Tetrahedron 72 (2016) 8603–8609

Contents lists available at ScienceDirect

Tetrahedron

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

Accurate quantification of modified cyclic peptides without the need for authentic standards

Rosemary I. Adaba [a], Greg Mann [b], Andrea Raab [c], Wael E. Houssen [a, d, e], Andrew R. McEwan [a, d], Louise Thomas [a, d], Jioji Tabudravu [a], James H. Naismith [b], Marcel Jaspars [a, *]

[a] Marine Biodiscovery Centre, Department of Chemistry, University of Aberdeen, Meston Walk, Aberdeen, AB24 3UE, UK
[b] Biomedical Sciences Research Complex, University of St Andrews, North Haugh, St Andrews, Fife, KY16 9ST, UK
[c] TESLA, Department of Chemistry, University of Aberdeen, UK
[d] Institute of Medical Sciences, University of Aberdeen, Aberdeen, AB25 2ZD, UK
[e] Pharmacognosy Department, Faculty of Pharmacy, Mansoura University, Mansoura, 35516, Egypt

ARTICLE INFO

Article history:
Received 4 September 2016
Received in revised form
31 October 2016
Accepted 14 November 2016
Available online 16 November 2016

Keywords:
Cyclic peptide
LC-MS
ICP-MS
NMR
Quantification without authentic standards
Cyanobacteria
qNMR

ABSTRACT

There is a growing interest in the use of cyclic peptides as therapeutics, but their efficient production is often the bottleneck in taking them forward in the development pipeline. We have recently developed a method to synthesise azole-containing cyclic peptides using enzymes derived from different cyanobactin biosynthetic pathways. Accurate quantification is crucial for calculation of the reaction yield and for the downstream biological testing of the products. In this study, we demonstrate the development and validation of two methods to accurately quantify these compounds in the reaction mixture and after purification. The first method involves the use of a HPLC coupled in parallel to an ESMS and an ICP-MS, hence correlating the calculated sulfur content to the amount of cyclic peptide. The second method is an NMR ERETIC method for quantifying the solution concentration of cyclic peptides. These methods make the quantification of new compounds much easier as there is no need for the use of authentic standards when they are not available.

1. Introduction

The growing application of peptides in drug discovery necessitates their accurate quantification in order to obtain the right metabolic, enzymatic, kinetic and pharmacokinetic data.[1–6] Several methods for peptide quantification have been reported to date, these include liquid chromatography combined with ultraviolet (UV) or fluorescence detection, capillary electrophoresis with UV detection, matrix-assisted laser-desorption/ionization mass spectrometry (MALDI-MS), surface-enhanced laser desorption/ionization (SELDI),[6–12] liquid chromatography-mass spectrometry (LC-MS),[7,8] inductively coupled plasma mass spectrometry (ICP-MS)[13,14] and quantitative nuclear magnetic resonance (qNMR).[9]

Limitations of these techniques for quantification of peptides vary by technique. Matrix effects limit optical techniques such as UV and fluorescence detection.[7,8] Different mass spectrometric methods suffer from different problems. Matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF) and electrospray ionization (ESMS) techniques both suffer drawbacks such as differential response of proteins and peptides depending on size, hydrophobicity, matrix, or solvents.[10] At low mass resolution, LC-MS data has a limited accuracy for reported intensity of the extracted ion currents due to contamination by nearby peptide signals, thereby affecting accurate quantification.[11,12] For quantification purposes, it is necessary to address these issues in particular ionization efficiency and matrix effects when using an ESMS or MALDI-MS direct measurements. For this reason various sample treatments for MS-based quantification are reported in the literature for peptides including; isotope-coded affinity tag reagents (ICATs),[13–15] isotope-coded protein labelling (ICPL),[16–18] stable isotope labelling by amino acid in cell culture (SILAC),[19–21] isotope-differentiated binding energy shift tag (IDBEST), chemical labelling, isobaric tagging (iTRAQ, TMT),[22,23] and

* Corresponding author.
E-mail address: m.jaspars@abdn.ac.uk (M. Jaspars).

http://dx.doi.org/10.1016/j.tet.2016.11.040
0040-4020/

absolute quantification with the use of synthetic labelled peptides (AQUA),[24,25] These methods require additional sample preparation and cost.

ICP-MS is a sensitive analytical tool for elemental analysis with advantages of having species independence and high ionization efficiency for most elements in the periodic table, high sensitivity of parts per billion to parts per trillion levels, together with affordable isotope distribution information.[26,27] For these reasons it has become a significant and complementary technique in bioanalysis for the determination of biomolecules and quantification of therapeutic agents.[28–36] Application of ICP-MS allows the quantification of elements independent of their molecular form, hence the analyte retains its original form during quantification. Coupled with molecular information obtained from ESMS or MALDI enables the compound identification simultaneously with its quantification. Sulfur has been successfully used for the quantification of proteins and peptide in biological samples by coupling the ICP-MS to different chromatographic systems.[37–40]

NMR produces a signal for any species that will have an area that is proportional to its concentration.[40] Complex mixtures can be analysed by NMR which provides the concentration of the chemical components in a mixture, hence allowing quantification of species for metabolomic and related studies.[41,42] Proton NMR quantification (qNMR) by ERETIC is a non-destructive and rapid way of providing accurate analyte concentrations[43] by using an indirect internal reference signal that represents a known concentration. This averts the need to determine a compound-specific response factor,[44] making qNMR an accurate and straightforward technique for quantification. The drawbacks to this method are that it requires relatively pure samples of large size that would allow sufficient signal to noise ratio (>150:1)[9] and an internal certified reference material.

Cyclic peptides show promise in many therapeutic areas, particularly in complex diseases such as auto-immune disorders.[45] Cyanobactins are a family of modified cyclic peptides that have interesting structural features including heterocycles, epimerized stereocentres and prenylated residues (Fig. 1).[46] Some of these modifications lead to better target affinity by constraining conformational flexibility, while others increase cellular permeability.[47,48] Members of cyanobactins are known to reverse multi drug resistance in human lymphoblasts by inhibiting the P-glycoprotein (Pgp) drug efflux pump.[49–51]

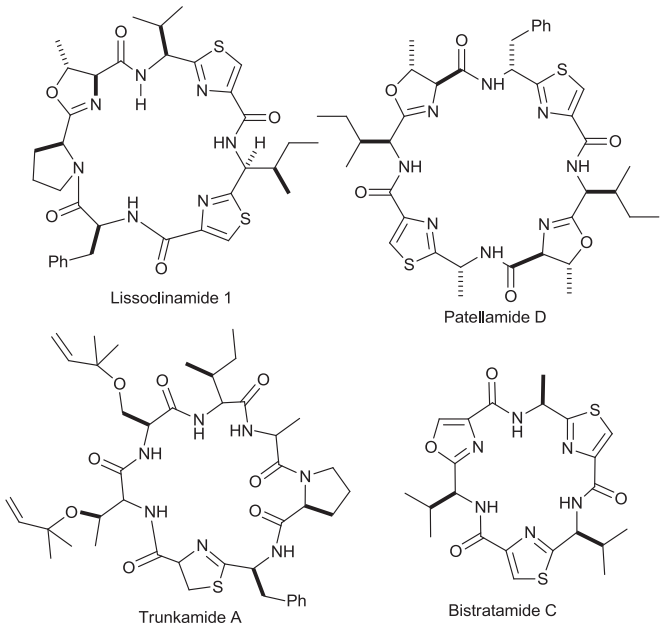

Fig. 1. Structures of some modified cyclic peptides in the cyanobactin family showing heterocycles, epimerized stereocentres and prenylated residues in trunkamide A.

Patellamides are the most studied members of the cyanobactins. They were originally isolated from extracts of the Indo-Pacific ascidian *Lissoclinum patella*, but shown later to be produced by its cyanobacterial symbiont *Prochloron* sp..[52,53] Genomic studies of *Prochloron* sp. delineated the gene cluster for the biosynthesis that directs the production of the patellamides.[54–59] Their biosynthesis occurs via the production of a ribosomally encoded precursor peptide, in which a core peptide sequence is modified by a series of processing enzymes.[52,60–64] We recently used these enzymes *in vitro* to generate natural and non-natural cyanobactins in milligram quantities.[61] Accurate quantification of the reaction products is essential to calculate yields before and after purification and for their downstream biological screening but is challenging due to the lack of authentic standards.

To overcome this, we herein report two quantification methods, the first relies on the quantification of the sulfur content in the products to estimate the concentration of these new heterocycle containing cyclic peptides in solutions, by coupling molecular electrospray mass spectrometry (ESMS) and elemental inductively coupled plasma mass spectrometry (ICP-MS) to a high pressure liquid chromatograph (HPLC) in parallel.[65] Using this approach we quantified sulfur containing peptides obtained after extraction and purification of these compounds from chemoenzymatic reaction mixtures and identified the most efficient extraction and purification strategy. While the second method describes an alternative quantification method using NMR and an ERETIC (electronic reference to access *in vivo* concentrations) reference for the quantification of non-sulfur containing cyclic peptides. ERETIC qNMR enabled us to obtain the concentration and identity of these new compounds simultaneously.

2. Results and discussion

2.1. Verification of sulfur quantification by HPLC

Two sulfur containing compounds **1** and **2** (Table 1 and SI Scheme 1) were used as calibration standards. The accuracy of the method was verified using, a known drug molecule containing sulfur; methylthioninium chloride **3**, commercially available sulfate standard solution and three certified reference materials (CRMs): RM8415 (whole egg powder); BCR-062 (olive leaves) and seronorm (trace elements in urine blank) whose total sulfur contents are known were analysed. The detection limits for sulfur by HPLC ranged from 1.00 to 2.03×10^{-4} mg/mL using either compound **1** or **2** as standard, with a correlation coefficient >0.99. There was no statistically significant difference in the results using either compound **1** or **2** for quantification of sulfur in the samples. Recovery of sulfur in the three certified reference materials was 101 ± 8% and compound **3** was 78 ± 2% (Table 2). The sulfur content of the HPLC calibration standards (**1** and **2**) was within the calculated range (±3%) allowing their use as standards in HPLC-ICP-MS/ESMS.

Quantification of compound **3** gave a recovery of 75 ± 3% (Table 2) of the theoretical value which is similar to the value achieved during total sulfur determination. This indicates that there was no loss of compound **3** on the column, that the standards used for quantification and the methods used are of sufficient accuracy.

2.2. Naturally occurring cyclic peptides

As a proof of concept, purified natural products **4** and **5** were