In-depth characterization and structure elucidation of non-ribosomal peptides related to adenopeptin using LC-HRAM-MS^n

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Abstract

Purpose: Characterization of adenopeptin and related peptaibol analogs in a crude fermentation broth extract using LC-HRAM-MS^n to identify adenopeptin congeners and elucidate their structures.

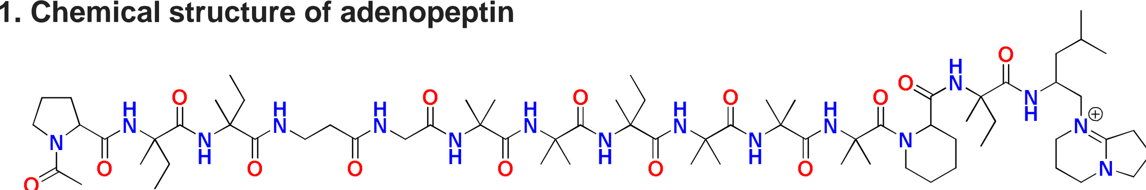
Methods: Adenopeptin and its analogs were separated using reversed phase chromatography and analyzed using multi-stage fragmentation on a Thermo Scientific™ Orbitrap IQ-X™ Tribrid™ mass spectrometer.

Results: Several analogs of adenopeptin differing by one or more methyl groups could be detected and the site of their modification could be narrowed to specific amino acid residues based on their fragmentation patterns.

Introduction

The non-ribosomal peptide adenopeptin was first isolated from *Acremonium persicinum* and reported in the literature in 1998.[1] It is of interest due to the reported antitumor effects of it and related peptaibols. Adenopeptin's structure is made up of 14 residues with an amidinium function on the C terminus and several non-proteinogenic amino acids, including aminoisobutyrate (Aib) and isovaline (Iva), as shown in Figure 1.

Figure 1. Chemical structure of adenopeptin



However, when isolated from fermentation broths of an isolated strain of *A. persicinum*, adenopeptin is produced in combination with several structurally related analogs. Here, we report the in-depth LC/MS^n investigation of the crude adenopeptin extract focused on the structure elucidation of its minor constituents.

Materials and methods

Sample Preparation

The adenopeptin fermentation broth was resin-extracted, followed by successive washes of deionized water and methanol. The methanol portion was concentrated and lyophilized to obtain the crude adenopeptin extract. An aliquot was reconstituted in methanol (20 µg/mL) for LC/MS analysis.

LC/MS Method

The separation of the crude sample was performed on a Thermo Scientific™ Hypersil Gold™ C18 column (2.1x150 mm, 1.9 µm) using 0.1% formic acid in water/acetonitrile as mobile phases and a 12-minute gradient elution (detailed in Table 1) on a Thermo Scientific™ Vanquish™ Horizon UHPLC system connected to a Thermo Scientific Orbitrap IQ-X Tribrid mass spectrometer. Data were acquired in positive ESI mode from Full Scan-dd-MS^n experiments as shown in Figure 3.

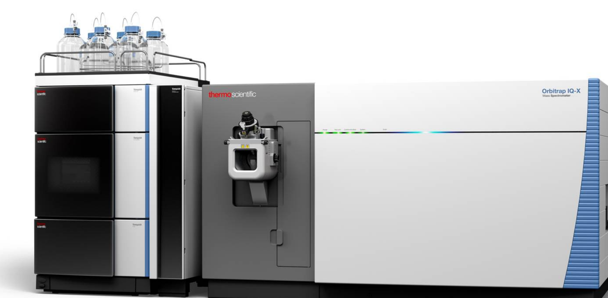
Data Analysis

The raw data were subsequently analyzed using the expected compounds workflow in Thermo Scientific™ Compound Discoverer™ 3.3 SP3 software to detect adenopeptin and its analogs, facilitating the elucidation of their structures.

Table 1. LC Gradient conditions

Time (min)	Mobile Phase B (%)
0.0	5
5.0	100
8.0	100
8.1	5
12.0	5

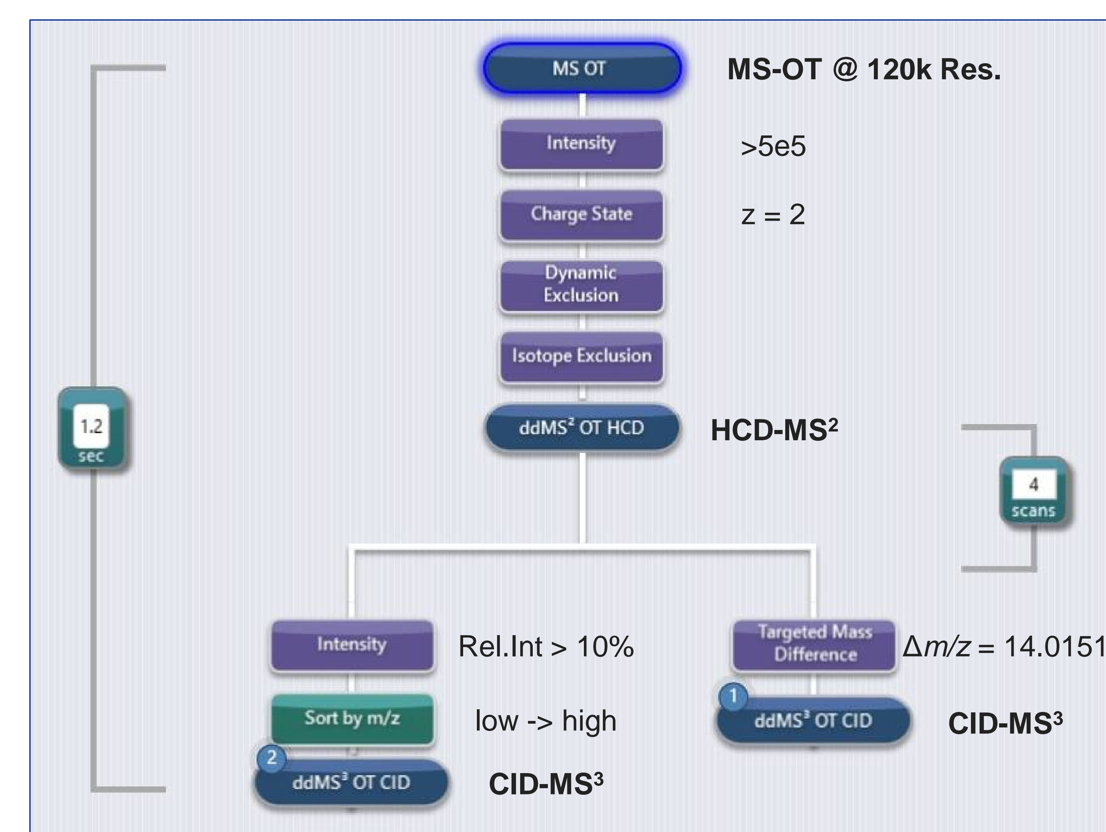
Figure 2. LC-HRAM-MS setup comprised of a Vanquish Horizon UHPLC system and the Orbitrap IQ-X Tribrid MS



Results

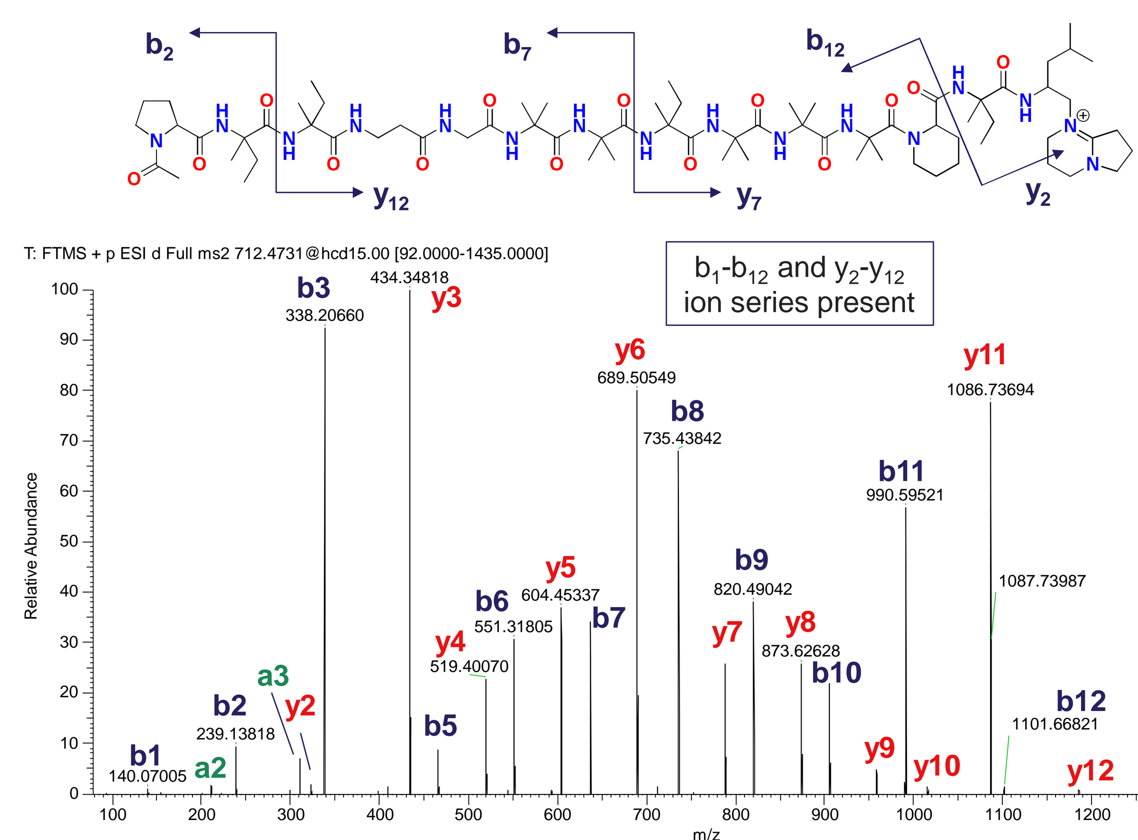
Initial compositional analysis of the crude extract using qNMR revealed adenopeptin, and structurally related analogs, to represent close to 50 wt%, with sucrose (from growth media) as the second most abundant component at approximately 18 wt%. To further investigate the minor components in the crude extract, it was analyzed using a multi-stage fragmentation method after LC separation using a C18 column, as described in the prior section.

Figure 3. Overview of the data-dependent MS^n decision tree targeting MS^3 scans on pairs of fragments with a mass difference corresponding to ± CH_2 prior to triggering other fragments



This allowed to confirm the amino acid sequence of adenopeptin (eluting at 4.0 min) using the HCD-MS^2 fragmentation spectrum, as shown in Figure 4. While the C-terminal isovaline-amidinium cleavage was not detected (y_1 or b_{13}), subsequent MS^3 spectra of the y_3 ion could be used to establish this structure (Figure 7).

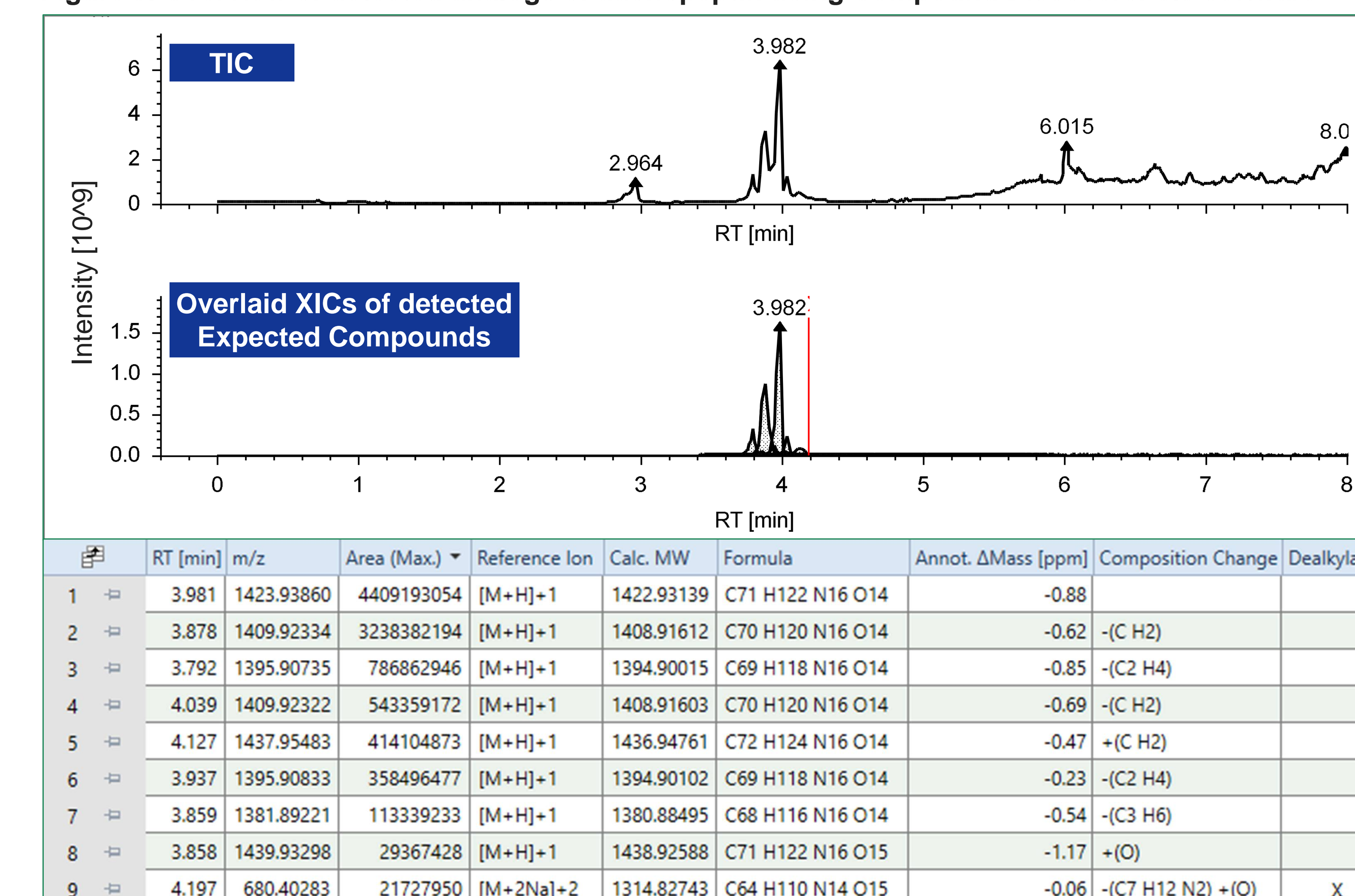
Figure 4. Sequence confirmation of adenopeptin based on its HCD-MS^2 fragmentation spectrum with b- and y-ion series annotated



Analysis of adenopeptin congeners in Compound Discoverer 3.3 software

Starting from the established structure of adenopeptin, the expected compounds workflow was used to detect analogs of adenopeptin based on a list of expected transformations (e.g., methylation, demethylation, hydration, oxidation) and dealkylation predictions. Figure 5 shows the resulting analogs detected by the software after background filtering and thresholding at 0.5% relative intensity, with their overlaid extracted ion chromatograms (XIC), including multiple isomers with one or two missing methyl groups (Compounds 2-4,6).

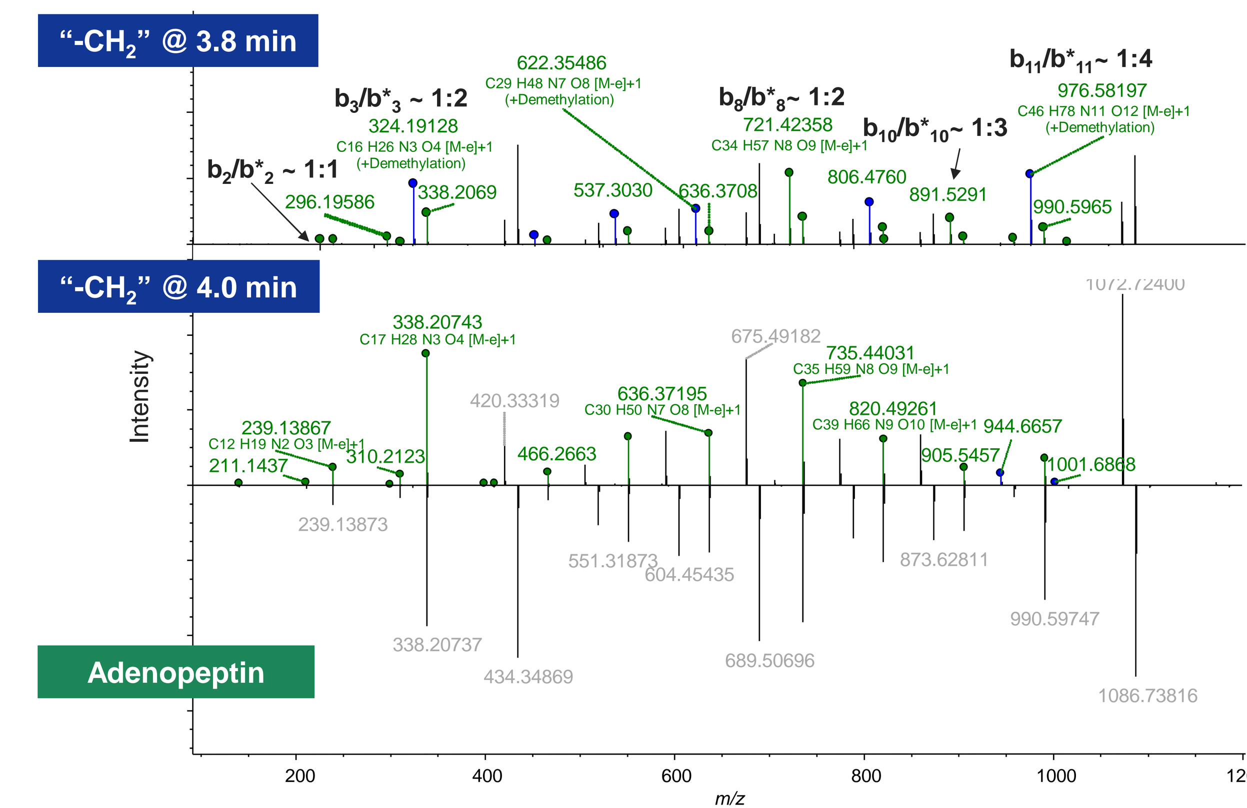
Figure 5. Overview of detected analogs of adenopeptin using Compound Discoverer 3.3 software



	RT [min]	m/z	Area (Max.)	Reference Ion	Calc. MW	Formula	Annot. ΔMass [ppm]	Composition Change	Dealkylated
1	3.981	1423.93860	4409193054	[M+H]+1	1422.93139	C71 H122 N16 O14	-0.88		
2	3.878	1409.92334	3238382194	[M+H]+1	1408.91612	C70 H120 N16 O14	-0.62	-(C H2)	
3	3.792	1395.90735	786862946	[M+H]+1	1394.90015	C69 H118 N16 O14	-0.85	-(C2 H4)	
4	4.039	1409.92322	543359172	[M+H]+1	1408.91603	C70 H120 N16 O14	-0.69	-(C H2)	
5	4.127	1437.95483	414104873	[M+H]+1	1436.94761	C72 H124 N16 O14	-0.47	+(C H2)	
6	3.937	1395.90833	358496477	[M+H]+1	1394.90102	C69 H118 N16 O14	-0.23	-(C2 H4)	
7	3.859	1381.89221	113339233	[M+H]+1	1380.88495	C68 H116 N16 O14	-0.54	-(C3 H6)	
8	3.858	1439.93298	29367428	[M+H]+1	1438.92588	C71 H122 N16 O15	-1.17	+(O)	
9	4.197	680.40283	21727950	[M+2Na]+2	1314.82743	C64 H110 N14 O15	-0.06	-(C7 H12 N2) +(O)	X

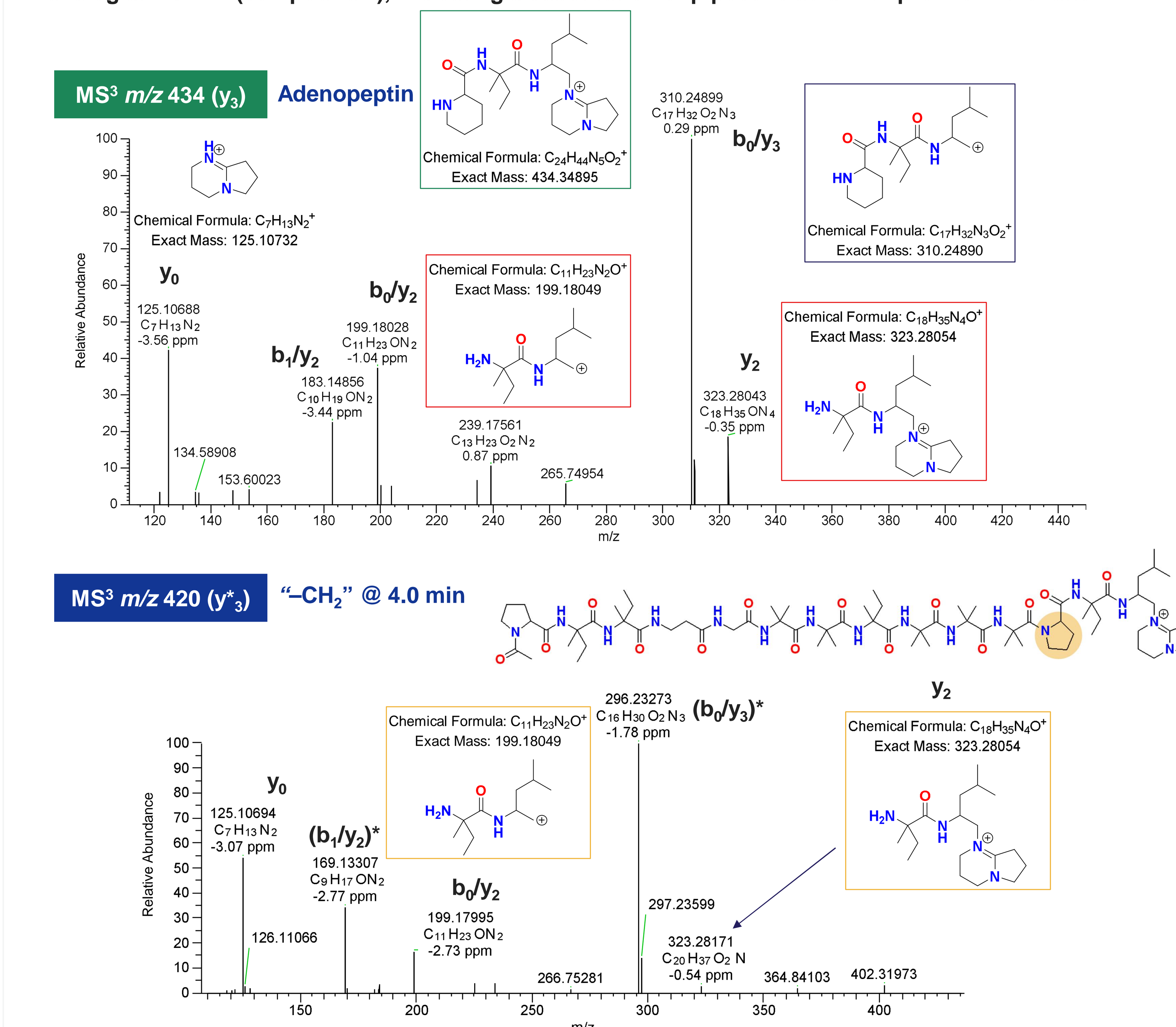
To aid in determination of the modification sites, the integrated fragment ion search (FISh) algorithm automatically annotated the respective fragmentation spectra of the expected compound hits based on the *in silico* predicted fragmentation of adenopeptin, with transformation-shifted fragments colored in blue.

Figure 6. Comparison of the fragmentation spectra of analogs 2 and 4 of adenopeptin and its parent compound 1, with FISh-annotated fragments shown in the Compound Discoverer 3.3 software



Comparison of the MS^2 spectra of the "-CH_2" analogs at 3.9 with its parent revealed the presence of co-eluting isomers. The differences in relative intensity of shifted and unshifted across the b-ion series indicated substitution of Iva with Aib in multiple positions (i.e. 2,3,8 and 13), as highlighted in the figure. Meanwhile, the shifted y-ion series for compound 4 indicated its modification to be on the C-terminus. The exact location could be revealed from the MS^3 spectrum of the shifted y_3 ion at m/z 420, as shown in Figure 7.

Figure 7. Annotated MS^3 spectra from the respective y_3 ions of adenopeptin and its "demethylated" analog at 4.0 min (compound 4), indicating a substitution of pipecolic acid with proline in the latter.



Conclusions

The LC/MS analysis of the crude adenopeptin extract revealed the presence of eight analogs above 0.5% relative MS-abundance, mainly resulting from "demethylations", i.e., substitution of Aib for Iva residues. Their modification sites could be determined based on shifts in the fragmentation pattern relative to adenopeptin. Additionally, a potentially crucial substitution of pipecolic acid with proline could be detected and verified based on MS^3 spectral evidence for compound 4.

References

1. Hayakawa, Y.; *et al.* "Adenopeptin, a new apoptosis inducer in transformed cells from *Chrysosporium* sp." *Tetrahedron*, **1998**, *54*, 15871–15878. (doi: 10.1016/S0040-4020(98)00996-X)

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Conflict of interest disclosure: SH, BB, SM and MD are employees of Thermo Fisher Scientific, whose instrumentation and software were used in acquiring and analyzing the presented data.