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# Methods for the comprehensive structural elucidation of constitution and stereochemistry of lipopeptides

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ABSTRACT

A panel of methods of general suitability for complete structural elucidation of the stereochemistry of cyclopeptides, depsipeptides and lipopeptides is presented and described in detail. The suitability of the proposed methods was exemplified on the lipopeptide poaeamide from *Pseudomonas poae*. Amino acid configurations have been assigned by direct LC enantiomer separation with Chiralpak ZWIX(+) and were confirmed by GC enantiomer separation on Chirasil L-Val. 3-Hydroxydecanoic acid absolute configuration was analyzed on Chiralpak ZWIX(+) and confirmed by injection on ZWIX(−) which showed opposite elution order. Plenty of D-amino acids have been found in this lipopeptide. It contained in total 5 Leu residues of which one had D-configuration. The position of the D-Leu in the peptide sequence was determined by pepsin and chemical digestions in combination with isolation of diagnostic peptide-fragments and subsequent identification of absolute configurations of the Leu residues. This allowed pinpointing the position of the D-amino acid. The complementarity of the peptide retention profiles on Chiralpak ZWIX column as compared to both RPLC and HILIC suggests its great utility as an alternative peptide separation tool.



## 1. Introduction

Lipopeptides are amphiphilic natural compounds constituted by an oligopeptide backbone and a fatty acid tail [1–3]. They often exist in macrocyclic form due to lactone-type ring closure between hydroxyl side chains and carboxylic functionalities, thus also termed depsipeptides. Lipopeptides are biosurfactants [4] and there is great interest in these biomolecules due to their potential bioactivities in particular antimicrobial and antitumor activities [1,5,6], usually by exerting their activity via specific interactions with the plasma membrane [7]. Interestingly, D-amino acids are commonly found in these biomolecules which make them more resistant against endopeptidase enzymes and proteolytic degradation, respectively [8].

Complete structure elucidation consequently requires the determination of the absolute configurations of the chiral building blocks. For this purpose, the peptide is hydrolyzed and the configurations of the residues are determined by enantioselective analysis [9]. For amino acid enantiomer separation and absolute configuration assignment, gas chromatography with pre-column derivatization has become a state-of-art methodology [10,11]. On the other hand, in liquid chromatography indirect methods [12,13] using either ortho-phthaldialdehyde (OPA)/chiral thiol as chiral derivatizating agent [14,15] or Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) [16–19] and subsequent achiral RPLC are commonly used. There are only a few methods which allow direct enantiomer separation of amino acids on chiral stationary phases in underivatized form, namely chiral ligand exchange chromatography [20], LC with macrocyclic antibiotics such as teicoplanin or teicoplanin aglycon based CSPs [21–24], LC with chiral crown ether CSPs [25–27], and LC on zwitterionic chiral ion exchangers [28–33] (for recent reviews on this topic cf. ref. [34]). Two-dimensional HPLC assays are sometimes implemented

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for the purpose of comprehensive enantioselective amino acid enantiomer analysis, also to overcome limited chemoselectivity of chiral stationary phases [35,36]. Since CLEC is incompatible with MS detection and chiral crown ethers typically require strongly acidic conditions (often perchloric acid in the mobile phase), the two MS compatible CSP classes, namely macrocyclic glycopeptide CSPs and zwitterionic cinchona alkaloid derived CSPs, were considered as first choice. Since the zwitterionic ion-exchangers based on cinchona alkaloids appear in two versions, Chiralpak ZWIX(+) and ZWIX(−) (see Fig. 1), with opposite elution orders [28,31], allowing validation of results by reversal of elution order, they were selected for our study and hyphenated with QTOF MS. Only few reports exist in the literature in which LC–MS in combination with chiral stationary phases was used for direct enantiomer analysis without pre-column derivatization [22] [37]. However, matrix effects, insufficient sensitivities, peak distortions and inadequate chemical selectivity for isobaric amino acids remained some of the unsolved problems complicating this endeavor [20–22,25,28–31,35–37].

Elucidation of the stereochemistry in a lipopeptide is straightforward if individual building blocks are present only once in the biomolecule or only in one configuration. The situation gets more complicated if chiral synthons of one sort are present more than once and both configurations are detected by enantioselective analysis of those constituents. In such cases, not only the sequence of the building blocks but also the position of the particular stereochemistry of the synthons has to be pinpointed for complete structural characterization. Analytically, this is more challenging because upon hydrolysis of the lipopeptide for enantioselective analysis of the building blocks the sequence information is lost. Different strategies have then to be adopted to solve the problem.

In this article, we suggest analytical strategies, methods and complementary stationary phases for the comprehensive structural elucidation of the constitution and stereochemistry of lipopeptides, depsipeptides and therapeutic peptides. Particular focus is given on the elucidation of the potential of a new chiral stationary phase, Chiralpak ZWIX (see Fig. 1), which is a chiral zwitterionic ion-exchanger, for its capability to resolve the enantiomers of the building blocks (amino acids, hydroxy acid) and serve as complementary stationary phase to RPLC and HILIC in separations of linearized and cyclic lipopeptide as well as of peptide fragments generated by enzymatic and chemical digestion. New selectivity profiles in such peptide separations and the stereoselectivity of this separation material for peptide enantiomers, epimers and diastereomers [30,38,39] might facilitate and support the structural elucidation.

It is documented for the comprehensive structural elucidation of a lipopeptide isolated from the endophytic *Pseudomonas poae* strain RE*1-1-14 (Fig. 2). Briefly, the target lipopeptide (LP) with a 10-amino acid peptide moiety was originally isolated from internal root tissue of sugar beet plants and shown to suppress growth of the fungal pathogen *Rhizoctonia solani*. The amino acid sequence and fatty acid side chains by which this LP was constituted could be readily confirmed by MS-based sequencing and NMR [40]. Uncertainty existed for the stereochemistry which has been clarified in this study by the use of Chiralpak ZWIX and a reference GC–MS method using Chirasil L-Val. Furthermore, a combination of enzymatic, chemical and chromatographic methods were utilized for unequivocally pinpointing which one of 5 Leu residues had D-configuration.

## 2. Materials and methods

### 2.1. Materials

Amino acid standards, 1-fluoro-2,4-dinitrobenzene (Sanger's reagent) as well as reagents and solvents for GC–MS analysis comprising trifluoroacetic anhydride (TFAA), ethyl trifluoroacetate (TFAEE), acetyl chloride, deuterium oxide, dichloromethane, deuterated ethanol (EtOD) were all purchased from Sigma-Aldrich (Munich, Germany).

Organic solvents, such as methanol and acetonitrile for LC–MS were of Ultra-MS grade and supplied by Carl Roth (Karlsruhe, Germany). Solvent additives (formic acid, ammonium formate) were purchased in trace metal analysis or UPLC-MS grade from Sigma-Aldrich. Ultra-MS-grade water was obtained from Water Purelab Analytics Purification System (from ELGA, Celle, Germany). The lipopeptide poae RE*1-1-14 was prepared and isolated as described previously [40].

### 2.2. Instrumentation

#### 2.2.1. GC–MS instrumentation

GC–MS analysis was performed as described previously [11] using an Agilent 7890 A GC-system coupled to a single quadrupole mass spectrometer 5975C inert MSD with Triple-Axis (Agilent Technologies, Waldbronn, Germany). A Chirasil L-Val column (20 m × 0.3 mm i.d.; 0.28 μm film thickness; C.A.T., Tübingen, Germany) was used for separation of the amino acid enantiomers after double derivatization as described below. The temperature of the split/splitless injector was set to 220 °C and the injector was operated in the splitless mode. Hydrogen was used as a carrier gas at constant pressure of 0.45 bar. The GC oven temperature was programmed as follows: 50 °C for 0.5 min, 50 °C/min to 77 °C, then 4 °C/min to 110 °C, then 10 °C/min to 140 °C, then 6 °C/min to 195 °C followed by a final hold for 7 min.

EI spectra were recorded in SIM mode determining the ions with a mass to charge ratio of 126, 138, 140, 152, 166, 168, 176, 180, 199, 182, 214, 228, 290, 315 and 421. All measurements were performed with an MS source temperature of 230 °C and a quadrupole temperature of 150 °C at 70 eV.

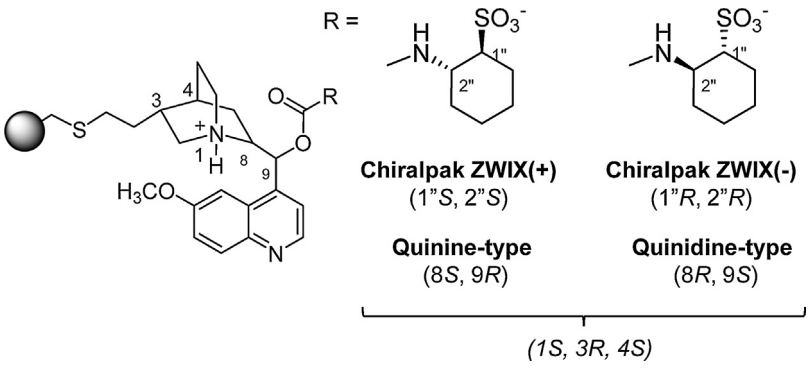


**Fig. 1.** Structure of utilized chiral stationary phases: (a) zwitterionic chiral ion-exchanger Chiralpak ZWIX(+) and ZWIX(−).