Supplemental information for

Effects of inactivation of D,D-transpeptidases of *Acinetobacter baumannii* on bacterial growth and susceptibility to β-lactam antibiotics

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Table S1. Names of genes, loci, length, and molecular weight (MW) of penicillin-binding proteins (PBPs) of *A. baumannii* CIP 70.10, and amino acid sequence identity (similarity) to *P. aeruginosa* PAO1 transpeptidases.

PBP	Gene[a]	ABCIP7010 locus	Number of amino acids	MW (Da)	Identity (similarity)[b] (%)
1a	*mrcA* (*ponA*)	3385	851	94,827	43.7 (73.2)
1b	*mrcB*	2471	798	88,256	42.2 (75.1)
2	*mrdA* (*pbpA*)	1009	672	74,452	47.0 (76.1)
3	*ftsI*	3391	610	67,659	40.1 (72.7)

[a] Gene names for the corresponding PBPs of *A. baumannii* and *P. aeruginosa*. In some databases, the genes for PBP1a and PBP2 have alternative names in *P. aeruginosa* PAO1, which are given in parentheses.
[b] Identity (similarity) given for TPs only.

Table S2. Bacterial strains and plasmid constructs used in this study

Strains	**Characteristics or genotype**	**Source**
E. coli DH10B	The host for recombinant plasmids	NEB
A. baumannii CIP 70.10	Parental wild-type strain	ATCC 15151
***A. baumannii* CIP 70.10 variants**		
ΔPBP1a	Deletion mutant of *mrcA* encoding PBP1a	This study
ΔPBP1b	Deletion mutant of *mrcB* encoding PBP1b	This study
PBP1a(S/A)	PBP1a mutant with Ser459Ala substitution	This study
PBP1b(S/A)	PBP1b mutant with Ser455Ala substitution	This study
ΔPBP2	Deletion mutant of *mrdA* encoding PBP2	This study
PBP1a(S/A)+PBP1b(S/A)	Double mutant	This study
ΔPBP1a+ΔPBP2	Double mutant	This study
ΔPBP1b+ΔPBP2	Double mutant	This study
PBP1a(S/A)+ΔPBP2	Double mutant	This study
PBP1b(S/A)+ΔPBP2	Double mutant	This study
PBP1a(S/A)+PBP1b(S/A)+ΔPBP2	Triple mutant	This study
Suicide Plasmids		
pMo130	Allelic exchange suicide vector	Addgene #27388
pMT308FW	Suicide vector for deletion of PBP1a	This study
pMT309FW	Suicide vector for deletion of PBP1b	This study
pNS257	Suicide vector for deletion of PBP2	This study
pMT310FW	Suicide vector for deletion of PBP3	This study
pMT321FW	Suicide vector for Ser459Ala substitution in the PBP1a TP domain	This study
pMT322FW	Suicide vector for Ser455Ala substitution in PBP1b TP domain	This study

Table S3. Steps of allelic exchange procedures, primers, and size of PCR products to generate and verify mutant derivatives of *A. baumannii* CIP 70.10

Steps	Primer's name and sequences	PCR product length (bp)
ΔPBP1a construct		
UR PCR amplification	oNS159 GACACGGTAACCTAATTTTTGC oNS158-Pho GGATAGCTTTTTCATGATAAGTAAGC	1116
DR PCR amplification	oNS160 AATCAAATTGAGTAAAAATAAAAAAGCC oNS161 CTATAATGCGGGGCAAAG	1046
PCR amplification of the ligated UR and DR	oNS163F TATATAGGATCCGACACGGTAACCTAATTTTTGC oNS164 TATATAGGATCCCTATAATGCGGGGCAAAG	2186
Sequencing of the insert cloned into the pMo130 vector	oMT306 GGTTTACGATATAACCTGAGCACACTA oMT307 GCATCTCCAGAACGTCGTATGCA oNS185 TTTACCACGACCGCATTCTC oNS186 CCTCTGACACATGCAGCTC	
PCR amplification to confirm integration into the chromosome	oNS185 TTTACCACGACCGCATTCTC oMT305 GCGAAGTCTTACCTCAAGTTGTGC	4850 or 2324
	oMT300 GAACGTCGACTTGAGGAGAAAAGC and oNS186 CCTCTGACACATGCAGCTC	2308 or 4834
Verification of the PBP1a gene deletion	oMT300 GAACGTCGACTTGAGGAGAAAAGC oMT305 GCGAAGTCTTACCTCAAGTTGTGC	2299
ΔPBP1b construct		
UR PCR amplification	oNS166 CGACATTGAGAGAGACACTATG oNS165-Pho ACGTTCAAACTTCATATAAATGAGAT	972
DR PCR amplification	oNS167 AGTTATAACAACTAAGCGTTAACAGG oNS168 CGAGAAGTAGAACAATTAACGG	1034
PCR amplification of the ligated UR and DR	oNS170 TATATAGGATCCCGACATTGAGAGAGACACTATG oNS171 TATATAGGATCCCGAGAAGTAGAACAATTAACGG	2029
Sequencing of the insert cloned into the pMo130 vector	oMT316 GATGACAAGCTATCGCATCATTAACG oMT317 CCGACCATAAGTACAGCACCAATAT oNS185 TTTACCACGACCGCATTCTC oNS186 CCTCTGACACATGCAGCTC	
PCR amplification to confirm integration into the chromosome	oNS185 TTTACCACGACCGCATTCTC oMT315 CTTATTCATCAGCCTTGGACATGGTA	4636 or 2269
	oMT310 GGTACGACAAGCCATATAGAAATCATAAC oNS186 CCTCTGACACATGCAGCTC	2186 or 4553
Verification of the PBP1b gene deletion	oMT310 GGTACGACAAGCCATATAGAAATCATAAC oMT315 CTTATTCATCAGCCTTGGACATGGTA	2278
ΔPBP2 construct		
UR PCR amplification	oNS175 CAGCTTTACGTGGCACAATAC oNS174 AAAGTGCTGTTTCATACGGATC	1039
DR PCR amplification	oNS172-Pho GAGGTCGATGAATAATGAAAAATC oNS173 CTTAGCATGTTACCTGCGTTAG	1145
PCR amplification of the ligated UR and DR	oNS177 TATATAGGATCCCAGCTTTACGTGGCACAATAC oNS176 TATATAGGATCCCTTAGCATGTTACCTGCGTTAG	2208
Sequencing of the insert cloned into the pMo130 vector	oMT326 GCGCTTGCGTTAGAATAAGCAGCTA oMT327 ATCGGGAGTTGGTCTTAATCCTTCA oNS185 TTTACCACGACCGCATTCTC	

	oNS186 CCTCTGACACATGCAGCTC	
PCR amplification to confirm integration into the chromosome	oNS184 AAATAGGCGTATCACGAGGC oMT320 GTTCAAATCCGACTTGCTCACCA	4447 or 2458
	oMT338 GATGTCATGGACCAAGATGCAAAACG oNS185 TTTACCACGACCGCATTCTC	2427 or 4416
Verification of the PBP2 gene deletion	oMT320 GTTCAAATCCGACTTGCTCACCA oMT338 GATGTCATGGACCAAGATGCAAAACG	2478
ΔPBP3 construct		
UR PCR amplification	oNS181 GAATTATGGCAGACTTAGGTGTG oNS180 TCGCTTATCTACCATACAGCAAG	999
DR PCR amplification	oNS178-Pho CCTATTCGCAGGTAAGCTATG oNS179 CAATGCCTGTAAATCGAAAC	987
PCR amplification of the ligated UR and DR	oNS183 TATATAGGATCCGAATTATGGCAGACTTAGGTGTG oNS182 TATATAGGATCCCAATGCCTGTAAATCGAAAC	2010
Sequencing of the insert cloned into the pMo130 vector	oMT336 ACCGATGACCTCAGAGCAAAATAAGTA oMT337 AGGCTGGCTATAACTTGTCAAAGCA oNS185 TTTACCACGACCGCATTCTC oNS186 CCTCTGACACATGCAGCTC	
PCR amplification to confirm integration into the chromosome	oNS185 TTTACCACGACCGCATTCTC oMT335 GGTGTATGCGCATAATCCACTACA	3961 or 2158
	oMT330 AGACCCTCAAGCTCTGGAAGTTG oNS186 CCTCTGACACATGCAGCTC	2205 or 4008
Verification of the PBP3 gene deletion	oMT335 GGTGTATGCGCATAATCCACTACA oMT330 AGACCCTCAAGCTCTGGAAGTTG	4009 and 2207
PBP1a(S/A) construct		
UR PCR amplification	oMT352 TATTGGATCCTTACTTATCATGAAAAAGCTATCCAGTTTG oMT351 GCCAGCCTTGTAAAGCACGGT	1382
DR PCR amplification	oMT350-Pho GTCAGCCAGGTGCAACCATCAAACCATTC oMT353 TATAGGATCCAGAACGTCGTATGCAACTAGAACAGA	1317
PCR amplification of the ligated UR and DR	oMT352 TATTGGATCCTTACTTATCATGAAAAAGCTATCCAGTTTG oMT353 TATAGGATCCAGAACGTCGTATGCAACTAGAACAGA	2700
Sequencing of the insert cloned into the pMo130 vector	oMT308 GCTGAGCTTGACGATATTGACTGT oMT309 CCAAATGGCCATGATTGCCG oNS185 TTTACCACGACCGCATTCTC oNS186 CCTCTGACACATGCAGCTC	
PCR amplification to confirm integration into the chromosome	oNS185 TTTACCACGACCGCATTCTC oMT305 GCGAAGTCTTACCTCAAGTTGTGC	3769
	oMT300 GAACGTCGACTTGAGGAGAAAAGC oNS186 CCTCTGACACATGCAGCTC	3918
Screening for the **Ala** Mutation	oMT355-Ala CGTCAGCCAGGT**GCA**AC oMT305 GCGAAGTCTTACCTCAAGTTGTGC	2310
Verification of the PBP1a(S/A) mutant	oMT306 GGTTTACGATATAACCTGAGCACACTA oMT305 GCGAAGTCTTACCTCAAGTTGTGC	3857
Sequencing primers	oMT301 GCCATAGAATCCCATCGGAAGTGA oMT304 GTGAGGAAATTGTGATTCCTTCCAAG oMT306 GGTTTACGATATAACCTGAGCACACTA oMT307 GCATCTCCAGAACGTCGTATGCA oMT308 GCTGAGCTTGACGATATTGACTGT oMT309 CCAAATGGCCATGATTGCCG	

PBP1b(S/A) construct		
UR PCR amplification	oMT358 ATATGGATC**C**ATTTATATGAAGTTTGAACGTGGTATCG oMT357 GCACCGACTTGGCGTTTTGCATCT	1378
DR PCR amplification	oMT356-Pho ATTATTGAAACCTGTCATTTATTTGAGCGCA oMT359 AATAGGATCCTGTTTCCTGTTAACGCTTAGTTGTTAT	1056
PCR amplification of the ligated UR and DR	oMT358 ATATGGATC**C**ATTTATATGAAGTTTGAACGTGGTATCG oMT359 AATAGGATCCTGTTTCCTGTTAACGCTTAGTTGTTAT	2437
Sequencing of the insert cloned into the pMo130 vector	oMT318 GGTTTGTTGTACGTTTAGACCATAACGA oMT319 GACCCTCACCCAACAGTTAGTCA oNS185 TTTACCACGACCGCATTCTC oNS186 CCTCTGACACATGCAGCTC	
PCR amplification to confirm integration into the chromosome	oNS185 TTTACCACGACCGCATTCTC oMT315 CTTATTCATCAGCCTTGGACATGGTA	3685
	oMT310 GGTACGACAAGCCATATAGAAATCATAAC oNS186 CCTCTGACACATGCAGCTC	3550
Screening for the **Ala** Mutation	oMT361Ala CGCCAAGTCGGT**GCA**TT oMT315 CTTATTCATCAGCCTTGGACATGGTA	2252
Verification of the PBP1b(S/A) mutant	oMT310 GGTACGACAAGCCATATAGAAATCATAAC oMT315 CTTATTCATCAGCCTTGGACATGGTA	4645
Sequencing primers	oMT311 GTCCCAGCGCTGTCCTTCAAAT oMT314 GGATGATAATACCGACAGTTATATTCGTGA oMT316 GATGACAAGCTATCGCATCATTAACG oMT317 CCGACCATAAGTACAGCACCAATAT oMT318 GGTTTGTTGTACGTTTAGACCATAACGA oMT319 GACCCTCACCCAACAGTTAGTCA	

Pho, phosphorylated at 5'; bp, base pairs; UR, upstream region; DR, downstream region; (S/A), the catalytic Ser to Ala substitution in the transpeptidase domain of bifunctional PBPs; Δ, deletion of a gene; the underlined GGATCC sequence represents the BamHI site; the **GCA** sequence in bold indicates the codon for alanine.

Table S4. Genomic mutations of *A. baumannii* CIP 70.10 mutants. Comparative genomic analysis of whole genome sequencing data was performed with a stringent quality threshold to verify gene inactivation and/or mutations.

ABCIP7010 locus	Position[a]	Read depth matrix[b]							Mutation	Mutation Type	Variant Type	Gene	Protein Product
		1a(S/A)	1b(S/A)	Δ2	1a(S/A) +1b(S/A)	1a(S/A) +Δ2	1b(S/A) +Δ2	1a(S/A) +1b(S/A) + Δ2					
3385	3597429	96	0	0	78	68	0	84	T>G	S459A	Complex[c]	*mrcA*	PBP1a
	3597431	96	0	0	78	68	0	84	T>A				
2471	2635740	0	54	0	64	0	74	46	T>G	S455A	Complex[c]	*mrcB*	PBP1b
	2635742	0	54	0	64	0	74	46	T>A				
0499	528612	0	0	54	0	72	68	66	T>G	I17S	SNP[d]		Gluconate permease
		Presence of in-frame deletion of *mrdA* gene in chromosome[e]											
1009	c1074099-1072111	–	–	+	–	+	+	+	Deletion of 1989 bp			*mrdA*	PBP2

[a] Position of mutation in the chromosome of *A. baumannii* CIP 70.10 (Accession number LN865143.1)
[b] Number of times each nucleotide in the genome was read
[c] Indicates two bases were mutated within the same codon
[d] Single nucleotide polymorphism
[e] (+) indicates in-frame deletion of *mrdA*, while (–) indicates no change
Abbreviations: PBP, penicillin-binding protein; 1a(S/A) and 1b(S/A), Ser to Ala substitution in the transpeptidase domain of PBP1a and PBP1b, respectively; Δ2, deletion of PBP2.

Table S5. The list of 20 muropeptide products generated from digestion of *A. baumannii* sacculus with mutanolysin.

Peaks[a]	Proposed muropeptide structure	Measured *m/z*[b] $[M + zH]^{z+}$	Theoretical *m/z* $[M + zH]^{z+}$	*z*[c]
1	Tri-Ala-Gly	999.4344	999.4364	1
Tetra	Tetra	942.4145	942.4150	1
2	Tri-Gly-Ala	999.4345	999.4364	1
3	Tri-Ala-Ala = Penta	1013.4504	1013.4521	1
TriTetra	TriTetra	897.8892	897.8911	2
4	TriPenta	933.4073	933.4097	2
Tetra2	Tetra2	933.4074	933.4097	2
5	TetraPenta	968.9255	968.9283	2
Tri2Tetra	Tri2Tetra	1324.0677 883.0475	1324.0712 883.0499	2 3
6	Tetra2*	923.3941	923.3966	2
TriTetra2	TriTetra2	1359.5851 906.7257	1359.5897 906.7289	2 3
7	Tetra2**	923.3939	923.3966	2
Tetra3	Tetra3	1395.1044 930.4051	1395.1083 930.4079	2 3
8	TetraPenta*	958.9123	958.9152	2
8	Tetra2Penta	1430.6229 954.0840	1430.6268 954.0870	2 3
TriTetraA	TriTetraA	887.8762	887.8780	2
TriTetraA'	TriTetraA'	887.8763	887.8780	2
9	TriPentaA	923.3941	923.3966	2
Tetra2A	Tetra2A	923.3939	923.3966	2
10	TriPentaA’	923.3943	923.3966	2

[a] Peaks labeled by number or by structure as shown Figure 2; [b] *m/z*, mass to charge ratio; [c] *z*, charge state.

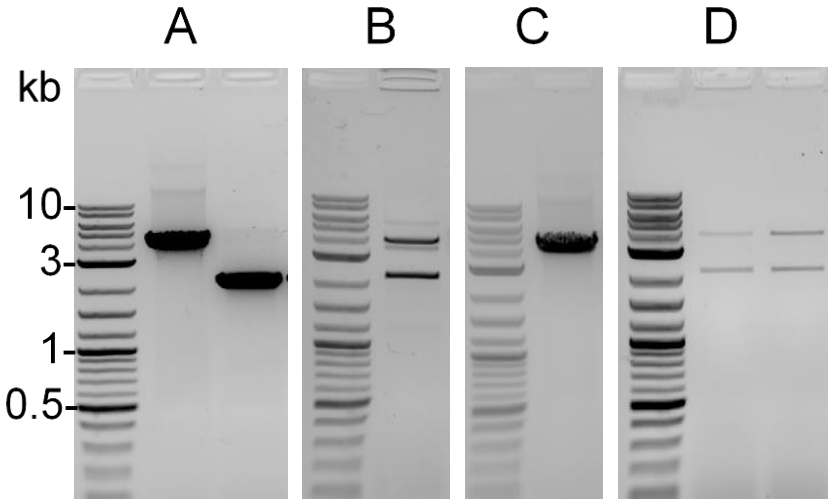

Figure S1. PCR fragments generated to verify integration and excision events during attempts to construct the *ftsI* gene deletion mutant of *A. baumannii* CIP 70.10. A. The size of PCR fragments (3961 bp and 2205 bp; see Table S3) confirms integration of the pMT310FW vector into the upstream homologous region of the chromosome (see Materials and Methods). B. The size of PCR fragments confirms excision from the chromosome generates both the *ftsI* gene deletion (2207 bp fragment) and the restoration of the wild-type chromosomal arrangement (4009 bp fragment). C. Analysis of hundreds of individual colonies obtained following selection on sucrose revealed the presence of only the wild-type chromosomal arrangement (4009 bp fragment). D. In a few colonies (two of them are shown), presence of both the wild type and the *ftsI* deletion mutant was observed. Following the subsequent round of sucrose selection, the DNA from all recovered colonies showed only the wild-type chromosomal arrangement as shown in panel C.

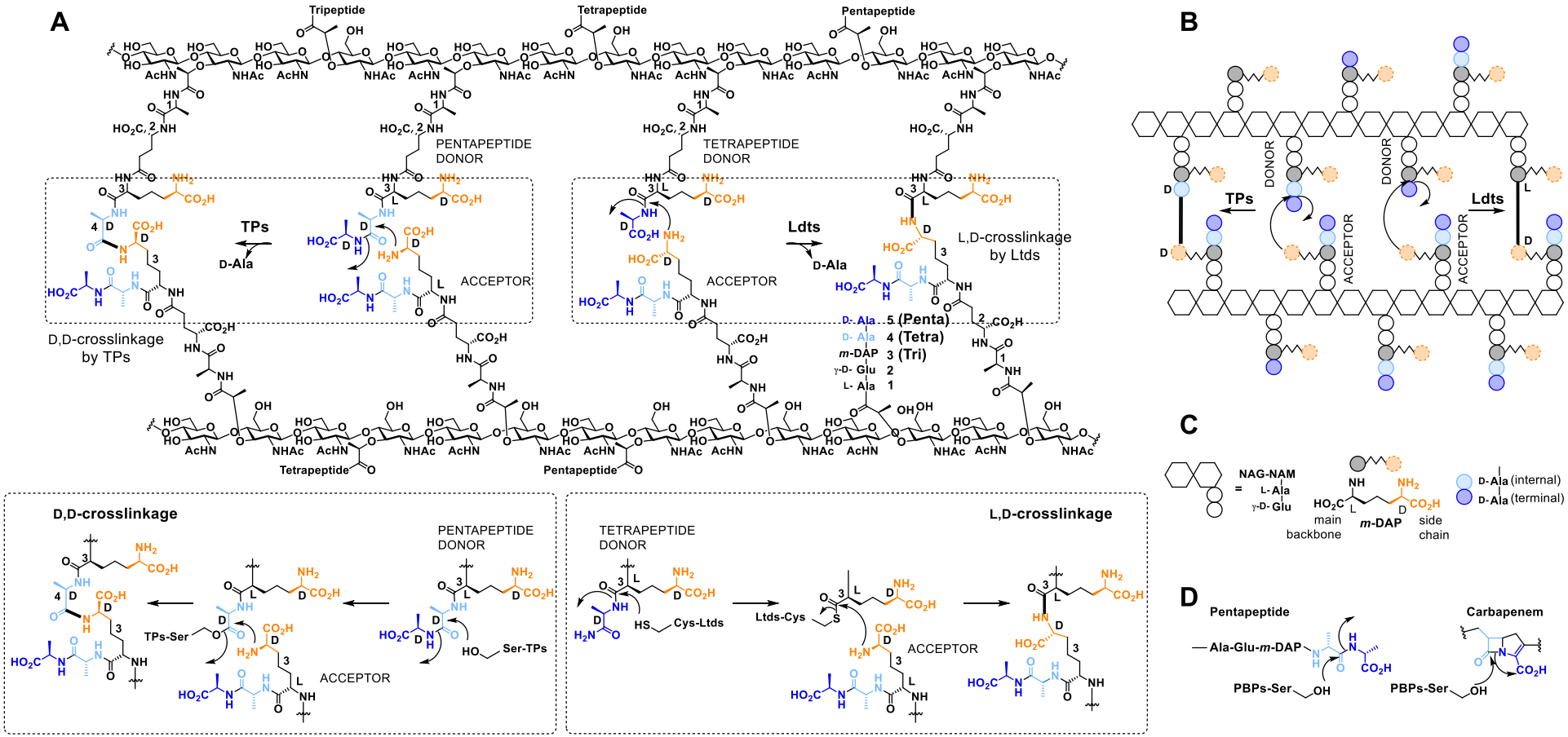

Figure S2. Peptidoglycan crosslinking. A. Crosslinking catalyzed by D,D-transpeptidases (TPs) and L,D-transpeptidases (Ldts). B. Cartoon representation of these reactions. C. Illustration of cartoon keys. D. Inhibition of a D,D-transpeptidase by a carbapenem.

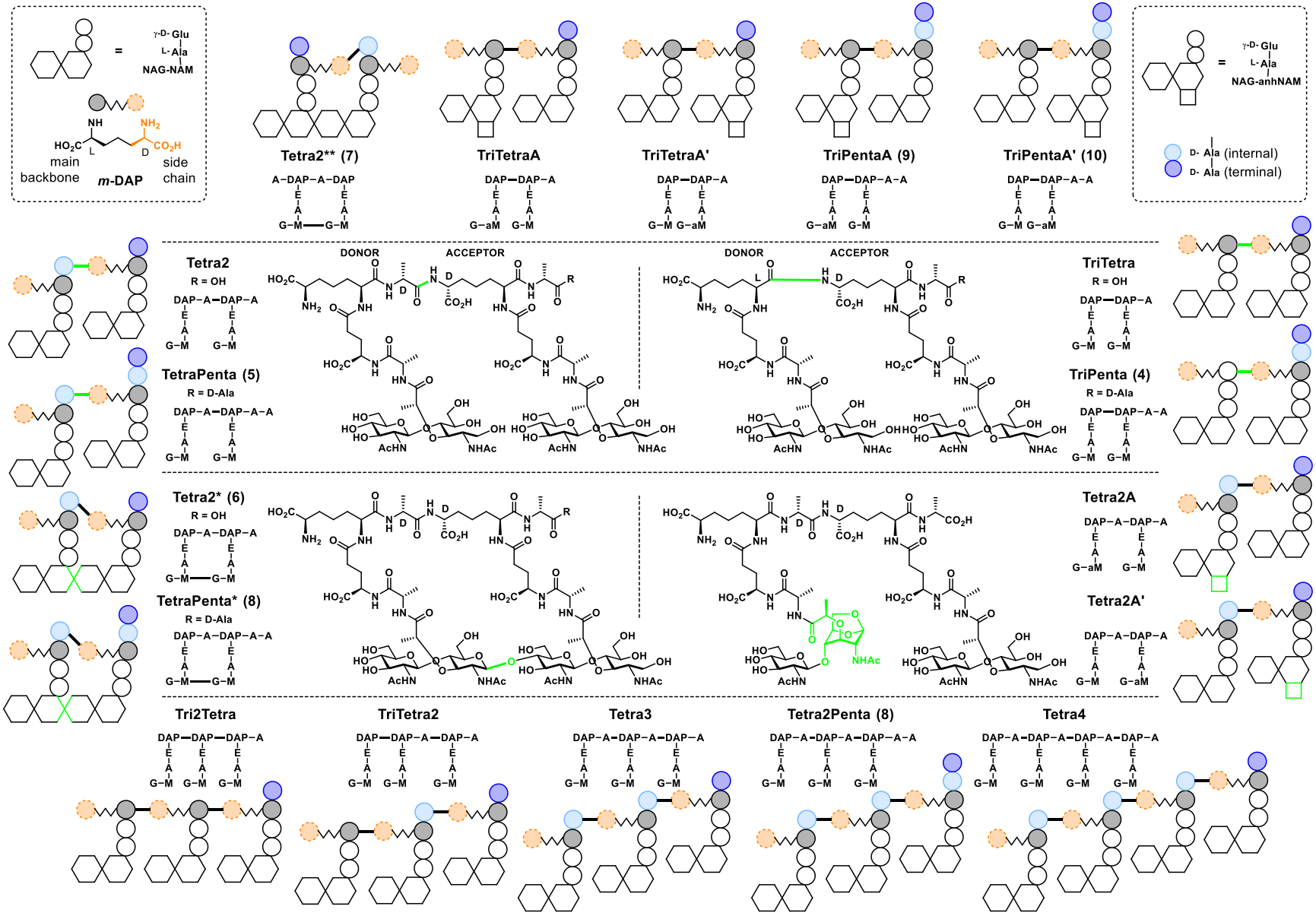

Figure S3. The proposed chemical structures and cartoon representation of crosslinked muropeptides indicated in Figure 2. The muramic acid at the reducing end was reduced to muramitol for simpler chromatograms. A distinctive feature in structures is colored in green. A = Ala, E = γ-D-Glu, DAP = *meso*-diaminopimelic acid, G-M = GlcNAc-MurNAc; G-aM = GlcNAc-1,6-anhydro-MurNAc. For muropeptides name's ending with A, one of G-M is replaced by G-aM.

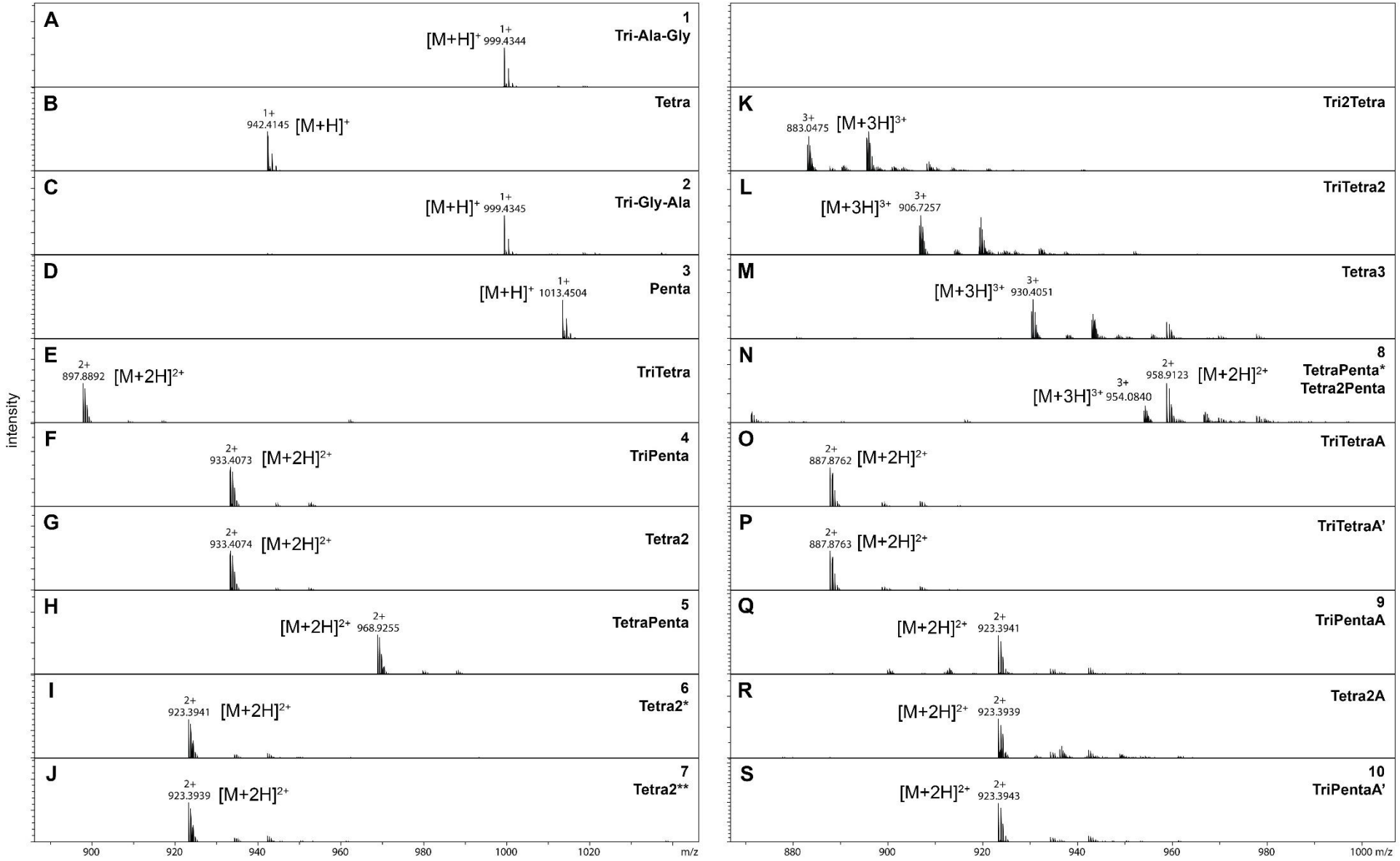

Figure S4. Mass spectra of 20 muropeptide products shown in Figure 2 and Table S5.

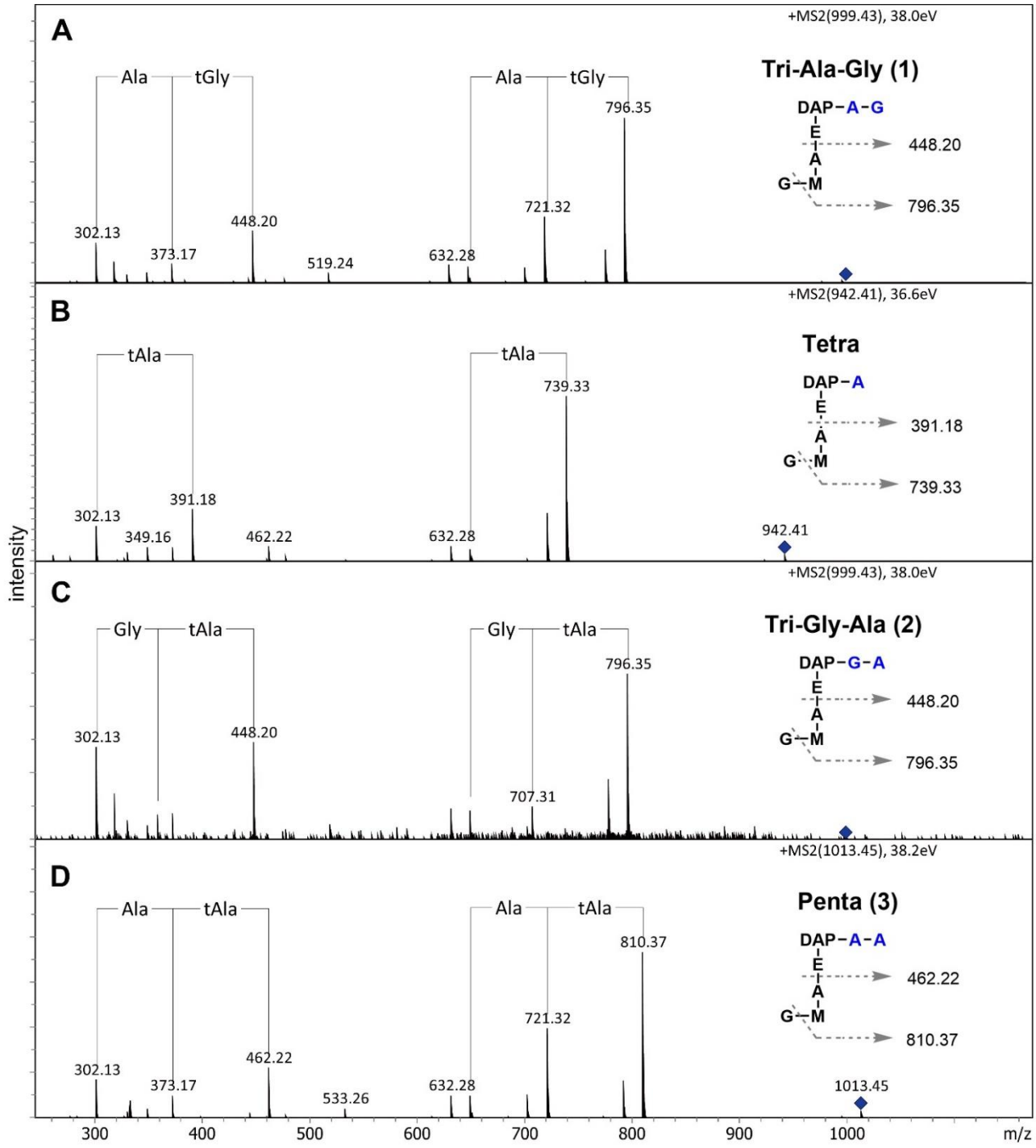

Figure S5. Collision induced dissociation (CID) mass spectra of protonated non-crosslinked muropeptides 1 (A), Tetra (B), 2 (C), and 3 (D). The fragment ion generated by the loss of dehydro NAG (203 Da) from the corresponding precursor ion is the most abundant ion. tAla represents a loss of terminal Ala (89.05 Da), while Ala indicates loss of internal Ala (71.04 Da). tGly represents a loss of terminal Gly (75.03 Da), while Gly indicates loss of internal Gly (57.03 Da). The peptide sequence of muropeptides was determined using signature ions generated by the concomitant loss of dehydroNAG and a terminal amino acid and/or an internal amino acid.

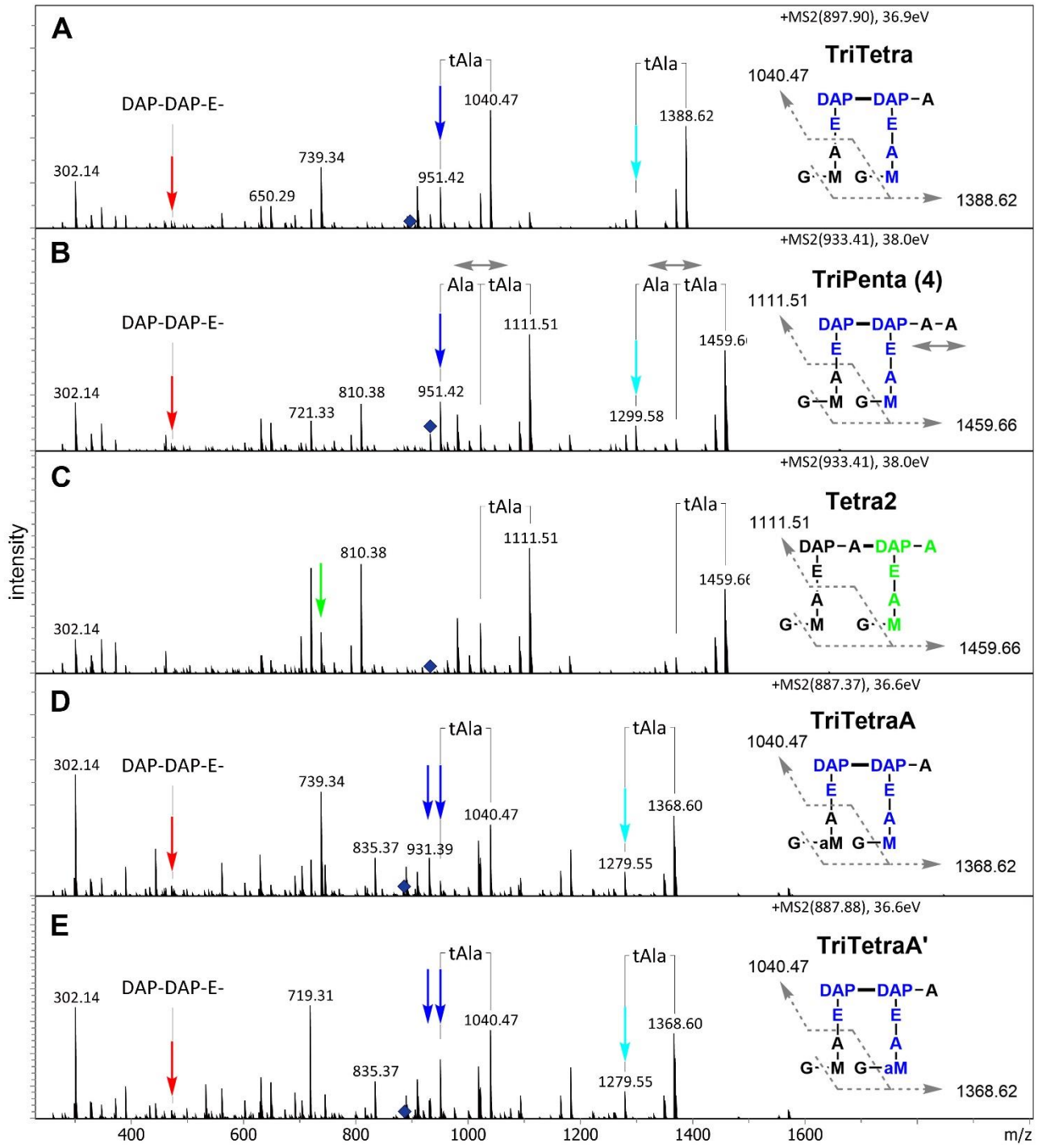

Figure S6. CID mass spectra of protonated L,D-crosslinked muropeptides TriTetra (A), 4 (B), TriTetraA (D), TriTetraA′ (E) and D,D-crosslinked muropeptide Tetra2 (C). The L,D-crosslink was confirmed by the presence of fragment ions only existing in the L,D-crosslink (not D,D-crosslink) – *m*/*z* 474.22 (DAP-DAP-E-, indicated with a red arrow), *m*/*z* 951.42 (-E-DAP-DAP-E-A-M-, indicated with a blue arrow) and/or *m*/*z* 931.38 (-E-DAP-DAP-E-A-aM-, indicated with a blue arrow), *m*/*z* 1299.58 (-M-A-E-DAP-DAP-E-A-M-, indicated with a cyan arrow) and/or *m*/*z* 1279.55 (-M-A-E-DAP-DAP-E-A-aM-, indicated with a cyan arrow). The peak at *m*/*z* 739.34 (-M-A-E-DAP-A, indicated with a green arrow) in panel (C) only exists from the precursor ion *m*/*z* 933.41 with a D,D-crosslink. The presence of -A-A was confirmed by the presence of fragment ions by the concomitant loss of Ala and tAla, indicated with a gray double arrow.

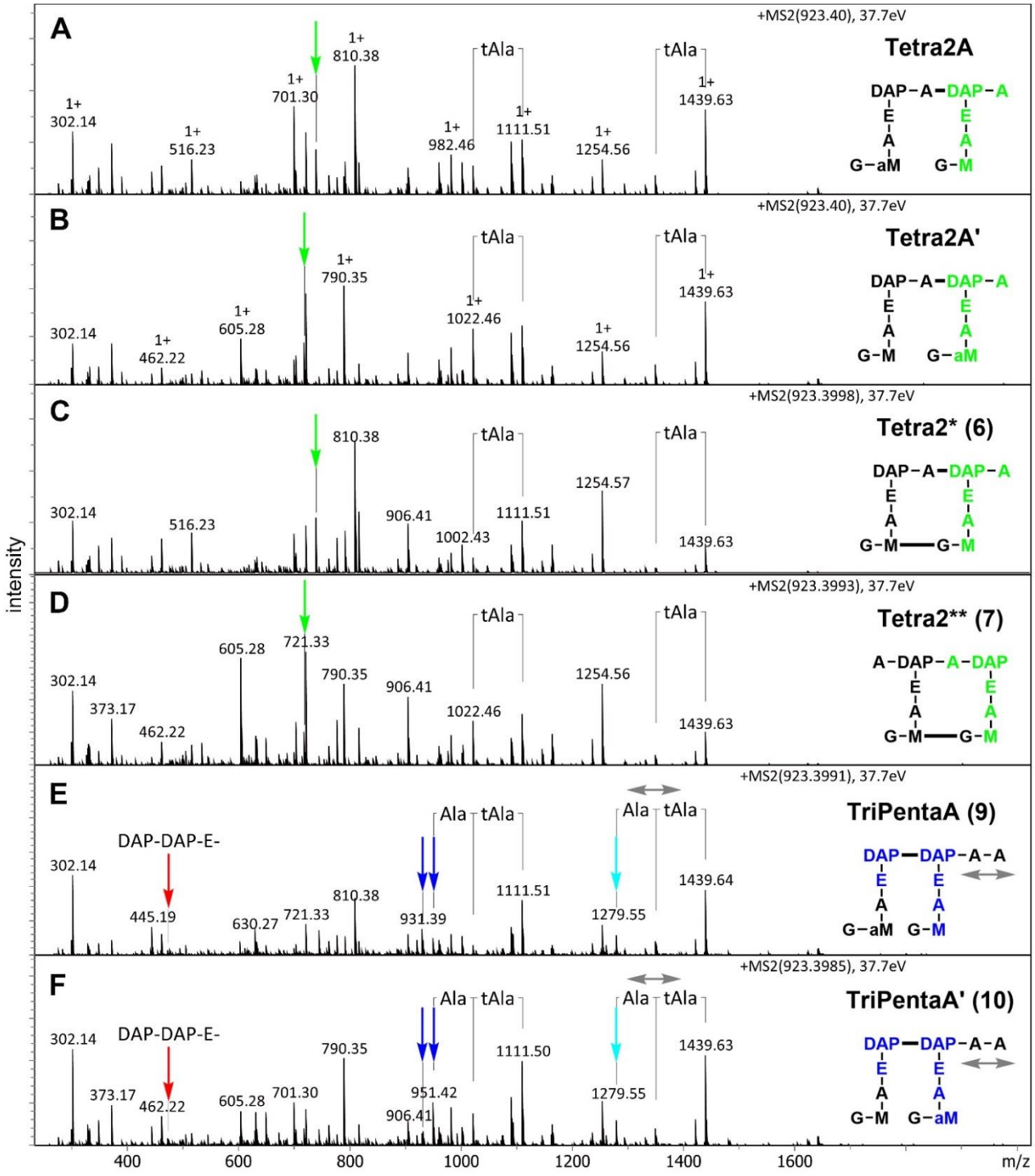

Figure S7. CID mass spectra of protonated crosslinked muropeptides with *m/z* 923.40. Peaks with green arrows only exist in structures with the D,D-crosslink, while peaks with blue, cyan, red and gray arrows support the structures with the L,D-crosslink as explained in Figure S6.

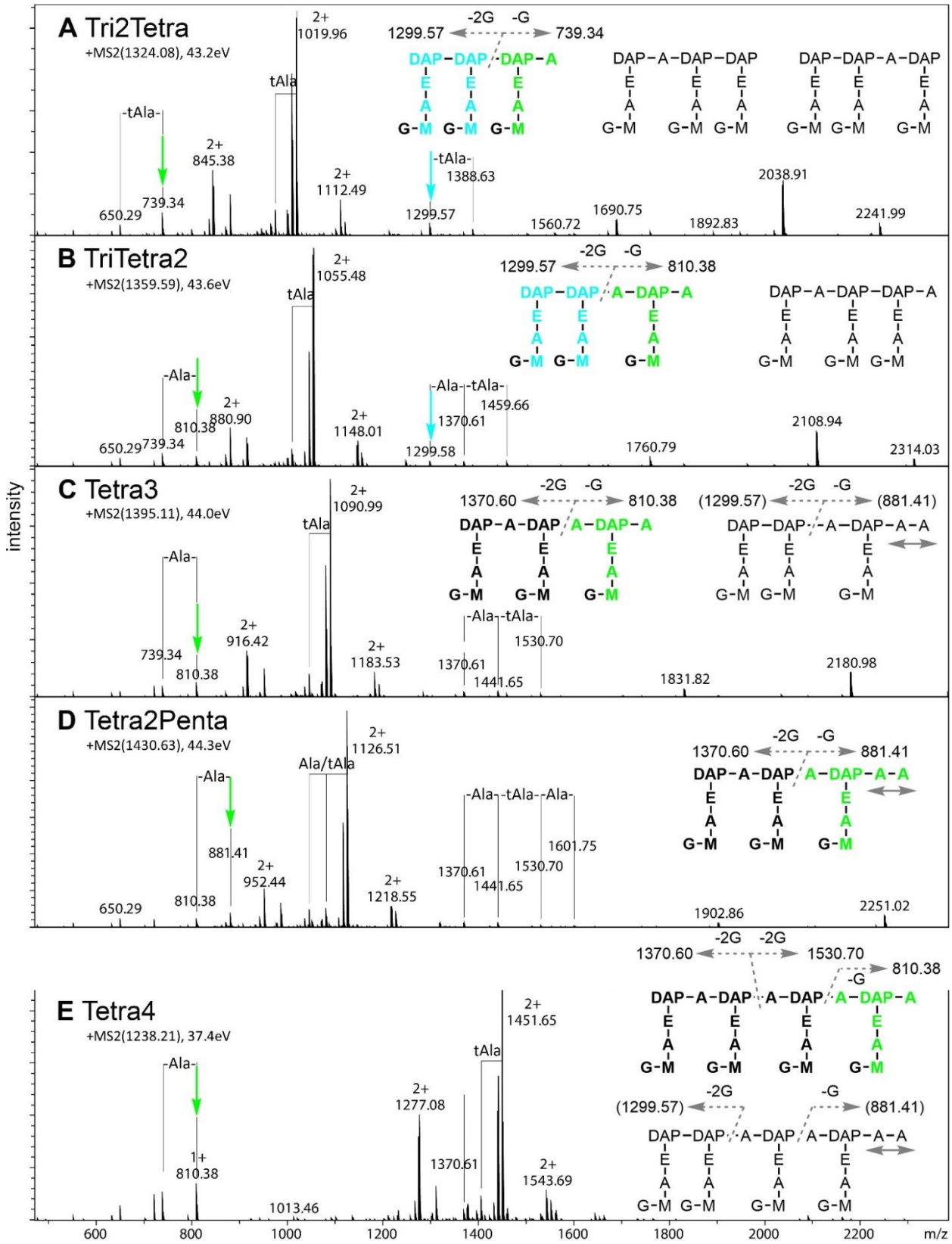

Figure S8. CID mass spectra of protonated crosslinked muropeptides (trimers and tetramer). All five muropeptides form fragment ions generated by a loss of a terminal Ala (tAla) at the acceptor strand of the corresponding crosslinked peptides. The structures were determined using the presence of signature ions (indicated with green arrows) existing only in the proposed structure and/or the absence of ions (*m*/*z* values in parentheses) existing only in alternative structures colored in dark gray.