Supplementary MS-data

Identification of chondroitin sulfate linkage region glycopeptides reveals prohormones as a novel class of proteoglycans

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Running title: Identification of chondroitin sulfate attachment sites

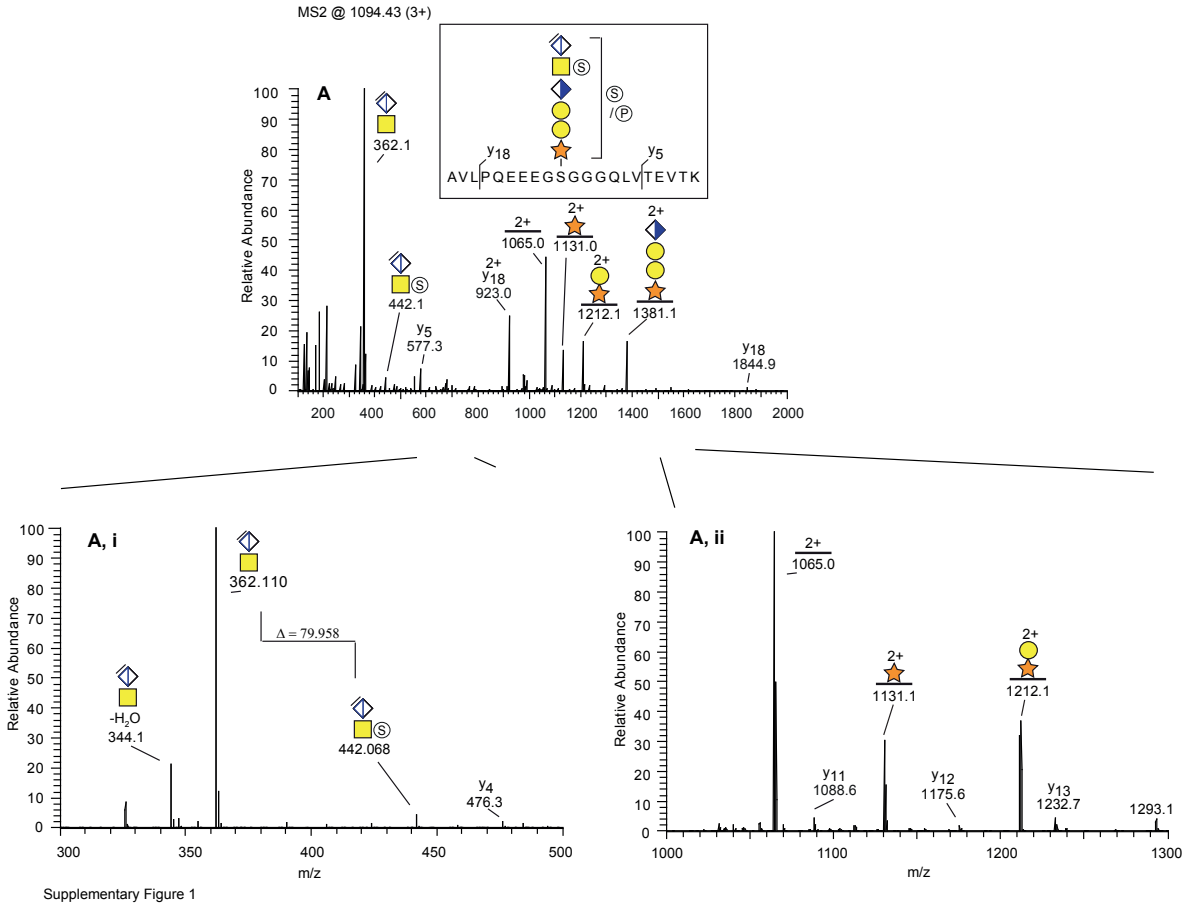
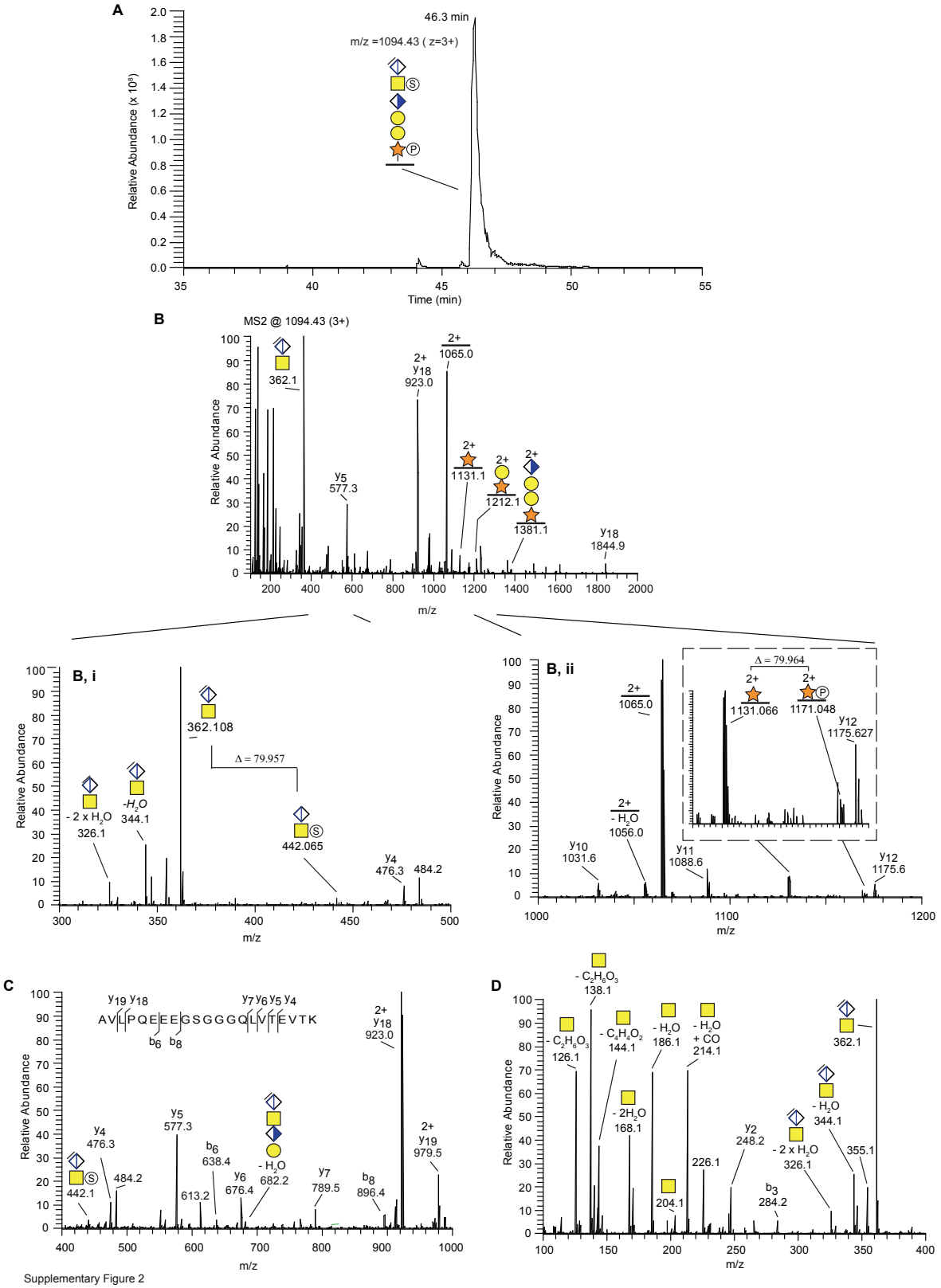
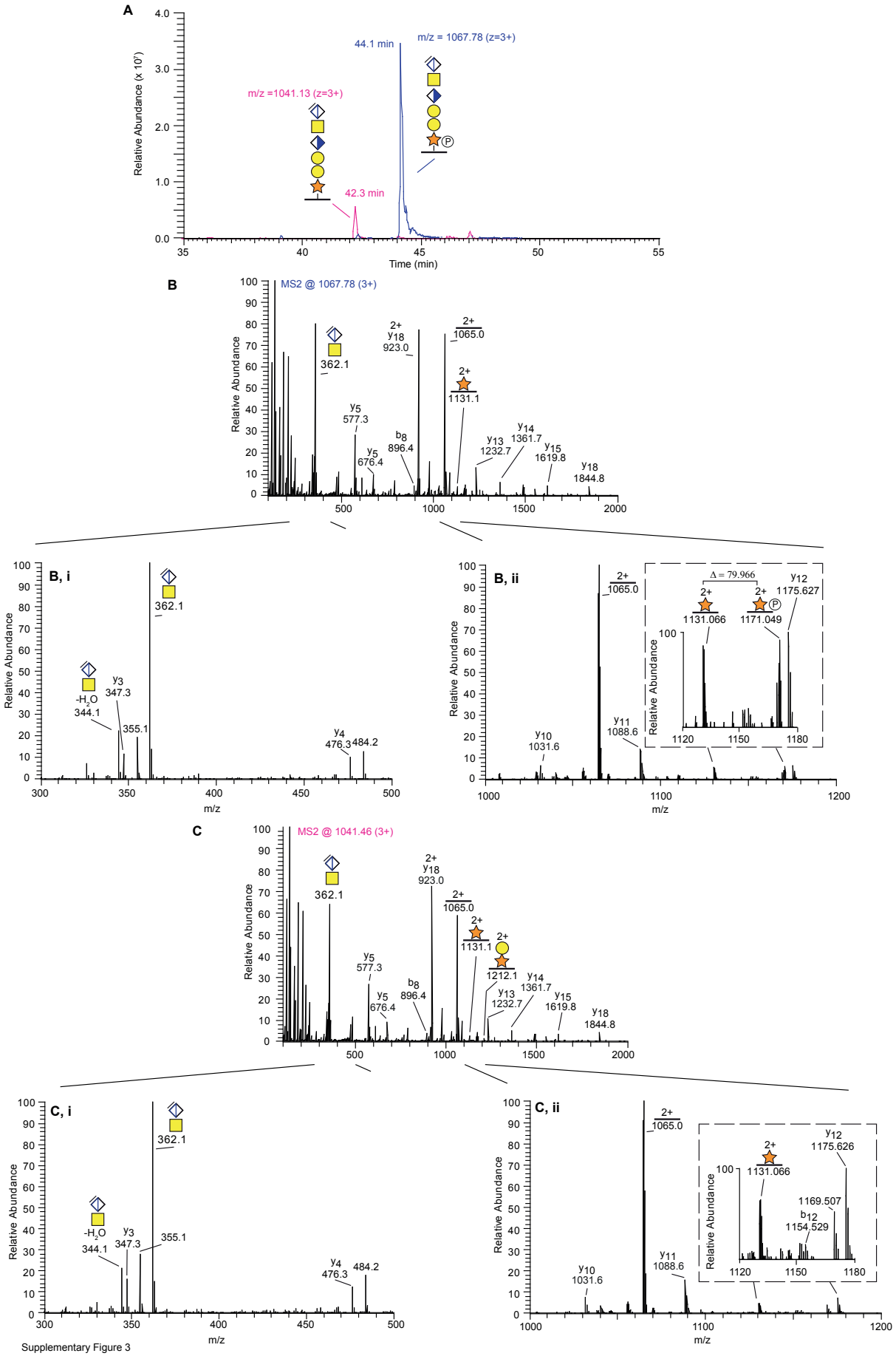


Fig S1. **Structural analysis of pharmaceutical grade bikunin.** (A) In addition to the CS-glycopeptide precursor ion (MS1 *m/z* 1094.43; 3+) presented in Fig. 1, an identical precursor ion (MS1 *m/z* 1094.43; 3+) was identified that co-eluted with the aforementioned structure (~ 18.5 min). (A, i) Similar to the first precursor ion, the MS2 fragment spectrum of the additional precursor ion displayed a mass shift of 79.958 Da between *m/z* = 362.110; 1+ and 442.068; 1+, demonstrating the presence of a sulfate group on the GalNAc-residue. (A, ii) However, in contrast to the first precursor ion, no additional mass shift was observed in the higher mass-range (*m/z* 1000-1300) that indicated either the presence of a phosphate- (79.966 Da) or sulfate modification (79.968) on the xylose or galactose residues.



Supplementary Figure 2

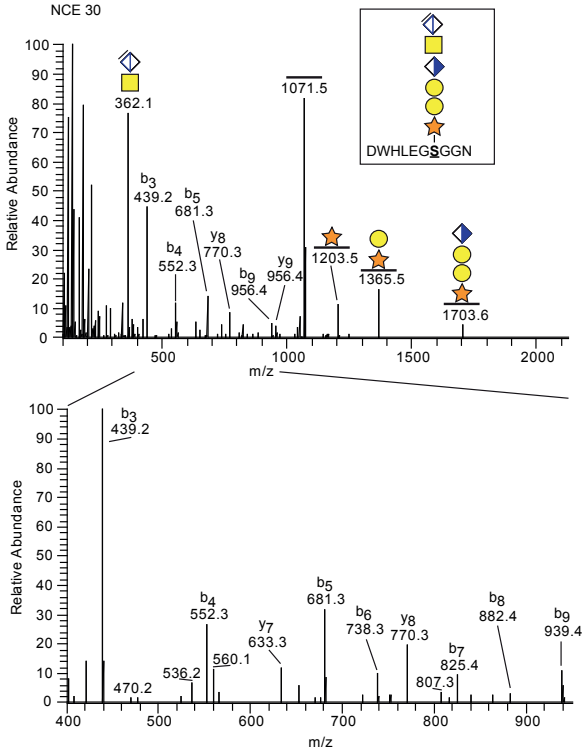
Fig. S2. **Structural analysis of the bikunin CS-linkage region in human urine** (A) LC-MS/MS analysis of a human urine sample revealed a bikunin-derived CS-glycopeptide precursor ion of *m/z* 1094.43; 3+. (B) The obtained MS2 spectrum displayed a fragmentation pattern similar to that of the pharmaceutical grade preparation (Fig. 1D). (B, i) A mass shift of 79.957 Da was observed between *m/z* = 362.108; 1+ and 442.065; 1+, demonstrating the presence of a sulfate group on the GalNAc-residue. (B, ii) A mass shift of 79.964 was observed between *m/z* = 1131.066; 2+ and *m/z* = 1171.048; 2+, which demonstrates the presence of a phosphate group (79.9663 Da) on the xylose-residue. (C) Expanding the spectrum between *m/z* 400 – 1000 enables the identification of several diagnostic b- and y-ions. (D) Expanding the spectrum in the low mass-range *m/z* 100 - 400 revealed several GalNAc-derived saccharide oxonium ions.



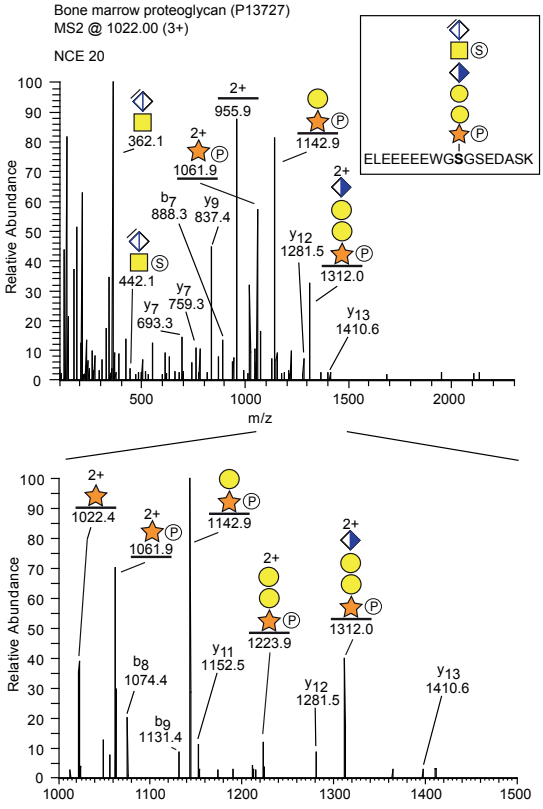
Supplementary Figure 3

Fig. S3. **Structural variation of the bikunin CS-linkage region in human urine.** (A) Minor additional variants of the bikunin CS-glycopeptide were identified in human urine, including the linkage region without modifications (1041.13; 3+) and with only one phosphate group (1067.78; 3+). (B) The late-eluting peak (44.1 min) displayed a mass shift of 79.666 Da between *m/z* = 1131.066; 2+ and 1171.049; 2+ (B, ii)**,** enlarged view, demonstrating the presence of a phosphate group on the xylose-residue. (B, i) No sulfate group was identified on the GalNAc-residue. (C) Accordingly, the early-eluting peak (42.3 min) representing the bikunin CS-glycopeptide without secondary modifications (1041.13; 3+), did neither display sulfate- nor phosphate modification at any positions (C, i and C, ii).

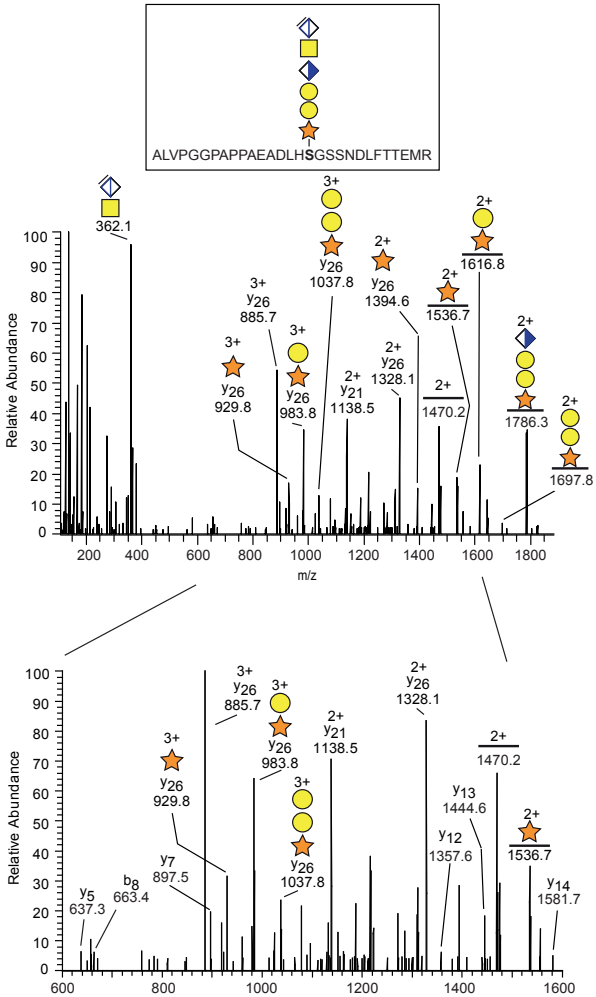
Basement membrane-specific heparan sulfate proteoglycan core protein (P98160)
MS2 @ 1032.88 (2+)
NCE 30



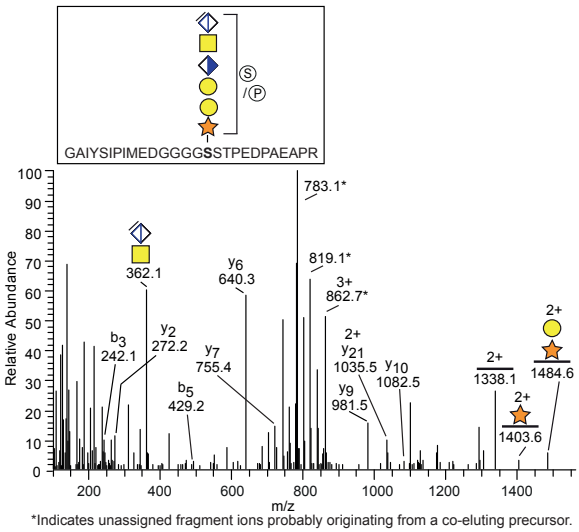
Bone marrow proteoglycan (P13727)
MS2 @ 1022.00 (3+)
NCE 20



Brain-specific angiogenesis inhibitor 2 (O60241)
MS2 @ 1311.58 (3+)
NCE 25



Brevican (Q96GW7)
MS2 @ 1250.5 (3+)
NCE 30



*Indicates unassigned fragment ions probably originating from a co-eluting precursor.

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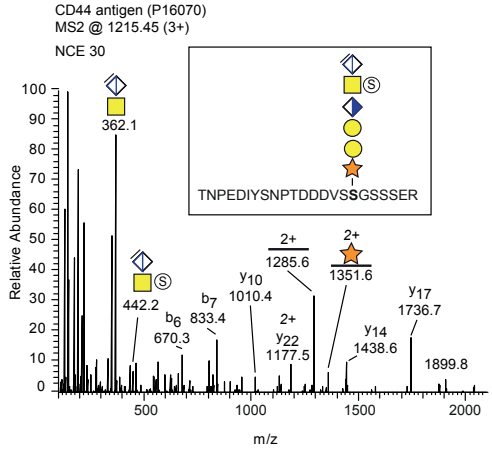

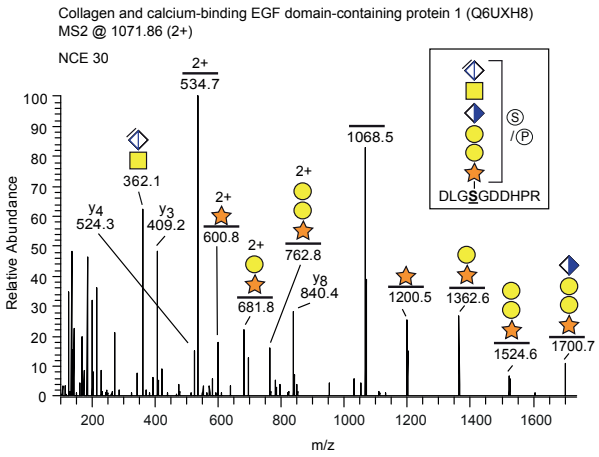

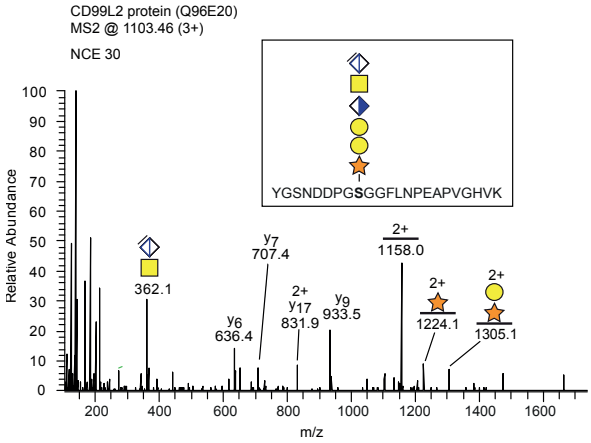

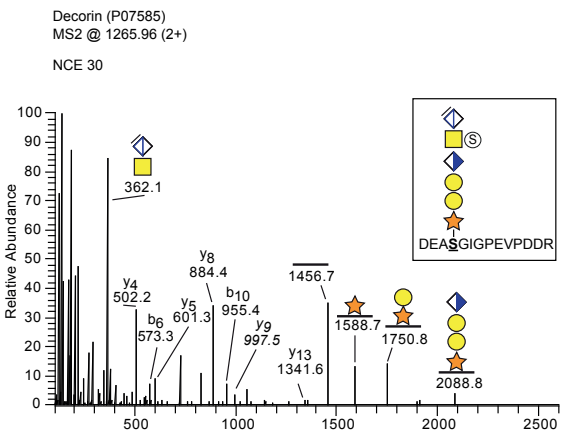

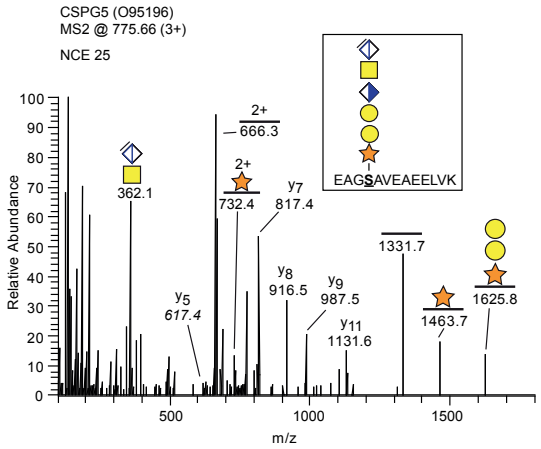

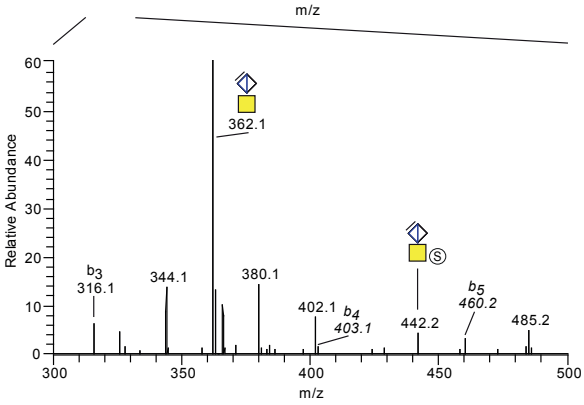

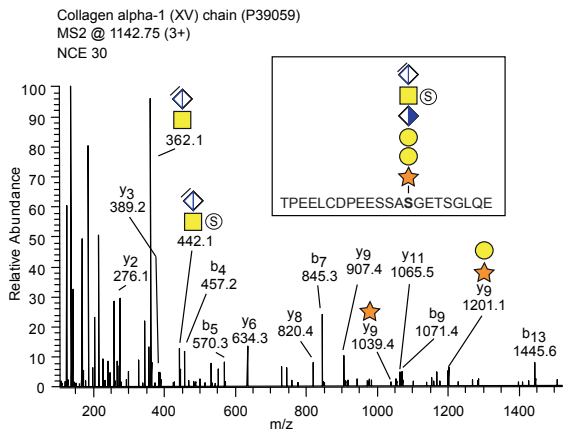

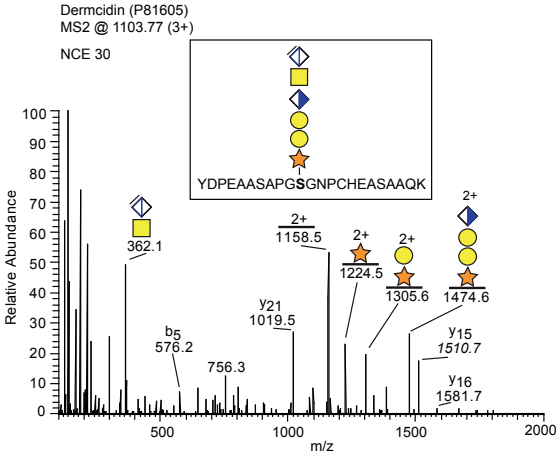

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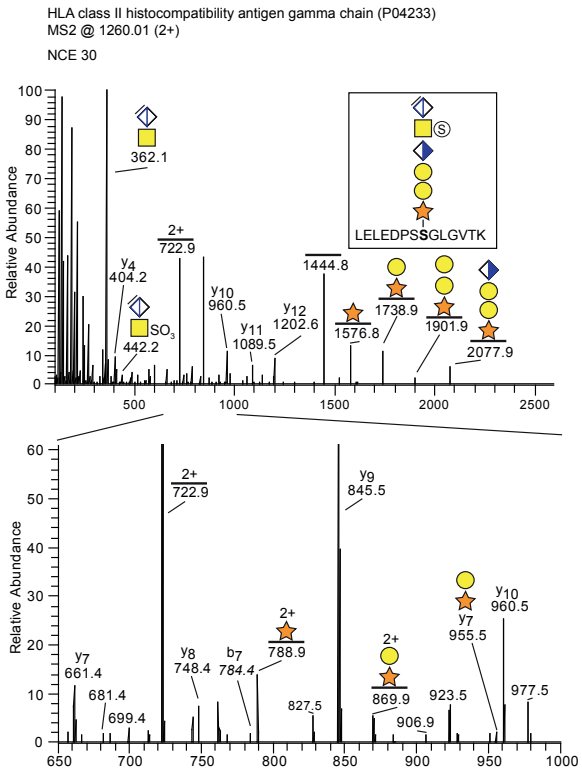

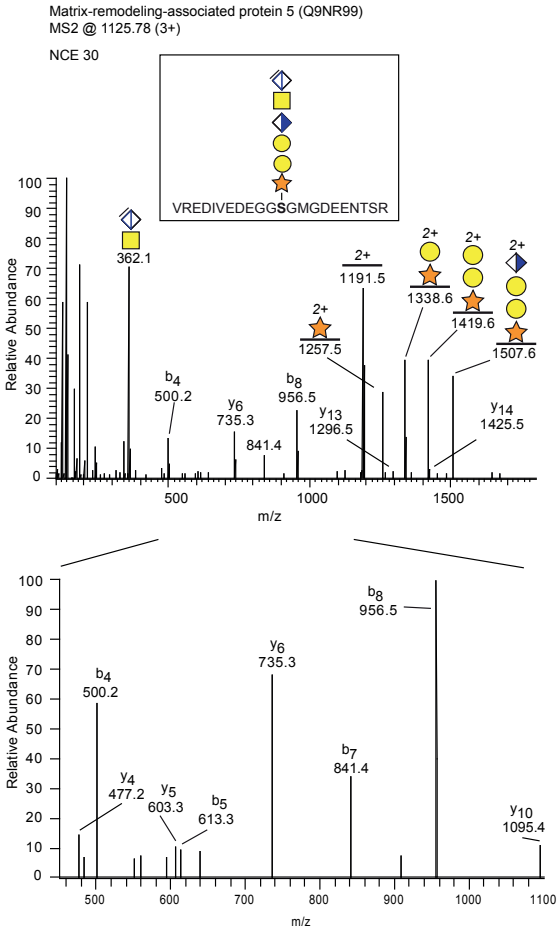

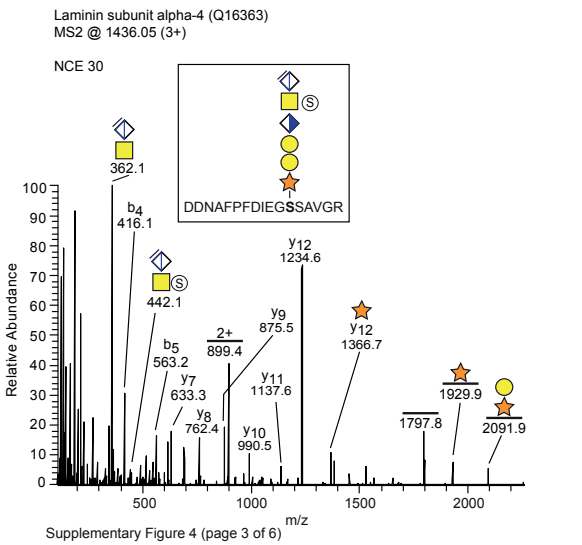

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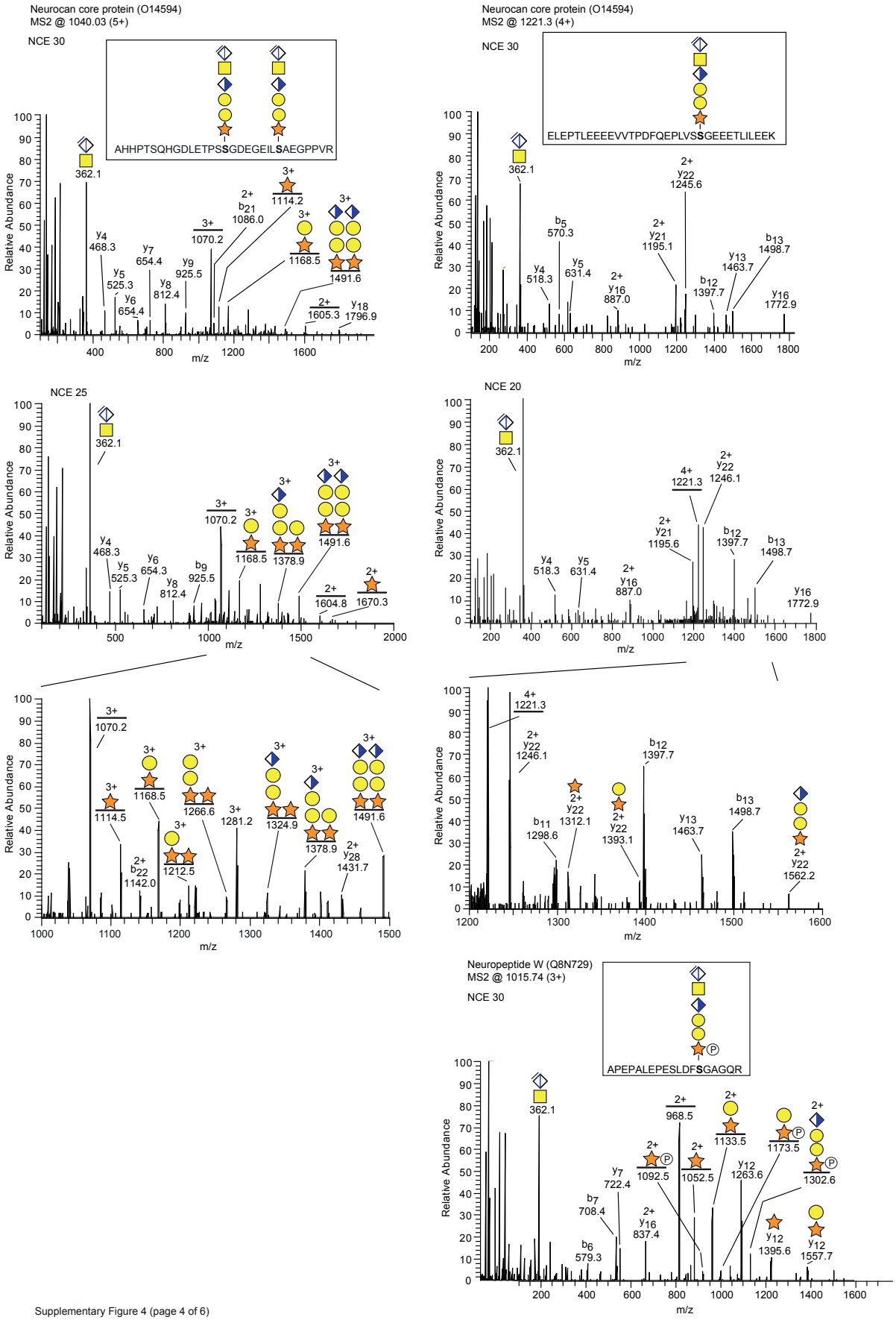

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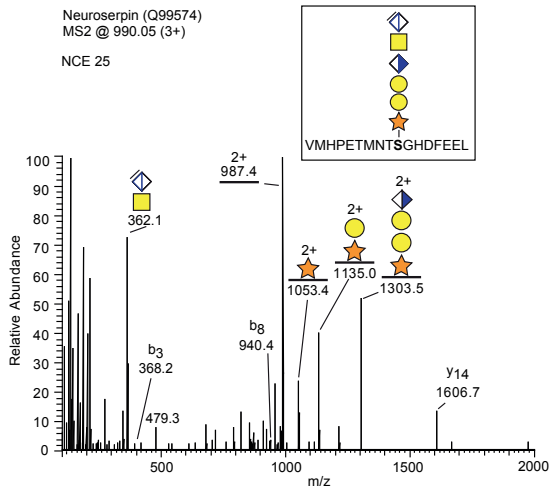

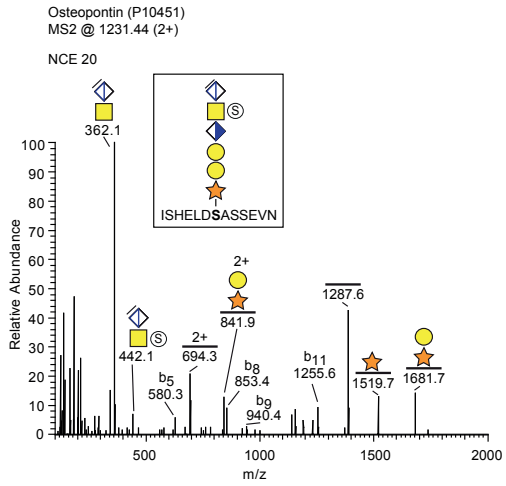

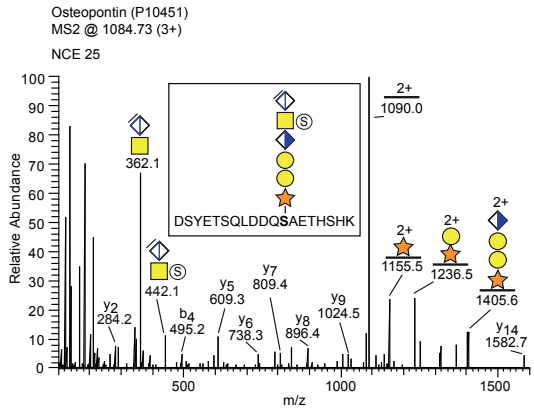

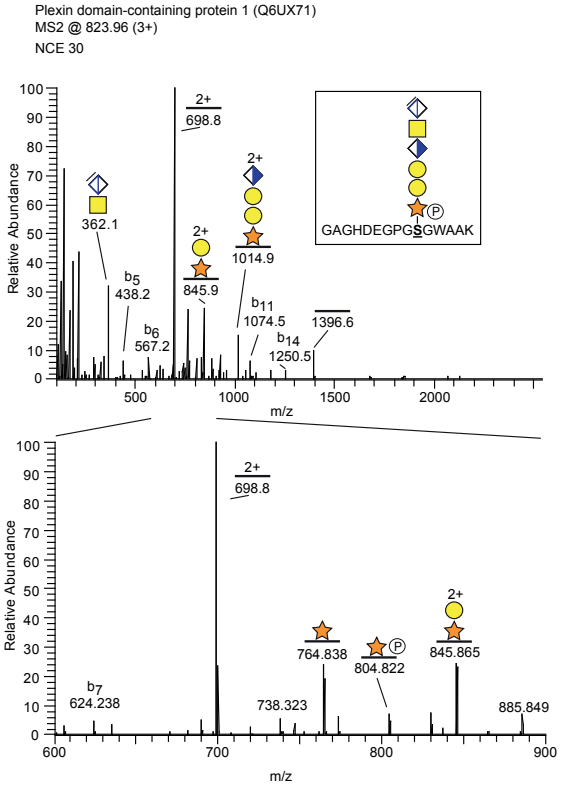

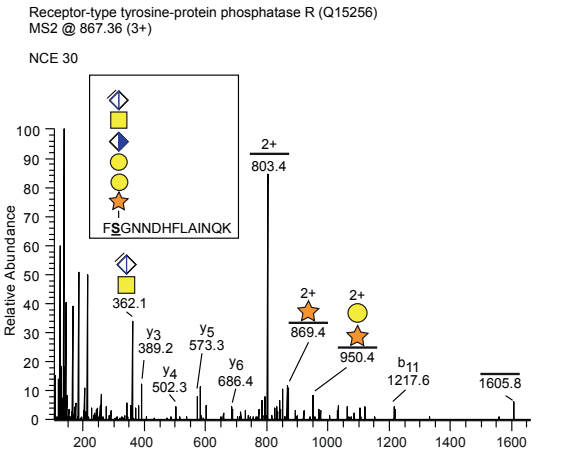

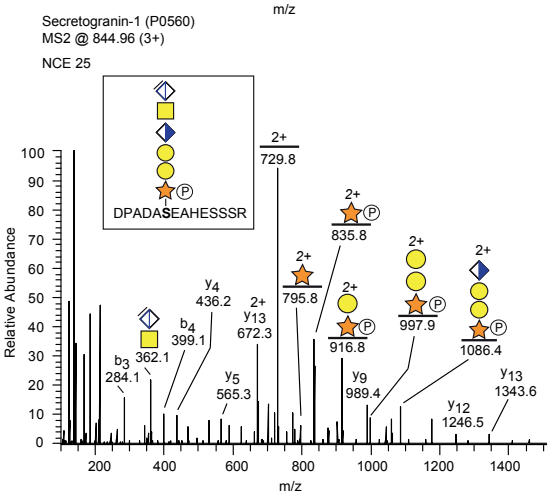

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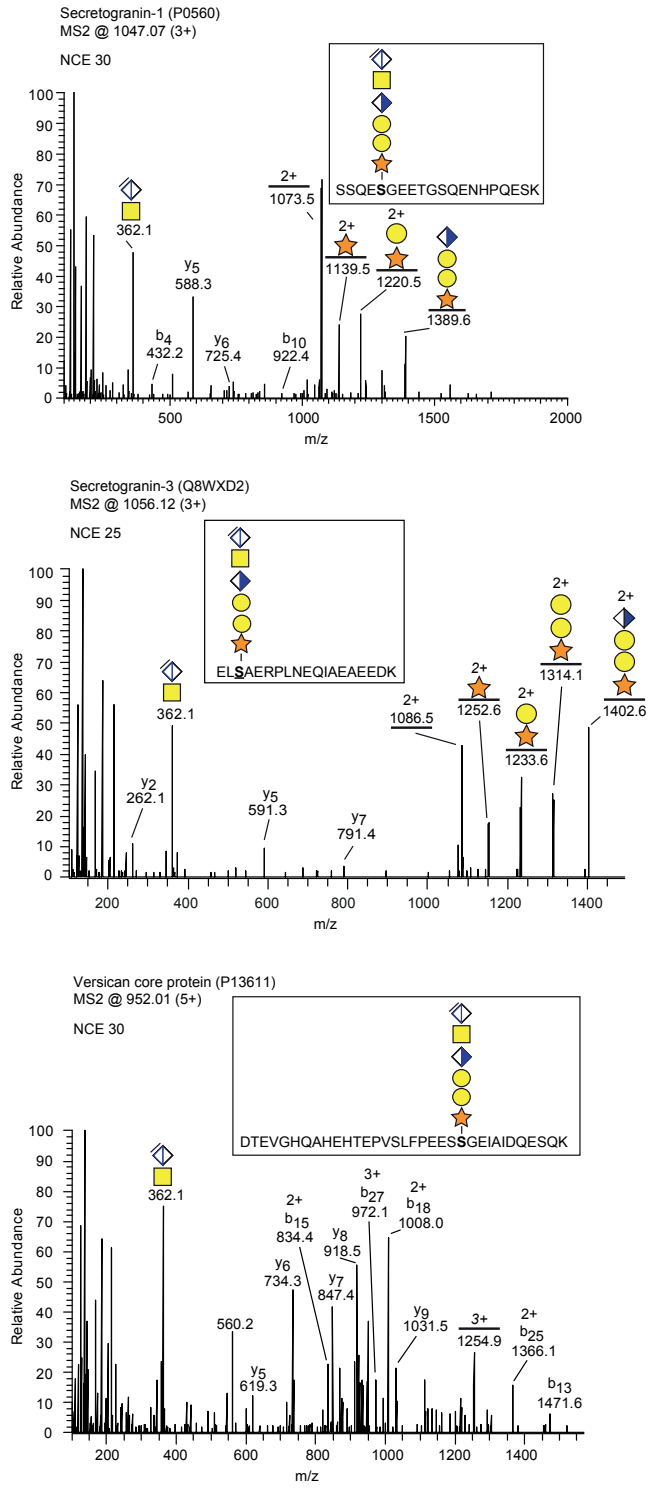

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Fig. S4**. Fragment mass spectra of all identified CS-glycopeptides.** A typical HCD spectrum of each identified CS-glycopeptide presented in Supplementary Table 1 is shown in alphabetical order. The fragmentation energy level for each precursor ion is indicated (NCE 20, 25 or 30%). The positioning and distinction of sulfate- (79.9663 Da) and phosphate- (79.9568 Da) modifications were made by manually evaluating the MS2-spectra for obtained hits. When the expected modification could not be identified in the MS2-spectra, a bracket including the whole hexasaccharide structure is presented as the expected modification could be, in theory, anywhere on the glycan. (Mass spectra of CgA and cholecystokinin are shown in Fig. 3).