Tetrahedron 68 (2012) 1622–1628

Contents lists available at SciVerse ScienceDirect

Tetrahedron

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

New nodulopeptins from *Nodularia spumigena* KAC 66

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ARTICLE INFO

Article history:
Received 22 August 2011
Received in revised form 4 November 2011
Accepted 21 November 2011
Available online 27 November 2011

Keywords:
Polypeptides
Peptides
Cyanobacteria
Structure determination
Spectroscopy
NF-κB

ABSTRACT

Investigation of aqueous methanol extracts of the cyanobacterium *Nodularia spumigena* KAC 66 led to the isolation of the nodulopeptins 899, 901 and 917. The chemical structures were determined using high-resolution NMR and MS-data. The stereochemistry of the amino-acid residues was realized by LC–MS analysis using Marfey's reagent. The bioactivity of the novel compounds was determined.

1. Introduction

Cyanobacteria (blue-green algae) have been often falsely classified as algae due to their relative size and the green colour. Independent from dissolved nitrogen sources in the water, these bacteria have been reported to cause a large number of animal and human poisonings throughout the world.[1,2] They are present in freshwater and salt water environments. The brackish water bacteria *Nodularia spumigena* KAC 66 produces the hepatotoxin Nodularin; this highly toxic cyclopentapeptide is a protein phosphatase inhibitor of type 1 and 2A.[3] The World Health Organization recommends a tolerable intake of 0.04 mg/kg, but even at lower levels, this toxin can cause severe damage to human health.[4] Recently, Sivonen and co-workers developed an efficient, reproducible and quick detection method by Real-Time PCR for hepatotoxic cyanobacteria.[5] Cyanobacteria are a rich source of biologically active secondary metabolites.[6,7]

In this publication, we examine the products isolated from *N. spumigena* KAC-66, which resulted in the isolation and identification of three new nodulopeptins.

2. Results and discussion

2.1. Isolation and structure determination

Wet biomass of *N. spumigena* was extracted using a water/methanol mix. The crude extract was pre-purified on a C18-cartridge; the 50%-methanol fraction contained three different polypeptides, which were purified by repeated reversed-phase HPLC. The masses of the isolated compounds were 12 mg for compound **1**, 4 mg for compound **2** and 6 mg for compound **3**.

For all three peptides, the key signatures in ^{1}H NMR were 10 aromatic protons, one tyrosine moiety, whose chemical shifts were present at 6.70 and 6.99 ppm and two methyl doublets at 0.99 and 0.96 ppm. This indicated they were variants of the same core structure.

From exact mass measurement the molecular formulae $C_{47}H_{63}N_7O_9S$, $C_{47}H_{63}N_7O_{10}S$ and $C_{47}H_{61}N_7O_{11}$ were determined for peptides **1**–**3**. Peptide **1** and **2** had 20 double bond equivalents and peptide **3** each had 21 double bond equivalents. Apart from this difference, the masses of peptide **1** and **2** differ by the mass of one oxygen.

The carbons were associated with their respective protons via a gradient-filtered HSQC experiment. The most abundant peptide **1** was analyzed first. The ^{1}H-resonances of the individual peptide residues were assigned using an HSQC–TOCSY spectrum. The most

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0040-4020/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tet.2011.11.056

deshielded α-proton at 4.78 ppm showed a correlation with an AB-system at 2.18/1.84 ppm and a γ-CH_2 at 2.36 ppm. HMBC correlations were used to complete this residue and identify it as a homotyrosine. HSQC–TOCSY and HMBC spectra were used to determine that peptide **1** contained HPhe, Phe, Met, Lys and Val residues. The assignments of the remaining quaternary carbonyls to their residues were completed using two and three bond correlations from the HMBC spectrum. In peptide **1**, the correlation between C=O (175.9 ppm) and Pheα and Pheβ indicated that it was the carbonyl of the phenylalanine residue; correlations from C=O (175.8 ppm) to Lysα/β identified it as the Lys amide carbonyl. A correlation from C=O (174.9 ppm) to HPheα placed it as the HPhe amide C=O; a correlation from C=O (174.8 ppm) to Valα/β indicated that it was the Val carbonyl. Correlations from C=O (174.4 ppm) to Metα/β showed that it was the carbonyl of the methionine residue; similarly correlations from C=O (172.0 ppm) to HTyrα/β made this the HTyr carbonyl. Finally the resonance at 159.5 ppm showed correlations to Pheα and Lysα showing that this was a urea carbonyl. The discrimination of the HTyr, HPhe and Phe aromatic moieties was achieved through the correlation of α,β,γ carbons of the residues to the aromatic positions. In addition, the correlation of Metγ to SCH_3 and Metβ clearly indicated the location of the methionine residue. The key HMBC correlations are shown in Fig. 1. The sequence of the amino-acid residues was determined from the HMBC-correlations LysC=O-Valα; HPheC=O-NMe; HTyrα-NMe; ValC=O-HPheα; MetC=O-Lysε; HTyrC=O-Metα. Finally, the proton signal at 9.03 ppm was assigned as HTyrNH.

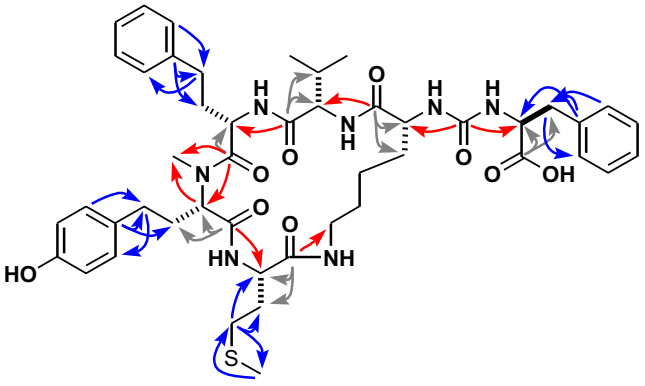

Fig. 1. Key HMBC correlations for polypeptide **1**. Correlation for sequence determination are coloured in red, whereas the correlation for the discrimination of HPhe, HTyr and Phe are coloured in blue.

Mass spectral fragmentation data confirmed these connections. For peptide **1**, predominant cleavage occurred at the Phe–urea bond, after which the loss of HPhe-HTyr-MEt took place. The loss of other larger fragments was also observed (Fig. 2.)

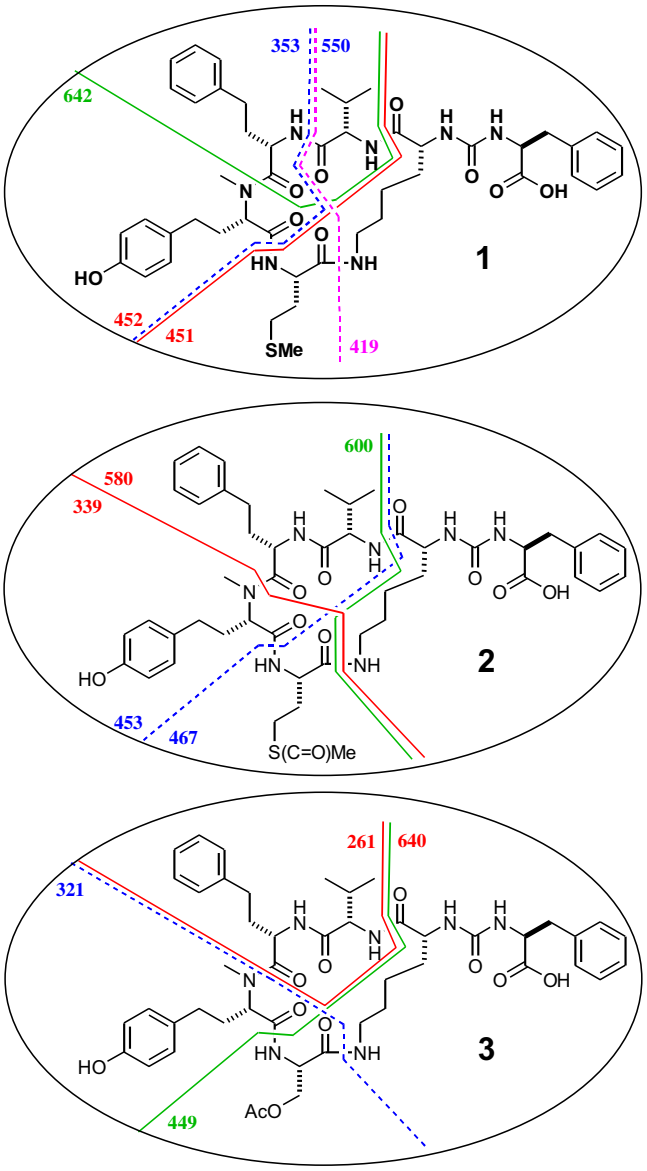

Fig. 2. Diagnostic fragmentations observed in the MS.

The acid hydrolysate of **1** was derivatised using Marfey's reagent followed by LC–MS analysis of the derivatised amino acids and the data was compared to derivatised standards. This chromatogram showed D-Lys-$(FDVA)_2$ at *m*/*z* 707, L-Met-FDVA at *m*/*z* 430, L-Phe-FDVA at *m*/*z* 446, L-HPhe-FDVA at *m*/*z* 460, L-Val-FDVA at *m*/*z* 398