Enhanced structural elucidation of microcystins by electron activated dissociation (EAD)

Using the ZenoTOF 7600 system and SCIEX OS software

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Introduction

In this study, electron activated dissociation (EAD) fragmentation in the ZenoTOF 7600 system from SCIEX enhanced the structural elucidation of microcystins (MCs) through the production of unique fragments that were not observed during collision-induced dissociation (CID). These unique fragments corresponded to additional fragmentation mechanisms—such as internal cyclic peptide cleavages and side-chain modifications—that could help distinguish different MC congeners (Figure 1).

Over 120 congeners of these cyanobacterially-derived hepatoxins have been identified to date,[1] where the structural diversity stemmed from variable amino acid substitutions within the cyclic peptide structures of MC and nodularin (NOD) (Table 1). Conventional immunoassay-based methods typically lack the specificity to unambiguously identify individual congeners,[2] while CID-based MS/MS analysis often produces non-selective fragments due to fragmentation primarily occurring at a common side chain present in all MC peptides. As a result, fragment-rich MS/MS spectra generated from unique and alternative dissociation pathways is critical for identifying and differentiating known and new microcystins, which may be numerous based on the possible amino acid configurations in the cyclic peptide.

Key features of the ZenoTOF 7600 system

- The ZenoTOF 7600 system offers EAD and CID fragmentation to collect complementary MS/MS data for structural elucidation
- EAD allows for fragmentation of singly and doubly charged molecules
- Tunable kinetic energy (KE) in EAD reveals additional dissociation pathways that would otherwise not be preserved in CID in favor of more labile fragmentation sites, which results in the production of more unique fragments in EAD MS/MS spectra

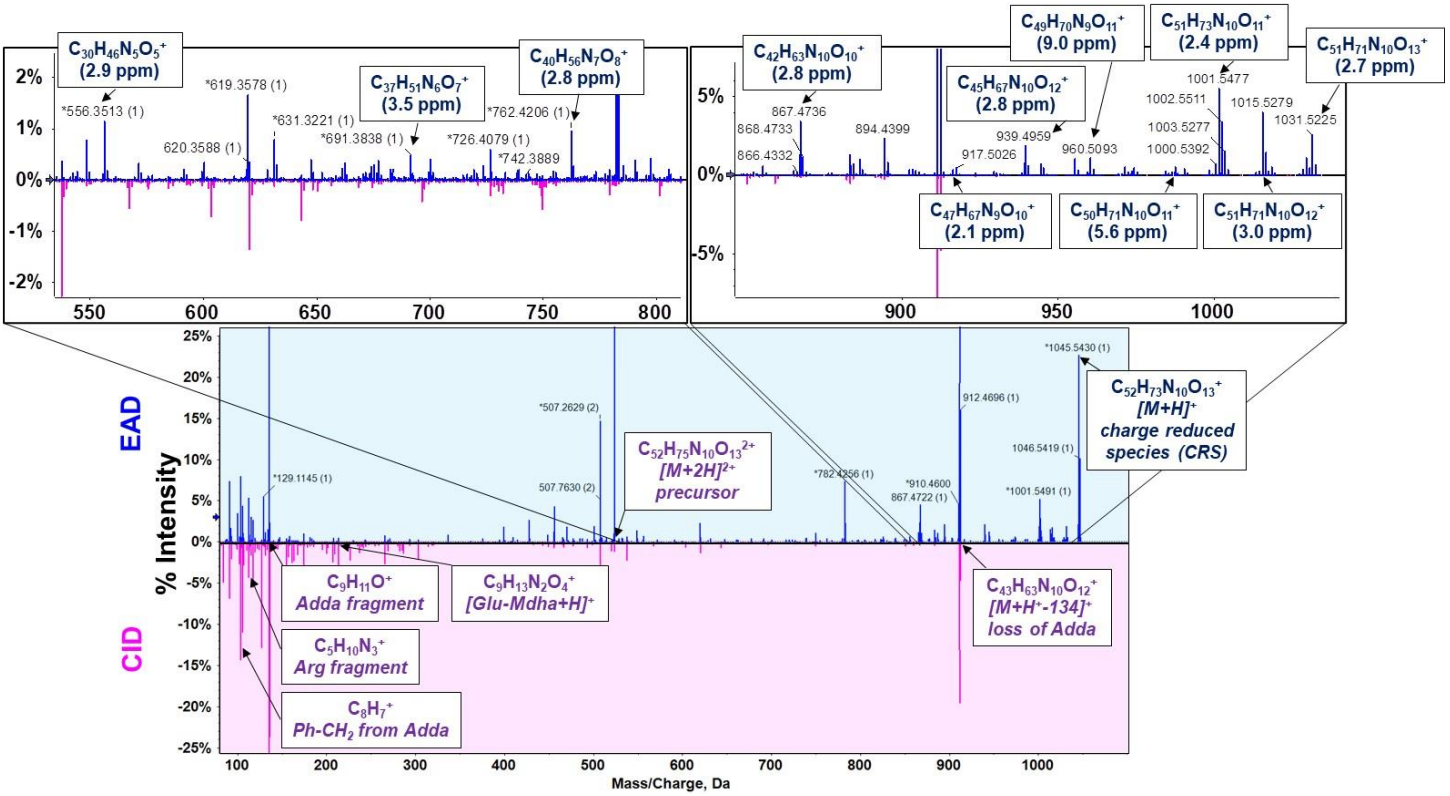

Figure 1. Comparison of EAD and CID MS/MS spectra for the MC-YR congener. In EAD, unique fragments were observed in the higher *m/z* range (blue text), while the CID spectrum exhibited fragments that were observable in both modes and primarily in the lower *m/z* range (purple text).

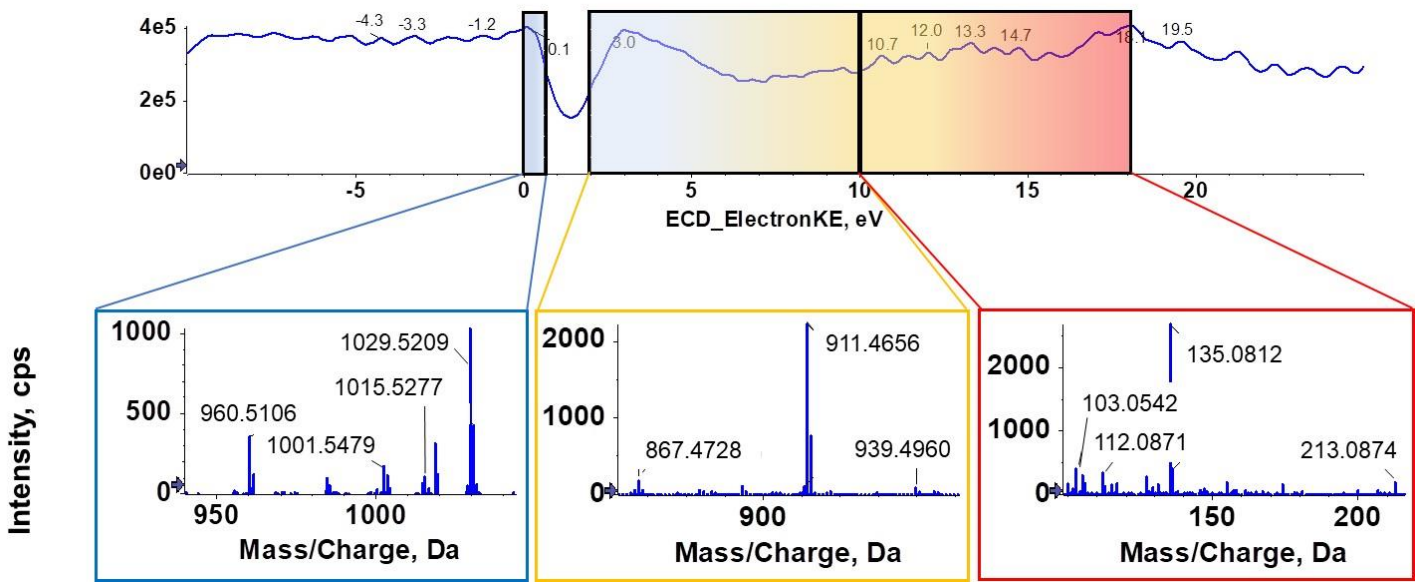

Figure 2. Kinetic energy ramping from -10 to 25 eV using EAD fragmentation mode for the MC-YR congener. Different fragments were generated in the ECD (0–2 eV), hot ECD (2–10 eV) and EIEIO (>10 eV) regions.

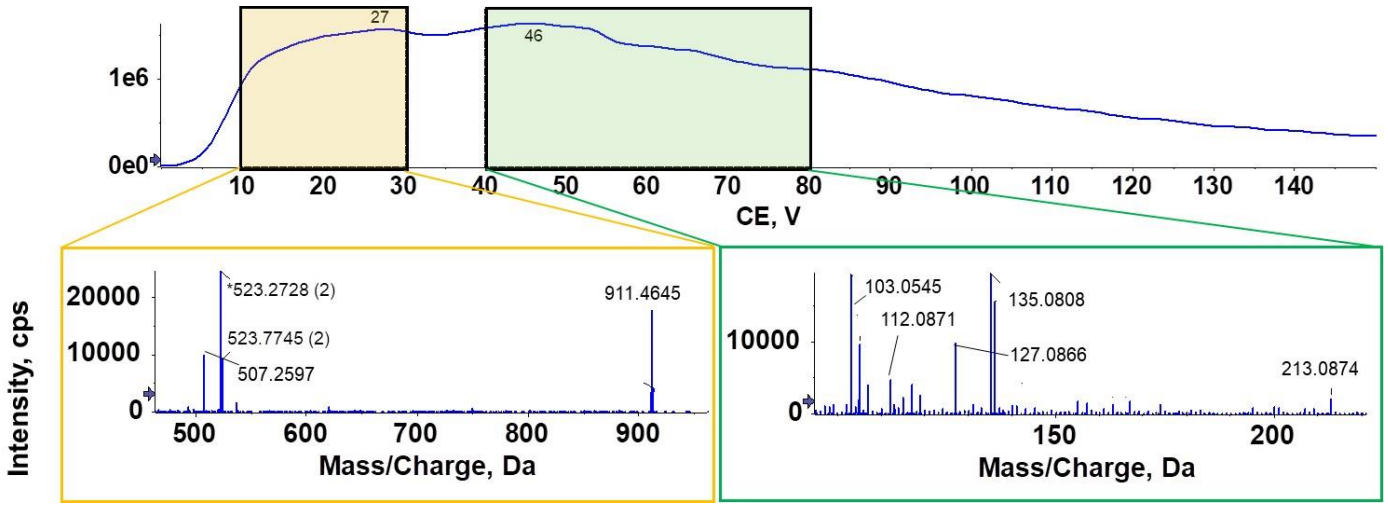

Figure 3. Collision energy ramping from 0 V to 150 V using CID fragmentation mode for the MC-YR congener. MS/MS spectra were generated by the two CE ramps, 10–30 V (yellow) and 40–80 V (green).

Experimental methods

Each MC congener is identified by a two-letter suffix that represents the two variable amino acids substituted at positions 2 and 4 of the cyclic heptapeptide structure (Table 1). MC-LR, MC-RR, MC-YR and nodularin standards were purchased from Enzo Life Sciences (Farmingdale, New York). The standards were introduced into the ZenoTOF 7600 system by infusion and acquired using both CID and EAD fragmentation modes. In EAD mode, the KE was ramped from -10 eV to 25 eV, while similar ramping experiments were performed to optimize the collision energy (CE) from 0 V to 150 V in CID mode. For some MC congeners, doubly charged $[M+2H]^{2+}$ parent ions were monitored as the precursor due to higher fragment intensities observed, as compared to those generated from singly charged $[M+H]^{+}$ precursor ions.

Structure .mol files were generated online using the ChemSpider database and imported into the Data Explorer module of SCIEX OS software 3.0, where theoretical and experimental fragmentation were compared.

KE ramping using EAD

For all the MC and NOD analytes, KE ramping showed a distribution of fragments that changed as the KE increased from

the electron capture dissociation (ECD) region (0–2 eV) to the hot ECD region (2–10 eV) and the electron impact excitation of ions from organics (EIEIO) region (>10 eV). For example, fragments of MC-YR with *m/z* ranges of 950–1030, 850–950 and 100–220 were observed in the ECD, hot ECD and EIEIO regions, respectively (Figure 2). In contrast, the MS/MS spectra for MC-YR generated from CE ramping in CID mode contained fewer fragments, with the majority occurring in the *m/z* range of 100–220 (Figure 3).

EAD fragmentation distinguishes MC congeners

During CID fragmentation, fragments at *m/z* 103, 135 and $[M-135]^+$ were consistently observed for all analytes and corresponded to cleavages in the Adda side chain present in all MCs (Figure 4, top).[5] The fragment at *m/z* 112 corresponding to the loss of an arginine residue can be used as a structural identifier for MCs containing this amino acid in their cyclic peptide structure, such as those analyzed here. Considering these fragments are formed from common features in the MC structure, additional fragments are necessary to distinguish different MC congeners structurally.

In general, the MS/MS spectra generated from EAD fragmentation showed additional fragments that were not observed during CID fragmentation, some of which were associated with modifications of the amino acid side chains and cleavages of the amide bonds throughout the peptide to open the ring structure (Figure 4, bottom). A closer examination of the insets in Figure 1 showed that some of the EAD-specific fragments corresponded to demethylation (*m/z* 1031) and loss of amino acid residues such as glutamic acid (*m/z* 1001) and tyrosine (*m/z* 939), the latter being specific to MC-YR (Figure 4, bottom). The expected *c* and *z* fragments from typical ECD cleavage of the N-C_α peptide bond were observed (*m/z* 1029, 762 and 556), although other fragment types corresponding to cleavages of the C-C(=O) and N-C(=O) peptide bonds also appeared with increasing KE in hot ECD and EIEIO (Figure 4, bottom).

While CID fragmentation typically consumes the majority of the precursor ions, the doubly charged $[M+2H]^{2+}$ precursor peak and its corresponding charge-reduced species (CRS) $[M+H]^+$, a product of ECD, were preserved in the EAD MS/MS spectra of MC-YR (Figure 1). The softer approach of EAD produces more diagnostic fragments that can supplement the lack of structural information whenever CID produces non-selective fragments. The fragments listed under the grey line at the bottom of Table 1 are considered common identifiers of MC and NOD due to their formation from the Adda side chain that is present in the cyclic peptide structure of all MC congeners. A comparison of the fragments above the grey line shows that EAD fragmentation consistently produces more unique fragments than CID fragmentation for the MCs tested.

Conclusions

- In this work, EAD fragmentation on the ZenoTOF 7600 system was used to provide complementary MS/MS data for the structural elucidation of MCs when CID MS/MS spectra were not sufficiently informative.
- KE ramping in EAD induced different dissociation pathways to produce unique fragmentation profiles in the ECD, hot ECD and EIEIO regions
- In addition to the common MC fragments—*m/z* 103, 135 and $[M-135]^+$—typically observed in CID fragmentation, EAD yielded unique product ions that revealed additional fragmentation pathways, such as side-chain modifications and ring-opening bond breakages within the cyclic peptide structures

References

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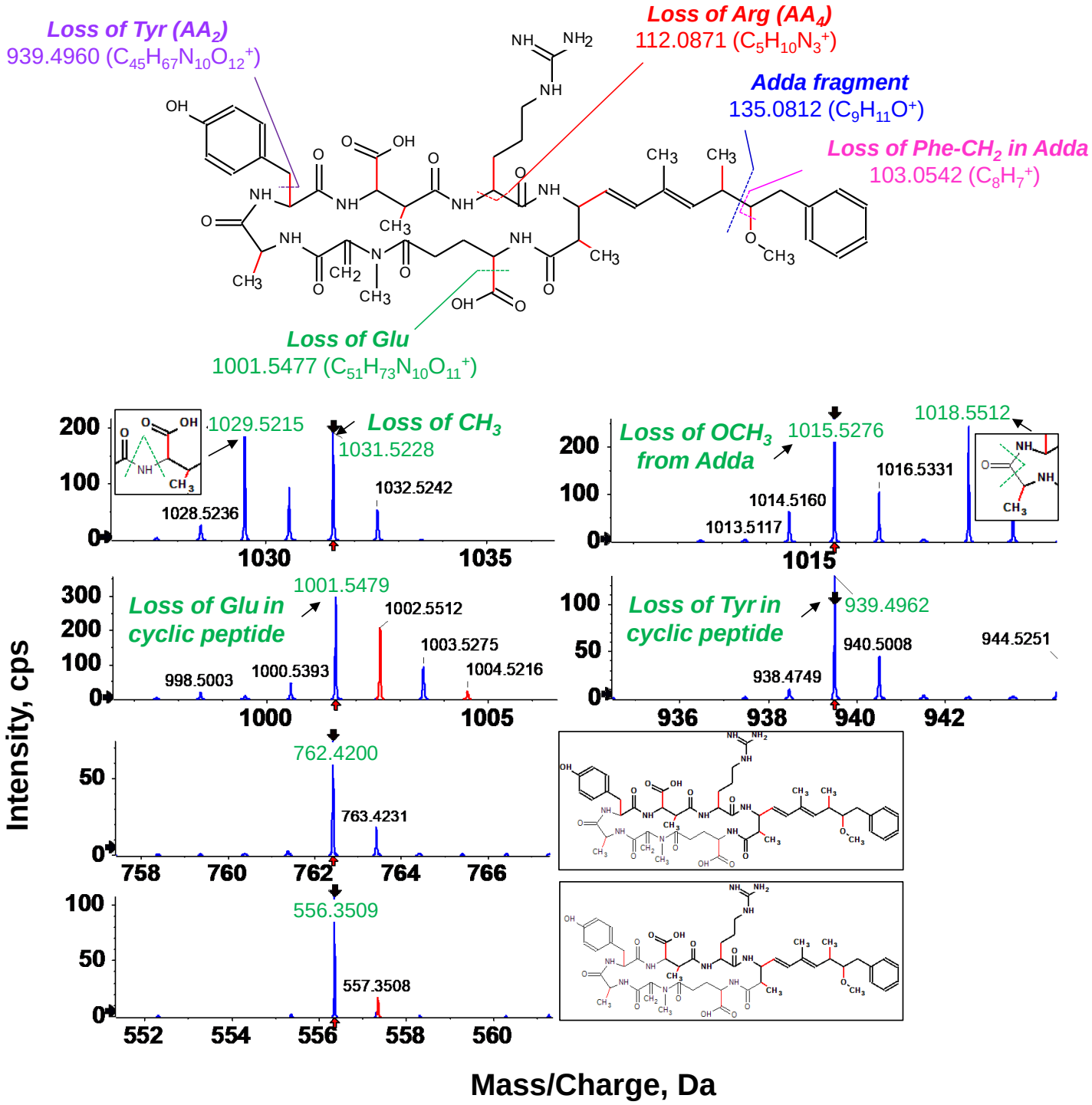
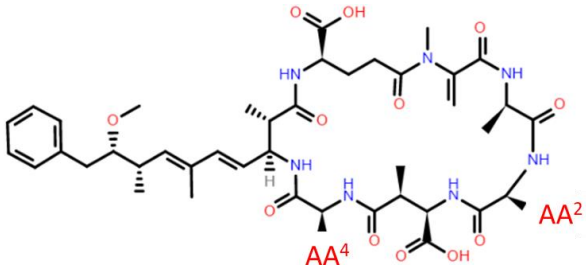
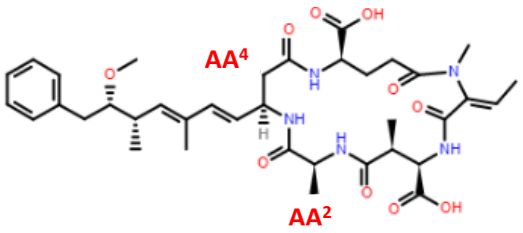


Figure 4. Fragmentation sites in the MC-YR congener (top) and closer examination of various unique fragments only observed in EAD MS/MS spectra of MC-YR (bottom). EAD-specific fragments corresponded to different modifications of the amino acid side chains and internal cleavages of the amide peptide bonds within the cyclic ring structure of MC-YR.





MC-LR AA² = Leu, AA⁴ = Arg			MC-RR AA² = Arg, AA⁴ = Arg			MC-YR AA² = Tyr, AA⁴ = Arg			Nodularin AA² = Arg, AA⁴ = Glu		
Predicted formula	**EAD**	**CID**	**Predicted formula**	**EAD**	**CID**	**Predicted formula**	**EAD**	**CID**	**Predicted formula**	**EAD**	**CID**
$C_{49}H_{75}N_{10}O_{12}^+$	✓	✓	**$C_{49}H_{76}N_{13}O_{12}^+$**	✓		**$C_{52}H_{73}N_{10}O_{13}^+$**	✓		**$C_{41}H_{61}N_8O_{10}^+$**	✓	✓
$C_{48}H_{75}N_{10}O_{11}^+$	✓	✓	$C_{48}H_{74}N_{13}O_{12}^+$	✓		$C_{51}H_{71}N_{10}O_{13}^+$	✓		$C_{40}H_{60}N_8O_8^+$	✓	✓
$C_{48}H_{74}N_{10}O_{10}^+$	✓	✓	$C_{49}H_{74}N_{13}O_{11}^+$	✓		$C_{52}H_{71}N_9O_{13}^+$	✓		$C_{39}H_{59}N_8O_6^+$	✓	
$C_{47}H_{73}N_{10}O_8^+$	✓		$C_{48}H_{77}N_{13}O_{11}^+$	✓		$C_{51}H_{74}N_{10}O_{12}^+$	✓		$C_{39}H_{57}N_8O_7^+$	✓	
$C_{47}H_{71}N_{10}O_9^+$	✓		$C_{48}H_{74}N_{13}O_{11}^+$	✓		$C_{51}H_{71}N_{10}O_{12}^+$	✓		$C_{32}H_{50}N_8O_9^+$	✓	✓
$C_{40}H_{63}N_{10}O_{11}^+$	✓	✓	$C_{48}H_{76}N_{13}O_{10}^+$	✓		$C_{51}H_{73}N_{10}O_{11}^+$	✓		$C_{31}H_{49}N_8O_7^+$	✓	✓
$C_{39}H_{63}N_{10}O_9^+$	✓		$C_{48}H_{74}N_{13}O_9^+$	✓		$C_{50}H_{71}N_{10}O_{11}^+$	✓				
			$C_{47}H_{74}N_{13}O_9^+$	✓		$C_{49}H_{70}N_{10}O_{12}^+$	✓				
			$C_{47}H_{72}N_{10}O_{10}^+$	✓		$C_{49}H_{70}N_9O_{11}^+$	✓				
			$C_{47}H_{70}N_{11}O_{10}^+$	✓		$C_{48}H_{68}N_{10}O_{11}^+$	✓				
			$C_{46}H_{70}N_{13}O_{11}^+$	✓		$C_{45}H_{67}N_{10}O_{13}^+$	✓				
			$C_{45}H_{71}N_{13}O_{10}^+$	✓		$C_{45}H_{67}N_{10}O_{12}^+$	✓				
			$C_{40}H_{66}N_{13}O_{11}^+$	✓	✓	$C_{43}H_{63}N_{10}O_{12}^+$	✓	✓			
			$C_{40}H_{65}N_{10}O_{12}^+$	✓		$C_{42}H_{63}N_{10}O_{10}^+$	✓				
			$C_{40}H_{57}N_8O_{10}^+$	✓		$C_{40}H_{56}N_7O_8^+$	✓				
			$C_{39}H_{64}N_{13}O_9^+$	✓		$C_{30}H_{46}N_5O_5^+$	✓				
			$C_{39}H_{59}N_9O_9^+$	✓							
			$C_{39}H_{59}N_9O_9^+$	✓							
$C_9H_{11}O^+$	✓	✓	$C_9H_{11}O^+$	✓	✓	$C_9H_{11}O^+$	✓	✓	$C_9H_{11}O^+$	✓	✓
$C_5H_{10}N_3^+$	✓	✓	$C_5H_{10}N_3^+$	✓	✓	$C_5H_{10}N_3^+$	✓	✓	$C_5H_{10}N_3^+$	✓	✓
$C_8H_7^+$	✓	✓	$C_8H_7^+$	✓	✓	$C_8H_7^+$	✓	✓	$C_8H_7^+$	✓	✓
$C_4H_{10}N_3^+$	✓		$C_4H_{10}N_3^+$	✓		$C_4H_{10}N_3^+$	✓		$C_4H_{10}N_3^+$	✓	

Table 1. Comparison of diagnostic fragments produced by EAD and CID for structural elucidation of different MC congeners. Molecular formulas in bold represent the parent ions.

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