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Communication

Mass spectrometric analysis of head-to-tail connected cyclic peptides★

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Tandem mass spectrometry is an extremly useful tool for high sensitive sequence identification of peptides. In the case of cyclic peptides fragmentation can easily be performed for sequence analysis. However, analysis is usually tedious due to the lack of a defined beginning and end of the sequence. Since cyclic peptides are a highly interesting class of compounds especially for the pharmaceutical industry, ways have to be found to identify their structures. In this work we demonstrate how software and dedicated analytical strategies can be used for detailed analysis of these substances.

Head-to-tail cyclic connected peptides are a class of compounds which have attracted permanently growing interest during the past years. They are naturally occurring, e.g. as bacterial, fungal or marine constituents and are of interest due to various physiological effects like high toxicity (microcystines), immunosuppressive effects (cyclosporin A), antitumor or ionophoric activity. Furthermore, there is interest in these substances as pharmaceuticals due to restricted mobility and enhanced proteolytic stability in comparison to linear peptides.

Tandem mass spectrometric analysis of these peptides is more difficult in comparison to linear peptides since the fragmentation of one single intracyclic bond does not lead to characteristic fragment ions. Therefore, mul-

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Abbreviations: CID, collision induced dissociation; DIPEA, diisopropylethylamine; Dmab, 4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]-amino} benzyl ester; DMF, dimethylformamide; Fmoc, 9-Fluorenylmethyloxycarbonyl; FTMS, fourier transform mass spectrometry; HBTU, 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; ISD, in-source decay; MALDI, matrix assisted laser desorption/-ionization; PSD, post source decay; TFA, trifluoro acetic acid.

tiple fragmentation has to be induced by appropriate methods. In addition, the interpretation of the spectra is more complicated due to the lack of defined N- and C-terminal ends. For a detailed structural analysis of the compound(s) MALDI-PSD and ion trap MS^n were applied.

MATERIALS AND METHODS

cyclo(YEAARdFPEDN) was synthesized on an Applied Biosystems ABI 433A peptide synthesizer (Applied Biosystems, F.R.G.) using standard FastMOC strategy. HBTU was used as activator, diisopropylethylamine (DIPEA) as coupling base. Cleavage was performed using piperidine and *N*-methylpyrrolidone was used as solvent. The sequence was synthesized on a Rink amide resin (Novabiochem, F.R.G.). Orthogonaly protected Fmoc-Asp-ODmab (Novabiochem, F.R.G.) was used for the cyclization. The cleavage of this protection group was done with 2% hydrazine in DMF. Cyclization was performed on resin with HBTU/DIPEA activation over 72 h at room temperature. Completeness of the cyclization was controlled by Kaiser test. Cleavage of the cyclic peptide from the resin was done with TFA/triethylsilane/water (95 : 2.5 : 2.5, by vol.) over 2 h at room temperature.

Matrix assisted laser desorption/-ionization was carried out on a Bruker Reflex III TOF mass spectrometer equipped with a SCOUT384 source and a nitrogen UV laser (Bruker, F.R.G.). One μl of sample solution was placed on the target and 1 μl of a freshly prepared saturated solution of α-cyano-4-hydroxy-cinnamic acid in acetonitrile/H_2O (2:1, v/v) with 0.1% trifluoroacetic acid was added. Post source decay (PSD) spectra were acquired in 14 steps (reflector voltages 28.7 to 0.9 kV) at an acceleration voltage of 25 kV.

Electrospray mass spectrometry was performed on an Esquire 3000 ion trap mass spectrometer (Bruker, F.R.G.). The sample was introduced using continuous flow injection at a flow rate of 2 μl/min. Samples were dissolved in isopropanol/0.2% acetic acid (1 : 1, v/v). Spray voltage was set to 4000 V and the collision energy was varied to receive

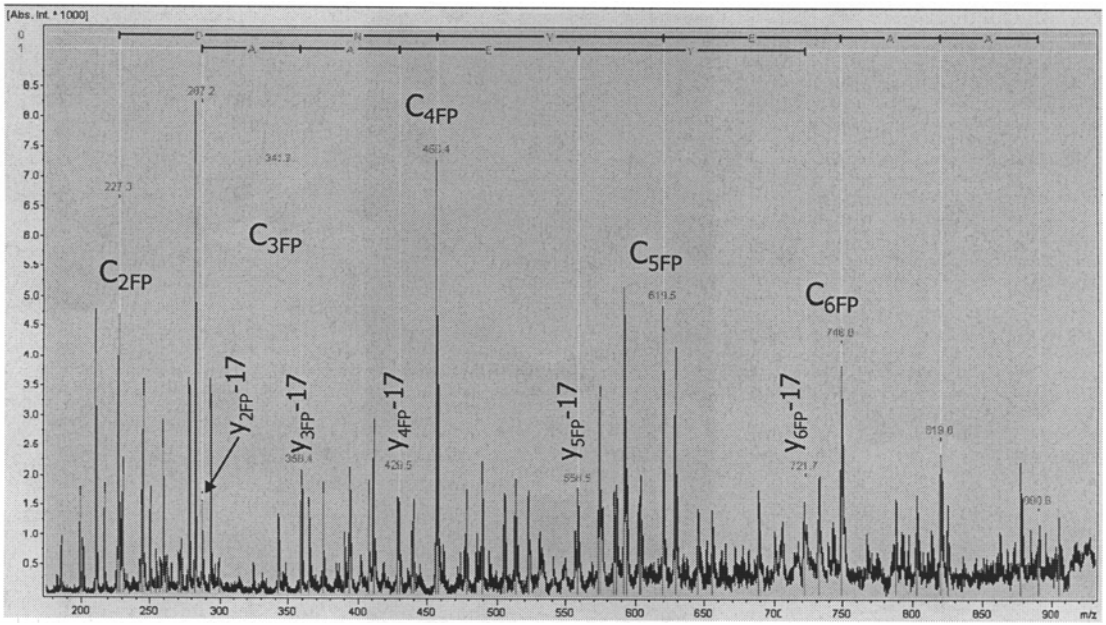

Figure 1. *De novo* interpretation of cyclo(YEAARdFPEDN) using the BioTools 2.0 software package (assignment of the fragment ion series was done manually).

The fragment labeling is according to [1].

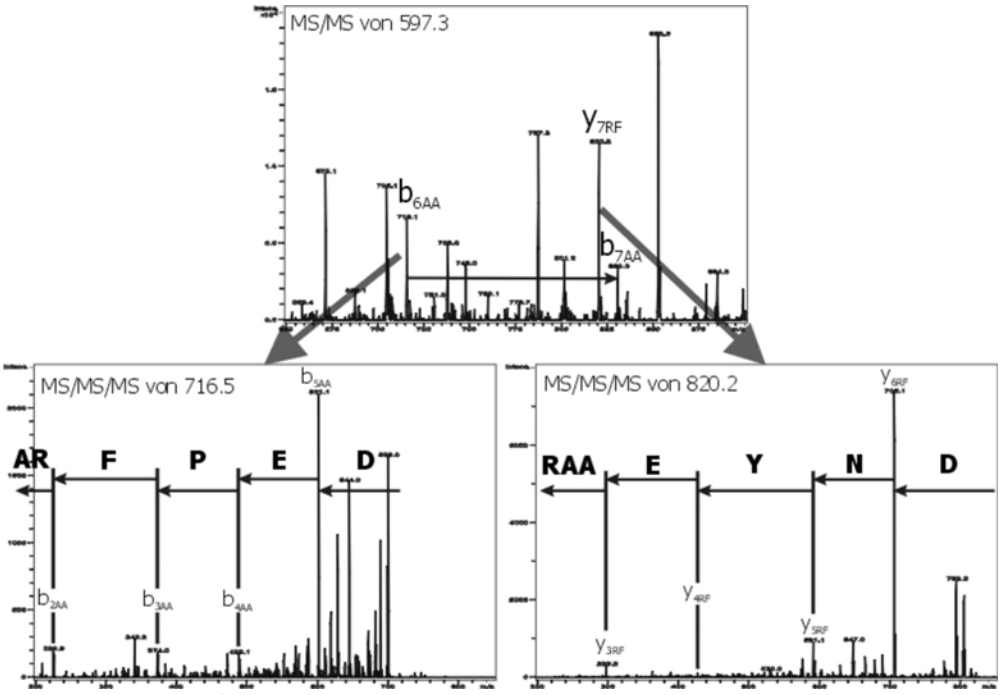

Figure 2. Correlation of MS^2 and MS^3 spectra of cyclo(YEAARdFPEDN).

The evaluation of MS^3 data identifies the fragments occuring in MS^2 and allows further interpretation of MS^2 spectra. Fragment labeling is according to [1].

good spectra quality. All spectra were acquired in the standard mode at a scan rate of 13 000 u/s.

RESULTS

While cyclic peptides show a very high stability towards proteases as well as upon storage (the peptide described here had been stored for 1.5 years at room temperature), it was observed that they undergo fragmentation under MS/MS conditions very easily. We assume that this is due to the fact that their structural rigidity hinders them to reduce their energy by rotation or vibration.

The resulting MS^n spectra are complex, however, they usually show preferences for distinct primary cleavage sites which may be comparable to those of linear peptides (e.g. preference of cleavage N-terminal to proline) but the primary cleavage can occur at other positions as well [2].

To simplify the interpretation, the correlation of MS^2 and MS^3 scans was evaluated with an ESI ion trap mass spectrometer. Figure 2 shows how the fragment ion signals in the MS^2 spectrum can be assigned to certain fragment ion series by using the MS^3 data, which makes *de novo* sequencing and interpretation of MS/MS spectra much easier [3, 4]. The application of these techniques is due to the MS^3 scan mode only possible on ion trap and FTMS type instruments which makes them extremely valuable instruments for *de novo* sequencing of peptides. The acquisition of these MS^2 and MS^3 data can be performed in a fully automated way, e.g., during LC-MS/MS runs of tryptic peptide mixtures using an Esquire 3000 instrument to provide the user with highly reliable *de novo* data for subsequent database search using tools like FASTA or MS-BLAST [6].

DISCUSSION

Cyclic head-to-tail connected peptides can easily be synthesized in high yields and very good purity using standard procedures and or-

thogonally protected amino-acid residues. However, the cyclization step is critical and can be very long depending on the peptide sequence, structural constraints and the resulting ring size. This step has therefore to be controlled by appropriate methods, e.g. the Kaiser test [5].

The observed fragment ions in MS^n spectra of cyclic peptides may vary from those usually observed in low energy CID or PSD mode. For example, in the PSD spectra of cyclo(YEAARdFPEDN), intensive c-ion series were observed, which are not typical for PSD spectra but are often observed in MALDI in-source-decay (ISD) spectra.

As already described by others [3, 4] and demonstrated herein as well, correlation of MS^2 and MS^3 spectra simplifies the identification of fragment ions which belong to a certain series and therefore enhances the confidence of the assignment of certain signals and fragment ion series in MS^2 spectra. This correlation of spectra turns out to be a very valuable tool for assisting *de novo*-sequencing not only of cyclic but generally of all kinds of peptides.

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