SUPPLEMENTAL MATERIAL

Table S1 is a summary of the mass spectra of molecules that differ only on the muramyl residue. Reduced GlcNAc-MurNAc-pentapeptide has a calculated monoisotopic mass of 1012.4 and a predicted $[M+H]^+$ of 1013.4. Molecules having molecular ions with *m/z* values of 1013.0 produced daughter ions **B-K** which were identical to those observed from authentic reduced MurNAc-pentapeptide (Table S1). In addition, a daughter ion (**A**) was observed with a *m/z* value of 810.1, which corresponds to the fragment generated by the loss of the GlcNAc residue from reduced GlcNAc-MurNAc-pentapeptide, as well as the molecular ion of the reduced standard. Consistent with this, the three ions **F**, **G** and **H** were double cleavage ions resulting from the neutral loss of GlcNAc and the cleavage indicated in Figure 6 of the manuscript. Thus, the *m/z* value of 1013.0 represents the molecular ion $[M+H]^+$ of GlcNAc-MurNAc-pentapeptide as predicted. Molecules having molecular ions with a *m/z* value of 1029.0 gave rise to fragments **A**, **G** and **H,** all of which retain the muramyl residue that were 16 amu larger than those of the standard compound. The daughter ions containing the lactyl and/or amino acid residues (**B**, **C**, **D**, **E**, **I**, **J** and **K**) had *m/z* values that were the same as those seen for the MurNAc-pentapeptide standard. Therefore the difference in mass is located in the muramic acid moiety. The calculated monoisotopic mass of GlcNAc-MurNGlyc-pentapetide is 1028.4 (16 greater than GlcNAc-MurNAc-pentapeptide). Thus, the *m/z* value of 1029.0 likely represents the molecular ion ($[M+H]^+$) of GlcNAc-MurNGlyc-pentapeptide. Similarly the *m/z* value of 971 is 42 mass units smaller than that corresponding to GlcNAc-MurNAc-pentapeptide and the *m/z* values of fragments **A**, **F**, **G** and **H** are also 42 amu lower, while fragments **C**, **D**, **E**, **I**, and **K** remain unchanged, again suggesting a conversion of the muramyl residue from MurNac to muramic acid ($MurNH_2$). The loss of 42 mass units represents the absence of the *N*-acetyl group since the calculated monoisotopic mass of GlcNAc-$MurNH_2$-pentapeptide is 970.4 (42 less than the monoisotopic mass of GlcNAc-MurNAc-pentapeptide). Therefore the *m/z* value of 971.0 represents the molecular ion of GlcNAc-$MurNH_2$-pentapeptide.

Table S2 represents the molecules of GlcNAc-MurNAc-pentapeptide in which the carboxylic acid groups of the peptide moiety were amidated. In cases where one

carboxylic acid residue of GlcNAc-MurNAc-pentapeptide is amidated the calculated monoisotopic mass of the molecule would be reduced by one amu from 1012.4 to 1011.4. Amidation of a second carboxylic acid would reduce the calculated monoisotopic mass by another amu and thus the $[M+H]^+$ values for these molecules would be 1012 and 1011 respectively. The interpretations of the fragmentation patterns of a molecule having an *m/z* value of 1012.0 and two non-identical molecules both having *m/z* values of 1011.0 are as follows.

The molecular ion having an *m/z* value of 1012.0 indicates the loss of a single amu suggesting the amidation of the D-Glu, DAP or the terminal D-Ala residue. The observation that fragments **B**-**K** are all reduced by one amu indicates that the modification was located in the peptide moiety. The decrease in **D** indicates that the loss could have occurred in the D-Glu, DAP or the terminal D-Ala residue. The loss of one amu from fragment **E** rules out D-Glu as the modified residue and the loss of one amu from both **J** and **K** rules out the terminal D-ala residue. Therefore, the loss of mass must be due to the modification of the DAP residue.

From the data summarized in Table S2 it is clear that there are two molecular ions with *m/z* values of 1011.0 that are not identical. We have interpreted these two species as 1) GlcNAc-MurNAc-pentapeptide which has been amidated on the DAP and D-Glu residues and 2) GlcNAc-MurNAc-pentapeptide which has been amidated on the DAP and terminal D-Ala residues. For the molecule interpreted to be amidated on the DAP and D-Glu residues (Table S2 second to last row), the loss of two amu from fragments **B**, **C**, **D**, and **I** indicates that the mass loss is from the peptide not the sugar residues. Loss of one amu from fragment **E** indicates that only one of the DAP or terminal D-Ala residues can be amidated. The loss of two amu from fragments **G**, **H**, and **K** rule out the terminal D-Ala residue, and therefore the DAP and D-Glu residues must be the sites of amidation. For the other molecular ion having a *m/z* value of 1011.0 (Table S2 last row) the loss of two amu from fragments **C**, **D**, and **I** confirms a mass loss in the peptide. The loss of two amu from fragment **E** indicates that the mass change occurs in the terminal three amino acids and the loss of one amu from fragments **G**, **J**, and **K** localizes the loss of one amu to the terminal D-Ala residue, suggesting that the loss of the other amu is due to modification of the DAP residue. An ion with a *m/z* of 1010 was also observed in the

averaged spectra (manuscript Figure 5), suggesting that tri-amidated species also exist. However, MS^2 data was not able to confirm this assignment.

Molecular ions with *m/z* values and fragmentation patterns that corresponded to molecules having various combinations of muramyl residue and peptide amidations are presented in Table S3.

Table S4 represents the molecules of GlcNAc-MurNAc-pentapeptide in which the carboxylic acid groups of the peptide moiety were methylated.

If GlcNAc-MurNAc-pentapeptide was methylated on one or more carboxylic acid residue the calculated monoisotopic masses for the resulting molecules would be 1026.4, 1040.4 and 1054.4. Molecular ions $[M+H]^+$ were observed with *m/z* values of 1027.2 and 1055.3, suggesting that the lipid II molecules can be mono- and trimethylated. Interestingly, these molecules appeared to give weak fragmentation patterns especially for fragments **F**-**K**. However, sufficient fragments were identified to be able to assign the methyl group on the molecule having an *m/z* value of 1027.2 to the terminal D-Ala residue (Table S4). The fully methylated molecule (*m/z* 1055.0) showed strong daughter ions corresponding to fragments **A**-**E** and **K** but fragments **F**-**J** were not observed. A molecular ion of 1071.0 was also observed, which could be obtained from a GlcNAc-MurNGlyc-pentapeptide that had been methylated on all three carboxylic acids (1013 +16+14+14+14). This molecular ion produced appropriate daughter ions corresponding to fragments **A**-**D** and **J.** As further evidence of methylation, an ion with a *m/z* value of 999.2 was observed in bands 1-3 (manuscript Figure 5), which, when subjected to MS^2 generated daughter ions suggesting that the terminal D-Ala had lost 14 amu. Since an ion having *m/z* of 999.2 was not seen in samples that were not reduced, this suggests that the ion is the result of chemical reduction of a methyl ester bond by the $NaBH_4$, thus strengthening the evidence that the terminal D-Ala residue was methylated.

Table S1. Summary of tandem mass spectral data of molecular ions inferred to differ only with regard to the muramyl moiety.

Reduced muropeptides derived from lipid II isolated from *M. smegmatis* were subjected to LC-tandem mass spectrometry as described in Experimental Procedures. The relative abundance of the fragment ions varied from scan to scan, therefore and the data shown may represent a composite of several scans which were used to identify the daughter ions, see Figure 6 for fragmentation patterns.

	Molecular Ion $[M+H]^+$	**Daughter Ions**											**Interpretation**
		A	B	C	D	E	F	G	H	I	J	K	
MurNAc-pentapeptide (reduced)	810.3		605.2	533.3	462.2	333.2	478.2	650.1	721.1	444.2	302.2	373.1	
Muropeptides Derived From Mycobacterial Lipid	1,013.0	810.1	605.2	533.0	462.0	333.1	478.5	650.1	721.1	444.2	301.9	373.1	GlcNAc-MurNAc-pentapeptide (lipid II)
	1,029.0	826.0	605.2	533.1	462.1	333.0	N/D	666.0	737.1	N/D	302.1	373.1	GlcNAc-MurNGlyc-pentapeptide
	971.0	768.2	N/D	533.0	462.0	332.9	435.8	608.1	679.2	444.1	301.9	373.1	GlcNAc-MurNH_2-pentapeptide

Table S2. Summary of tandem mass spectral data of molecular ions inferred to differ only with regard to amidation of the peptide moiety. Reduced muropeptides derived from lipid II isolated from *M. smegmatis* were subjected to LC-tandem mass spectrometry as described in Experimental Procedures. The relative abundance of the fragment ions varied from scan to scan, therefore and the data shown may represent a composite of several scans which were used to identify the daughter ions, see Figure 6 for fragmentation patterns.

	Molecular ion $[M+H]^+$	**Daughter Ions**											**Interpretation**
		A	B	C	D	E	F	G	H	I	J	K	
GlcNAc-MurNAc-pentapeptide (from lipid II, reduced)	1,013.0	810.1	605.2	533.0	462.0	333.1	478.5	650.1	721.1	444.2	301.9	373.1	
Muropeptides Derived From Mycobacterial Lipid	1,012.0	809.1	604.1	532.1	461.0	332.0	N/D	649.0	720.2	443.1	N/D	372.1	GlcNAc-MurNAc-pentapeptide, DAP amidated
	1,011.0	808.1	602.9	531.1	460.0	331.8	N/D	648.1	719.1	442.0	300.3	371.1	GlcNAc-MurNAc-pentapeptide, DAP and D-Glu amidated
	1,011.0	808.1	N/D	531.0	460.0	331.0	N/D	649.1	N/D	442.0	301.0	372.1	GlcNAc-MurNAc-pentapeptide, DAP and D-Ala amidated

Table S3. Summary of tandem mass spectral data of molecular ions inferred to differ with regard to the muramyl moiety and amidation of the peptide.

Reduced muropeptides derived from lipid II isolated from *M. smegmatis* were subjected to LC-tandem mass spectrometry as described in Experimental Procedures. The relative abundance of the fragment ions varied from scan to scan, therefore and the data shown may represent a composite of several scans which were used to identify the daughter ions, see Figure 6 for fragmentation patterns.

	Molecular Ion $[M+H]^+$	**Daughter Ions**											**Interpretation**
		A	B	C	D	E	F	G	H	I	J	K	
GlcNAc-MurNAc-pentapeptide (from lipid II, reduced)	1,013.0	810.1	605.2	533.0	462.0	333.1	478.5	650.1	721.1	444.2	301.9	373.1	
Muropeptides Derived From Mycobacterial Lipid	1,028.0	825.1	604.0	532.2	461.2	332.0	N/D	664.9	736.1	443.1	301.0	372.1	GlcNAc-MurNGlyc-pentapeptide, DAP amidated
	1,027.0	824.1	603.0	531.0	460.0	332.0	N/D	664.0	735.1	442.1	300.0	371.0	GlcNAc-MurNGlyc-pentapeptide, D-Glu and DAP amidated
	970.0	767.2	N/D	532.1	461.2	331.9	436.1	607.11	678.1	N/D	N/D	372.1	GlcNAc-MurNH_2-pentapeptide, DAP amidated
	969.0	766.2	603.0	531.1	460.0	332.0	N/D	606.1	677.2	442.4	300.1	371.0	GlcNAc-MurNH_2-pentapeptide, DAP and D-Glu amidated

Table S4. Summary of tandem mass spectral data of molecular ions inferred to differ with regard to methylation of the peptide moiety. Reduced muropeptides derived from lipid II isolated from *M. smegmatis* were subjected to LC-tandem mass spectrometry as described in Experimental Procedures. The relative abundance of the fragment ions varied from scan to scan, therefore and the data shown may represent a composite of several scans which were used to identify the daughter ions, see Figure 6 for fragmentation patterns.

	Molecular Ion $[M+H]^+$	**Daughter Ions**											**Interpretation**
		A	B	C	D	E	F	G	H	I	J	K	
GlcNAc-MurNAc-pentapeptide (from lipid II, reduced)	1,013.0	810.1	605.2	533.0	462.0	333.1	478.5	650.1	721.1	444.2	301.9	373.1	
Muropeptides Derived From Mycobacterial Lipid	1,027.2	824.1	N/D	547.1	476.1	347.0	N/D	650.0	721.0	457.8	302.2	373.0	GlcNAc-MurNAc-pentapeptide, D-Ala methylated
	1,055.0	852.2	647.3	575.0	504.1	361.0	N/D	N/D	N/D	N/D	N/D	401.0	GlcNAc-MurNAc-pentapeptide, D-Glu, DAP and D-Ala methylated
	1,071.0	868.3	647.1	575.1	504.1	N/D	N/D	N/D	N/D	N/D	330.1	N/D	GlcNAc-MurNGlyc-pentapeptide, D-Glu, DAP and D-Ala methylated