology Laboratory, Department of Microbiology, School of Biology, College of Science, University of Tehran, PO Box 14155-6455, Tehran, Iran
[c] DSMZ—German Collection of Microorganisms and Cell Cultures GmbH, Inhoffenstraße 7B, D-38124 Braunschweig, Germany

ARTICLE INFO

Article history:
Received 12 August 2011
Revised 22 October 2011
Accepted 27 October 2011
Available online 17 November 2011

Keywords:
Streptomyces
N-methylated cyclic peptides
Persipeptides

ABSTRACT

Two new N-methylated cyclopeptides, persipeptide A (**1**) and B (**2**), have been isolated from *Streptomyces* sp. UTMC1154. Their structures were established using 1D and 2D NMR experiments. 2D TOCSY experiments were applied to identify the amino acid residues, while HMBC correlations were used to determine their sequence. According to Marfey's method, all amino acids had the L-configuration. The two cyclic peptides had the same ring size and amino acid composition, but differed in their sequence; they did not show activity against the tested bacteria, fungi and algae. Molecular identification experiments placed the strain in the genus *Streptomyces* closely related to *Streptomyces coerulescens* DSM40146^T (99.45%) and *Streptomyces varsoviensis* DSM40346^T (99.25%).

1. Introduction

Peptides have actually found tremendous attention in diverse aspects of science ranging from rational drug design[1] to nanomaterials.[2] Among them, many cyclopeptides exhibiting unique structures and interesting pharmacological activities have been isolated from marine[3] and terrestrial microorganisms[4,5] and higher plants[6] and have received considerable attention. One of them is streptogramin B, an antibiotic from the genus *Streptomyces*. These bacteria and further actinomycetes have been the source of many antibiotics including those which are presently the 'last line of defense' against multi-resistant bacteria.[7]

During our ongoing search for new antimicrobial natural products, *Streptomyces* sp. isolate UTMC1154 was subjected to a chemical and biological screening leading to the isolation of new cyclic peptides named persipeptide A (**1**) and B (**2**). Details of isolation, structure elucidation and antimicrobial activity of these new cyclopeptides will be reported here.

2. Results and discussion

Persipeptide A (**1**) was isolated as a colorless oil of the molecular formula $C_{35}H_{49}N_5O_5$, determined by HRESIMS. Analysis of its ^1H and ^{13}C NMR and HSQC data (Table 1) revealed three amide protons (δ_H 5.91–8.36), eight methyl groups including two *N*-methyls, two methylene groups, five methines, two mono-substituted phenyl rings and five carbonyl carbons (δ_C 169.9–173.6). These data accounted for 13 of the 14 double bond equivalents calculated from the empirical formula, indicating that **1** is a monocyclic pentapeptide.

Detailed interpretation of ^1H–^1H COSY, TOCSY, HSQC and HMBC data of **1** established the amino acids as two valines, one *N*-methylvaline, one phenylalanine and one *N*-methylphenylalanine residue and allowed the complete shift assignment of all atoms (Table 1). Their sequence was determined using HMBC correlations between the NH or *N*-CH_3 protons, respectively, with the two amide carbons in their vicinity (see Fig. S1, Supplementary data), except for valine 2, whose NH proton showed no HMBC correlations.

The *N*-methyl protons (δ_H 2.86) of *N*-methylvaline displayed an HMBC correlation to the amide carbon signal (δ_C 171.0) of phenylalanine indicating an acylation by this amino acid. In the same way, the amide protons of phenylalanine (δ_H 7.57), valine 1 (δ_H 5.91), and the *N*-CH_3 signal (δ_H 3.03) of *N*-methylphenylalanine correlated with the amide carbons in positions C-16, 21 and 31, respectively (see Fig. S1, Supplementary data). The α-methine protons in positions 2, 8, 17, 22 and 32 showed in all cases correlations to both adjacent amide carbonyl groups. The amide proton of valine 2 (δ_H 8.36) showed only a weak HMBC correlation to the amide carbon C-1 of the methylvaline; however, the position of MeVal was finally confirmed by the HMBC correlations of the *N*-methyl group C-6 with carbons C-2 and C-7.

Marfey's method[8] was applied to assign the absolute configuration of persipeptide A (**1**). The amino acids obtained by hydrolysis of **1** and in parallel the respective D- and L-reference amino acids were treated with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-

* Corresponding author. Tel.: +49 551 393211; fax: +49 551 399660.
E-mail address: hlaatsc@gwdg.de (H. Laatsch).

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doi:10.1016/j.bmc.2011.10.076

Table 1
NMR data of persipeptide A (**1**) in $CDCl_3$

Amino acid	Position	δ_C	mult.	δ_H, (*J* in [Hz])	HMBC
N-Methylvaline	1	171.9	C_q		
	2	79.9	CH	2.77, d (11.4)	1, 3, 4, 5, 6, 7
	3	27.2	CH	3.00, m	2, 4, 5
	4	19.6	CH_3	0.83, d (6.8)	2, 3, 5
	5	18.9	CH_3	0.42, d (6.8)	2, 3, 4
	6	39.9	*N*-CH_3	2.86, s	2, 7
Phenylalanine	7	171.0	C_q		
	8	52.6	CH	5.06, m	7
	9	38.0	CH_2	2.90, 3.29, dd	7, 8, 10, 11, 15
	10	137.4	C_q		
	11,15	129.5	CH	7.29, m	9, 10
	12,14	129.1	CH	7.30, m	10
	13	127.5	CH	7.23, m	
	NH			7.57, d (8.3)	16
Valine 1	16	169.9	C_q		
	17	59.3	CH	4.18, dd (3.6, 7.5)	16, 18, 19
	18	28.9	CH	2.41, m	17, 19, 20
	19	16.5	CH_3	0.69, d (6.9)	17, 18, 20
	20	19.2	CH_3	0.60, d (7.1)	17, 18, 19
	NH			5.91, d (7.5)	21
N-Methylphenylalanine	21	170.1	C_q		
	22	67.5	CH	3.80, dd	21, 23, 24, 30, 31
	23	33.6	CH_2	3.34, m	21, 22, 24, 25, 29
	24	136.2	C_q		
	25,29	128.5	CH	7.19, m	
	26,28	128.1	CH	7.20, m	24
	27	126.3	CH	7.11, m	29
	30	40.3	*N*-CH_3	3.03, s	31
Valine 2	31	173.6	C_q		
	32	54.4	CH	4.56, dd (6.8, 9.6)	1, 31, 33, 34, 35
	33	30.4	CH	1.85, m	32, 34, 35
	34	19.5	CH_3	0.89, d (6.8)	32, 33, 35
	35	17.9	CH_3	0.96, d (6.8)	32, 33, 34
	NH			8.36, d (9.6)	

5-L-alanine amide, FDAA) and the products were compared by HPLC–MS: All the amino acid residues were determined to have the L-configuration; therefore, peptide **1** was established as *cyclo*(L-Val, L-Phe, *N*-Me-L-Val, L-Val, L-MePhe) (Fig. 1), which we named persipeptide A.

Persipeptide B (**2**) was isolated as colorless oil as well. It was determined by HRESIMS to be an isomer of **1**. Analysis of the NMR data (Table 2) revealed the same amino acid composition as for **1**, but in a different sequence. Due to the higher polarity, dimethyl sulfoxide (DMSO) had to be used for NMR analysis. Although the NH signals of contaminating **1** were visible, the NH protons of **2** seemed to be hidden under broad signals at δ 8.61 (2H) and 7.38 under these conditions. Therefore the amino acid sequence was determined using solely the HMBC correlations from the *N*-CH_3 protons and the α-methines in positions 2, 7, 13, 18, and 28 (see Fig. S2, Supplementary data) to the respective amide carbons in their vicinity. The ambiguous connection of Val with MeVal was confirmed in this way by an HMBC correlation of the *N*-methyl protons of *N*-methylvaline (δ_H 2.61) to the amide carbon C-12 (δ_C 169.9) of valine 2. *N*-Methylphenylalanine (δ_H 2.79) showed a similar correlation of C-26 to the amide carbon C-27 (δ_C 169.6) of phenylalanine. These HMBC correlations (see Fig. S2, Supplementary data) confirmed the amino acid sequence in persipeptide B finally as *cyclo*(Val, *N*-MeVal,Val, Phe, *N*-MePhe) (Fig. 2). As in the case of **1**, all amino acid residues in persipeptide B were determined to be L-configured.

An interesting feature in the ^{13}C NMR spectrum of **1** is the strongly deshielded α-carbon of the MeVal residue (C-2, δ_C 80.0), which is in contrast to the small proton value of H-2 (δ 2.77) and also to C/H-22 (δ 67.8/3.80) in *N*-MePhe. Similar values of α-protons have

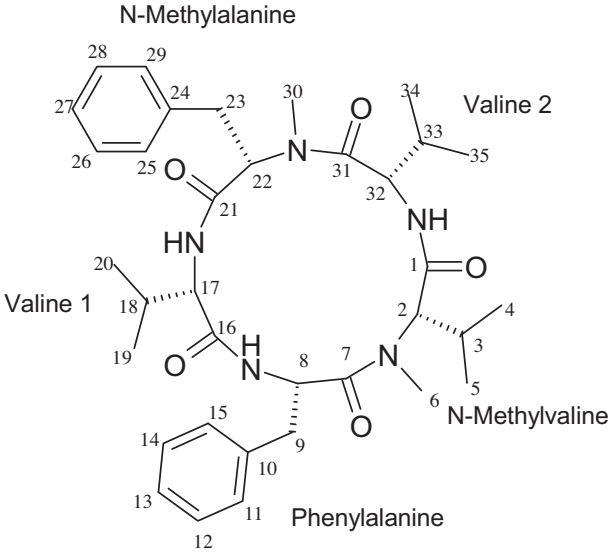

Figure 1. Persipeptide A (**1**).

been described, however, for a few other *N*-methyl amino acids, for example for *N*-MeIle in clavariopsin A.[9] *N*-Methyl residues can influence the flexibility of peptides and abnormal shift values may be due to conformational changes and resulting anisotropy effects, as in the case of, for example teleocidin A[10] and 14-*O*-(*N*-acetylglucosaminyl)-teleocidin A:[11] For these compounds, each two conformers

Table 2
NMR data of persipeptide B (**2**) in DMSO-d_6

Amino acid	Position	δ_C	mult.	δ_H, (J in [Hz])	HMBC
Valine 1	1	169.6	C_q		
	2	52.9	CH	3.86, t (8.3)	1, 3, 4, 5, 6
	3	30.1	CH	1.69, m	2, 4, 5
	4	18.9	CH_3	0.32, d (6.6)	2, 3, 5
	5	18.2	CH_3	0.61, d (6.8)	2, 3, 4
	NH				
N-Methylvaline 2	6	168.1	C_q		
	7	64.1	CH	3.65, d (7.1)	6, 8, 9, 10, 11, 12
	8	28.5	CH	2.26, m	6, 7, 9, 10
	9	18.6	CH_3	0.55, d (7.1)	7, 8, 10
	10	21.1	CH_3	0.81, d (6.6)	7, 8, 9
	11	33.3	*N*-CH_3	2.61, s	7, 12
Valine 2	12	169.7	C_q		
	13	61.1	CH	4.07, t (10.5)	12, 17, 14, 15, 16
	14	30.6	CH	1.80, m	13, 15, 16
	15	19.0	CH_3	0.81, d (6.5)	13, 14, 16
	16	19.4	CH_3	0.93 d (6.6)	13, 14, 15
	NH				
N-Methylphenylalanine	17	167.6	C_q		
	18	62.2	CH	4.28, dd (2.5, 11.5)	17, 19, 26, 27
	19	33.5	CH_2	3.42, dd (2.5, 10,14.3)	18, 20
	20	137.5	C_q		
	21,25	129.1	CH	7.20, m[a]	19
	22,24	127.9	CH	7.25, m[a]	20
	23	126.1	CH	7.15, m	25
	26	30.6	*N*-CH_3	2.79, s	27
Phenylalanine	27	169.9	C_q		
	28	51.5	CH	4.65, dd (2.9, 11.6)	27, 1
	29	37.5	CH_2	2.70, dd (3.1, 9.6, 13.3)	27, 28, 30, 31, 35
	30	137.8	C_q		
	31,35	129.3	CH	7.31, m[a]	29
	32,34	128.5	CH	7.34, m[a]	30
	33	126.6	CH	7.21, m	31
	NH				

[a] Values tentatively assigned.

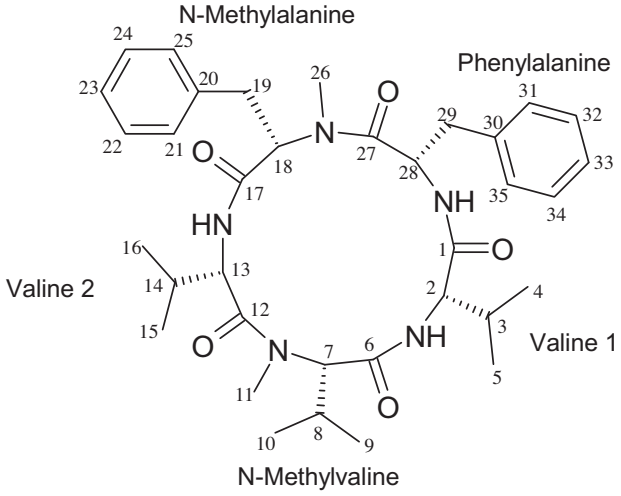

Figure 2. Persipeptide B (**2**).

were described with α-methine values for the *N*-Me-Val unit at δ ~71–72 and ~77, respectively. At least 26 cyclic pentapeptides have been reported from microbial sources.[16] The main difference between persipeptides A and B and related cyclopeptides reported in literature lies in the amino acid composition, whereby the persipeptides have basically only two types of amino acids, if the N-methylation is not considered, those reported in literature are composed of at least three or more amino acids. Among them, the closest similarity is found in bingchamide A [*cyclo*(Phe, Phe, *N*-Me-Phe, Leu, Ile)[12] or cotteslosin A[13] [cyclo(*N*-MeTyr, Tyr, Pro, Val, Val)].

The N-methylation of peptides results in modified pharmacokinetic properties: Hydrogen bonding and aggregation are reduced, and the increased lipophilicity allows a facile penetration of plasma membranes. N-methylation also affects the backbone of peptides by stabilizing the s-*cis* amide configurations and enhances the stability of the amide bonds against endopeptidases.[14,15] Many N-methylated peptides are therefore showing a pronounced bioactivity, for example as insecticides, enzyme inhibitors or antimicrobial compounds.[16] However, in the agar diffusion test, persipeptide A (**1**) and B (**2**) showed no activity against *Staphylococcus aureus* and multi-resistant *S. aureus* (MRSA) up to 100 μg/ml. Further tests were not yet performed due to the lack of material.

Based on 16SrRNA sequence similarity and the result of DNA–DNA hybridization results, strain UTMC1154 may be a new species in the *Streptomyces* genus. The results of our ongoing chemotaxonomical and biochemical characterization will be reported elsewhere.

3. Experimental

3.1. Instrumental analysis

NMR experiments were performed on a Bruker AMX 300 (300.135 MHz), a Varian Unity 300 (75.145 MHz), and a Varian Inova 600 (150.820 MHz) spectrometer. The chemical shifts were expressed in δ values using $CDCl_3$ and DMSO as solvent and TMS as internal standard. UV/vis spectra were recorded on a Perkin–Elmer

Lambda 15 UV/VIS spectrometer. IR spectra were measure using a Jasco 810 spectrometer. Optical rotation was measured on a Perkin–Elmer polarimeter, model 241. The ESI mass spectra were measured on a Finnigan LCQ with quaternary pump Rheos 4000 (Flux Instrument). TLC was carried out on pre-coated silica gel sheets Polygram SIL G/UV_{254} (Macherey-Nagel and Co. Düren, Germany). Size-exclusion chromatography was performed on Sephadex LH-20 (Lipophilic Sephadex, Amersham Biosciences Ltd; purchased from Sigma–Aldrich Chemie, Steinheim, Germany). Amberlite XAD-16 resin was obtained from Rohm and Haas, Frankfurt, Germany.

3.2. Isolation of the source strain

Strain UTMC1154 was isolated from a soil sample collected from sandy soil taken at a depth of 10 cm from Fars province, Iran. The soil sample was dried[17] and then heat treated at 120 °C for 10 min.[18] The strain was isolated by a dilution plating method on GAC[19] agar supplemented with cycloheximide (100 μg/ml) after 21 days incubation at 28 °C. The strain is maintained on M2 agar slants at 4 °C and 20% (w/v) glycerol suspensions in liquid nitrogen.

3.3. Identification of the source strain

Chromosomal DNA was prepared using a Jetflex DNA Purification kit (GENOMED 600100). The 16S rRNA gene was amplified with oligonucleotide primers 10–30F (5′-6GAGTTTGATC-CTGGCTCA-3′) and 1500R (5′AGAAAGGAGGTGATCCAGCC-3′), as described by Rainey et al.[20] PCR products were purified with the PCR product purification kit (Qiagen GmbH, Germany). The 16S rRNA gene sequences were aligned with POA v2[21] in the progressive mode in conjunction with BLASTN.[22] DNA for hybridization test was isolated using a French pressure cell and was purified by chromatography on hydroxylapatite as described by Cashion et al.[23] DNA–DNA hybridisation was carried out as described by De Ley et al.[24] under consideration of the modifications described by Huss et al.[25]

The 16S rRNA gene sequence strain UTMC 1154 showed the highest degree of 16S rRNA gene sequence similarity to members of the genus *Streptomyces* especially those closely related to *Streptomyces coerulescens* DSM40146 (99.45%) and *Streptomyces varsoviensis* DSM40346 (99.25%). DNA–DNA hybridization with *S. coerulescens* DSM40146^T resulted in only 11.0 ± 0.1% genome homology in replicated experiments, indicating that UTMC 1154 might be the type strain of a novel not yet named species within the genus *Streptomyces* (to be evaluated in a follow-up analysis). The GenBank accession number for the 16S rRNA gene sequence of strain UTMC1154 is JF917242; the strain was also deposited in a culture collection (University of Tehran Microorganisms Collection as UTMC1154).

3.4. Fermentation and isolation

The *Streptomyces* sp. strain UTMC1154 was cultured on M2 agar (0.4% glucose, 0.4% yeast extract and 1% malt extract and 1.8% agar pH 7.2 ± 0.2) for 10 days at 28 °C. Grown agar plates were used for inoculation of 20 of 1L-Erlenmeyer flasks containing 250 ml of M2 medium. This pre-culture was incubated at 28 °C (180 rpm) for 48 h and used for inoculation of 30 L of M2 fermentation medium in a fermentor (Biostat U, Braun Melsungen, Germany; stirrer at 200 rpm, 28 °C, pH 7.0 ± 1.0, aeration 1.5 vvm). After 7 days fermentation, the culture was filtered with the aid of Celite to give the aqueous filtrate and the mycelium. The mycelium was extracted several times with EtOAc and MeOH. The solvents were removed under reduced pressure to afford concentrated extracts, which were combined to give 0.2 g of crude extract.

The aqueous filtrate was passed through an Amberlite XAD-16 column (8 × 120 cm) and the adsorbed material eluted with methanol. The MeOH eluate was concentrated under reduced pressure and the resulting extract freeze–dried. The resulting powder was extracted with EtOAc to give 0.1 g of crude extract. The combined crude extracts were subjected to column chromatography on silica gel using a CH_2Cl_2/MeOH gradient. The peptides **1** and **2** were isolated from the fractions with 5% and 10% MeOH, respectively. They were purified on Sephadex LH20 (MeOH) and further by repeated preparative TLC (CH_2Cl_2/MeOH, 9:1) to yield 12 mg of **1** and 10 mg of **2**, respectively.

3.4.1. Persipeptide A (1)

Colorless oil, R_f = 0.74 (CH_2Cl_2/MeOH, 90:10); $[\alpha]_D^{25}$ −139° (*c* 0.1, MeOH); λ_{max} (log ε) 202 (4.51) nm; IR spectrum (neat) see Fig. S11, Supplementary data; 1D and 2D NMR data see Tables 1 and S1 (Supplementary data); HRESIMS *m/z* 642.3630 $[M+Na]^+$, (calculated for $C_{35}H_{49}N_5O_5Na$, *m/z* 642.3626).

3.4.2. Persipeptide B (2)

Colorless oil; R_f = 0.63 (CH_2Cl_2/MeOH, 90:10); $[\alpha]_D^{25}$ −214° (*c* 0.1, MeOH); λ_{max} (log ε) 203 (4.61) nm. IR spectrum (neat) see Fig. S12, Supplementary data; 1D and 2D NMR data see Tables 2 and S2 (Supplementary data); HRESIMS *m/z* 642.3636 $[M+Na]^+$, (calculated for $C_{35}H_{49}N_5O_5Na$, *m/z* 642.3625).

3.5. Absolute configuration of the amino acids

Samples of each 0.4 mg of **1** and **2** were hydrolyzed by heating in 1 ml 6 N HCl for 24 h at 110 °C. After cooling, the solution was evaporated to dryness and the residue redissolved in H_2O (50 μL). To each of these hydrolyzate solutions, or to a solution of the reference amino acid (50 μL; 50 mM), a solution of FDAA (Marfey's reagent, *N*-(2,4-dinitro-5-fluorophenyl)-L-alaninamide) in acetone (100 μL of 1% (w/v) solution) was added. After addition of $NaHCO_3$ solution (20 μL; 1 M), the mixture was incubated for 1 h at 40 °C. The reaction was stopped by addition of HCl (10 μL; 2 M), the solvents were evaporated, and the residue was redissolved in acetonitrile (1 ml). An aliquot of this solution (20 μL) was analyzed by HPLC (Phenomenex Luna C18, 250 4.6 mm, 5 μm; solvents: A is H_2O + 0.05% HCOOH, B is MeOH + 0.05% HCOOH; linear gradient from 10% B in A at *t* = 0 min to 90% B in A within 20 min; 25 °C; 300 μL min^{-1}). Configuration of the amino acid units were determined by comparing the chromatograms with those of derivatives of commercially available amino acids. The retention times (min) were as follows: L-Val (15.1), D-Val (16.9), *N*-Me-L-Val (16.0), *N*-Me-D-Val (17.0), L-Phe (16.4), D-Phe (18.1), *N*-Me-L-Phe (15.9), and *N*-Me-D-Phe (11.6).[26] In independent experiments, all **1**/**2**-derived amino acids were shown to have the L-configuration.

Acknowledgments

The authors would like to thank the German Academic Exchange Service (DAAD) and the Alexander von Humboldt Foundation (Germany) for financial support to FM and JM, respectively. We thank Prof. Dr. Dr. H. Eiffert (Med. Microbiology, Göttingen) for performing the activity tests.

Supplementary data

Supplementary data (1D and 2D NMR spectra and antimicrobial test result of Persipeptide A and Persipeptide B) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.10.076.

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