

# Supporting Information

## Platform to discover protease-activated antibiotics and application to siderophore-antibiotic conjugates

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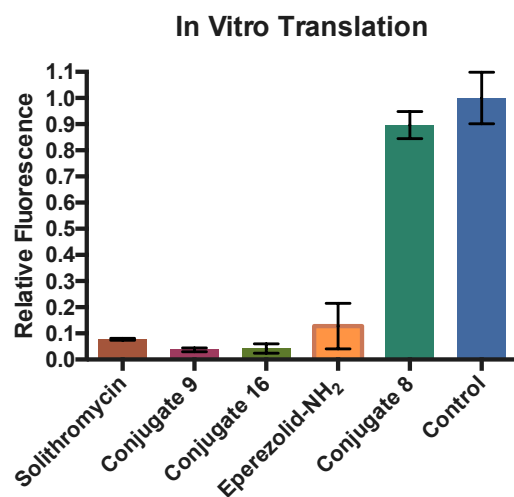
## Table of Contents

<b>I. SUPPLEMENTARY TABLES and FIGURES.....</b>	<b>S2</b>
<b>II. GENERAL INFORMATION.....</b>	<b>S18</b>
<b>III. EXPERIMENTAL PROCEDURES AND COMPOUND CHARACTERIZATION.....</b>	<b>S20</b>
<b>IV. MIC, PERIPLASMIC CLEAVAGE ASSAYS, AND CELL-FREE TRANSLATION.....</b>	<b>S73</b>
<b>V. SUBSTRATE PHAGE DISPLAY.....</b>	<b>S78</b>
<b>VI. REFERENCES CITED.....</b>	<b>S82</b>
<b>VII. SELECT NMR SPECTRA.....</b>	<b>S84</b>
<b>VIII. HPLC TRACES FOR CLEAVAGE IN PERIPLASMIC EXTRACT.....</b>	<b>S115</b>

## I: Supplementary Tables and Figures

**Table S1A.** Antimicrobial activity (MIC in  $\mu\text{M}$ ) of solithromycin conjugate **9** and derivatives thereof. ND = not determined.

Solithromycin Conjugates and Controls	Periplasmic Transport in Pathogenic Species (Cleavage Required)					Cytoplasmic Transport in <i>E. coli</i> (Cleavage not Required)			Poor Diffusion Through Cytoplasmic Membrane	
	<i>A. nosocomialis</i> pathogenic	<i>E. aerogenes</i> pathogenic	<i>K. pneumoniae</i> multidrug resistant	<i>S. typhi</i> pathogenic	<i>S. enterica</i> pathogenic	<i>E. coli</i> K12 wild type	<i>E. coli</i> $\Delta$ bamB $\Delta$ tolC efflux knockout	<i>E. coli</i> DCO wild type	<i>E. coli</i> $\Delta$ surA outer-membrane knockout	<i>S. aureus</i> Newman Gram-positive
Solithromycin ( <b>6</b> )	5	9	9	1	1	5	1	2	1	1
<i>L</i> -Linker conjugate ( <b>9</b> ) [cleavable]	7	7	13	7	7	3	0.4	7	>27	>27
<i>D</i> -Linker conjugate ( <b>16</b> ) [Non-cleavable]	>27	>27	>27	>27	>27	3	0.8	13	27	>27



**Figure S1A.** In vitro translation shows the ability of conjugates **8** (38  $\mu\text{M}$ ), **9** (10  $\mu\text{M}$ ), and **16** (10  $\mu\text{M}$ ) to inhibit the 70S *E. coli* ribosome relative to the parent antibiotics eperezolid-NH<sub>2</sub> (**5**, 38  $\mu\text{M}$ ) and solithromycin (**6**, 10  $\mu\text{M}$ ).

for their ribosomes, the data in Figure S1A would suggest that **9** and **16** have an equal ability to inhibit protein synthesis if they reached their targets in the cytoplasm, indicating that proteolysis in the periplasm is likely responsible for the activity differences in the pathogenic strains.

The *D*-linker analogue **16** was inactive (MIC > 27  $\mu\text{M}$ ) against ten pathogenic strains of Gram-negative species (see Table S1B on page S3). However, the *L*-linker variant **9** was active in five of these pathogenic strains (Table S1A), with activities similar to solithromycin (**6**) in *A. nosocomialis*, *E. aerogenes*, and *K. pneumoniae*, which is consistent with proteolytic activation of **9** in these pathogens. In contrast, the *D*-linker variant **16** was active in *E. coli*, with similar activity to its *L*-linker analogue **9**. These results were consistent with ribosomal inhibition by **9** and **16**, suggesting that cleavage was not required for these conjugates in *E. coli*. To investigate this possibility, we conducted an in vitro translation assay with *E. coli* ribosomes (Figure S1A), which showed that conjugates **9** and **16** inhibited translation comparable to solithromycin (**6**) at a concentration of 10  $\mu\text{M}$ . However, this discrepancy between *E. coli* and other Gram-negative species likely results from inherent differences in siderophore transport mechanisms (refer to reference 13 in the manuscript). Given the activity of both *D*- and *L*-linker variants in *E. coli*, it is likely that **9** and **16** are directly transported to the cytoplasm, enabling the conjugates to inhibit the ribosomes without cleavage. In the pathogenic species (*A. nosocomialis*, *E. aerogenes*, *K. pneumoniae*, *S. typhi*, and *S. enterica*), conjugates **9** and **16** are likely transported to the periplasm, without proceeding to the cytoplasm, thereby requiring linker cleavage for solithromycin to reach its ribosomal target. This scenario is also supported by the fact that **9** and **16** do not readily diffuse through the cytoplasmic membrane since they are not active against *E. coli*  $\Delta$ surA, a mutant that lacks outer-membrane proteins (Table S1A). Indeed, diffusion of either **9** or **16** through the inner membrane in *E. coli*  $\Delta$ surA would lead to ribosomal inhibition. We also rule-out premature release of solithromycin prior to conjugate uptake (see Table S3B).

Despite structural differences between ribosomes in different species that may contribute to activity variations, solithromycin inhibits protein synthesis in many Gram-negative species, suggesting that activity would be observed upon release of the warhead from an inactive uncleaved conjugate. Although we cannot rule-out that the activity differences observed for conjugates **9** and **16** in pathogenic strains may be due to different affinities






## Full MIC Table

Minimum Inhibitory Concentration (μM)	Gram-Negative Strains														
	<i>E. coli</i> K12 MG1655 wild type	<i>E. coli</i> DCO wild type	<i>E. coli</i> Δ <i>bamB</i> Δ <i>tolC</i> BW25113 <sup>a</sup> efflux-knockout	<i>A. baumannii</i> ATCC BAA-1797 multidrug resistant	<i>A. nosocomialis</i> M2 pathogenic	<i>E. aerogenes</i> ATCC 13048 pathogenic	<i>K. pneumoniae</i> MGH 78578 multidrug resistant	<i>S. typhi</i> ATCC 700831 pathogenic	<i>S. enterica</i> 14028s pathogenic	<i>E. cloacae</i> ATCC13047 multidrug resistant	<i>P. aeruginosa</i> ATCC 10145 multidrug resistant <sup>f</sup>	<i>P. aeruginosa</i> PA01 pathogenic	<i>P. aeruginosa</i> PA14 multidrug resistant	<i>E. coli</i> Δ <i>surA</i> <sup>d</sup> outer-membrane knockout	<i>S. aureus</i> Newman Gram-positive
<b>Conjugates and Controls</b>															
Daptomycin (4)	>39	>39	>39	>39	>39	>39	>39	>39	>39	>39	>39	>39	>39	0.6	0.6
<i>L</i> -Linker Daptomycin Conjugate (7)	11	21	11	5	1	>21	>21	>21	>21	>21	>21	21	>21	>21	>21
<i>D</i> -Linker Daptomycin Conjugate (13)	>23	>23	23	23	11	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	>23	23	>23
Conjugate Without Antibiotic, Acid (11)	>48	>48	48	>24	>48	>48	ND <sup>a</sup>	>48	ND <sup>a</sup>	>48	>48	>48	>48	>48	>48
Conjugate Without Antibiotic, Ester (12)	>48	ND <sup>a</sup>	24	>24	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	>48	ND <sup>a</sup>	ND <sup>a</sup>	>48	>24	>48	48	>48
Eperezolid-NH <sub>2</sub> (5)	>171	>171	>171	>171	>171	ND <sup>a</sup>	ND <sup>a</sup>	>171	ND <sup>a</sup>	>171	>171	>171	>171	48	48
<i>Ent</i> -Eperezolid-NH <sub>2</sub>	>171	>171	>171	>171	>171	>171	ND <sup>a</sup>	>171	ND <sup>a</sup>	>171	>171	>171	>171	>171	>171
Eperezolid-OH	170	>170	5	>170	>170	ND <sup>a</sup>	ND <sup>a</sup>	>170	ND <sup>a</sup>	>170	>170	>170	>170	3	5
<i>Ent</i> -Eperezolid-OH	>170	>170	>170	>170	>170	ND <sup>a</sup>	ND <sup>a</sup>	>170	ND <sup>a</sup>	>170	>170	>170	>170	170	>170
<i>L</i> -Linker Eperezolid-NH <sub>2</sub> Conjugate (8)	>38	>38	1	>38	>38	ND <sup>a</sup>	ND <sup>a</sup>	>38	ND <sup>a</sup>	ND <sup>a</sup>	>38	>38	>38	38	>38
<i>D</i> -Linker Eperezolid-NH <sub>2</sub> Conjugate (14)	>38	>38	19	>38	>38	>38	ND <sup>a</sup>	>38	ND <sup>a</sup>	>38	>38	>38	>38	>38	>38
Conjugate With Inactive Enantiomer (15)	>38	>38	9	>19	>38	ND <sup>a</sup>	ND <sup>a</sup>	>38	ND <sup>a</sup>	ND <sup>a</sup>	>38	>38	>38	>38	>38
Conjugate With WSWG Linker (17)	>47	ND <sup>a</sup>	37	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>
Conjugate Without Siderophore (18)	>77	ND <sup>a</sup>	>77 <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	>77	ND <sup>a</sup>	ND <sup>a</sup>	>77	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	>77
Solothromycin (6)	4	2	1	5	5	9	9	1	1	9	19	38	19	1	1
<i>L</i> -Linker Solothromycin Conjugate (9)	3	7	0.4	>27	7	7	13	7	7	>27	>27	>27	>27	>27	>27
<i>L</i> -Linker Solothromycin Conjugate Epimer (S27A) <sup>f</sup>	3	13	0.8	>27	>27	13	13	13	13	27	>27	>27	>27	24	>27
<i>D</i> -Linker Solothromycin Conjugate (16)	3	13	0.8	>27	>27	>27	>27	>27	>27	>27	>27	>27	>27	27	>27
<i>D</i> -Linker Solothromycin Conjugate Epimer (S27B) <sup>g</sup>	3	7	0.8	>27	>27	13	>27	>27	>27	>27	>27	>27	>27	13	>27

MIC Color Scale    1    3    5    11    19    23    48    >48

**Table S1B.** MICs for conjugates and controls in 14 Gram-negative strains and one Gram-positive strain. All MICs were evaluated in biological duplicate and technical triplicate. All MIC assays were conducted in Mueller-Hinton-II (MH-II) broth with DP (600 μM for *P. aeruginosa* and 200 μM for all other strains). <sup>a</sup>ND = Not Determined. <sup>b</sup>*E. coli* Δ*bamB*Δ*tolC* is an efflux knockout and lacks the lipopeptide BamB, which results in a functional, albeit less efficient, BamACDE protein complex for outer-membrane protein (OMP) assembly. <sup>c</sup>Sewell, A.; Dunmire, J.; Rowe, T.; Bouhenni, R. *Mol Vis.* **2014**, *20*, 1182–1191. <sup>d</sup>*E. coli* Δ*surA* is an outer-membrane knockout that lacks the chaperone SurA, which compromises the primary pathway of OMP assembly. <sup>e</sup>Evaluated in *E. coli* Δ*tolC*. <sup>f,g</sup>During the coupling reaction to attach solithromycin to the WSPKYM linker, racemization resulted in an epimerized adduct for both the *L*-linker and *D*-linker conjugates, **S27A** and **S27B** respectively. In addition to differences in activity between the *L*- and *D*-linker solithromycin conjugates **9** and **16**, the activities of the diastereomers **S27A** and **S27B** further supported that proteolysis of solithromycin conjugate **9** was responsible for its activity in five pathogenic strains. abbreviations: DP=dipyridyl (iron scavenger), *Ent*-eperezolid = enantiomer of eperezolid (inactive molecule).

### Potential for Scarless Cleavage in the Bacterial Periplasm

Strain <sup>b</sup>	L-Linker Eperezolid-NH <sub>2</sub> Conjugate (8) [MIC (μM)] <sup>c</sup>	% Eperezolid-NH <sub>2</sub> (5) release in Periplasmic Extract <sup>d</sup>	%Error <sup>e</sup>
<i>E. coli</i> K12 MG1655	>38		3.8 ± 0.4
<i>E. coli</i> DCO	>38		6.8 ± 1.3
<i>E. coli</i> Δ <i>bamB</i> Δ <i>tolC</i> BW25113	1		33.9 ± 1.5
<i>A. baumannii</i> ATCC BAA-1797	>19		30 ± 8.4
<i>A. nosocomialis</i> M2	>38		3.1 ± 2.1
<i>P. aeruginosa</i> PA01	>38		ND <sup>a</sup> ND <sup>a</sup>
<i>P. aeruginosa</i> ATCC 10145	>38		ND <sup>a</sup> ND <sup>a</sup>
<i>E. cloacae</i> ATCC 13047	ND <sup>a</sup>		ND <sup>a</sup> ND <sup>a</sup>
<i>S. enterica</i> 14028s	ND <sup>a</sup>		ND <sup>a</sup> ND <sup>a</sup>
<i>E. aerogenes</i> ATCC 13048	ND <sup>a</sup>		ND <sup>a</sup> ND <sup>a</sup>

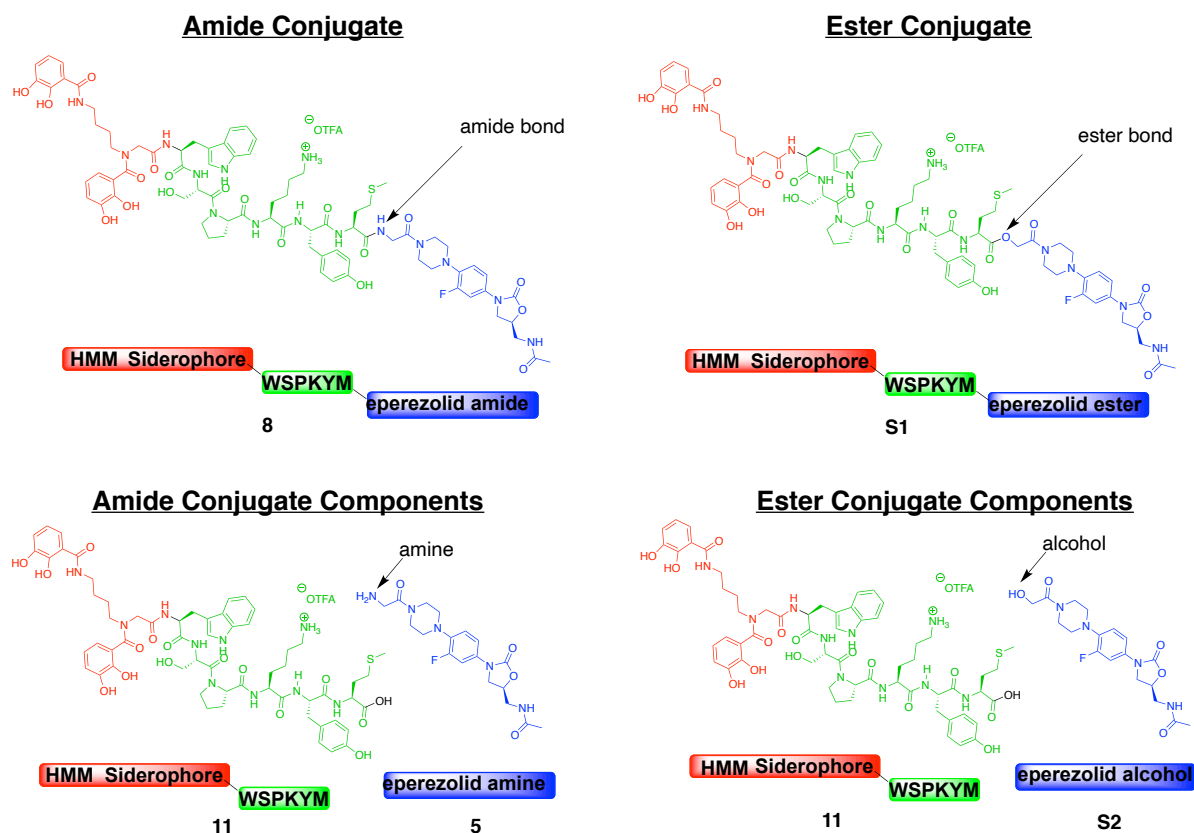
MIC Color Scale: 1 (green), 3 (light green), 5 (yellow-green), 11 (yellow), 19 (orange), 23 (red-orange), 48 (red), >48 (dark red)

**Table S2.** <sup>a</sup>ND = Not Determined. <sup>b</sup>The periplasmic extract was isolated by osmotic shock from each strain grown in LB media and in MH-II media. <sup>c</sup>The MICs were evaluated in biological triplicate. <sup>d</sup>The release of eperezolid-NH<sub>2</sub> (5) was monitored by HPLC after 11 h of incubating substrate and periplasmic extract at 37 °C with mixing at 1050 rpm. The antibiotic peak was confirmed by MS and by retention time comparison to a standard at a concentration corresponding to the theoretical yield of eperezolid-NH<sub>2</sub> release. The following equation was used to calculate the % yield: area of antibiotic peak/area of standard, which was then averaged over two biological replicates. <sup>e</sup>The error was calculated from the following equation: standard deviation/sqrt(# of replicates). Please refer to HPLC data on pages S133-136 for analysis of scarless eperezolid-NH<sub>2</sub> (5) release in the periplasmic extract of five strains listed in Table S2.

For analysis of daptomycin release from conjugate 7 in the periplasmic extract of all 10 strains listed in Table S2, please refer to HPLC data on pages S115-S134.

**Discussion of Table S2:** The MICs for conjugate 8 are compared to the % eperezolid-NH<sub>2</sub> release in periplasmic extract. **A measurable % yield confirms the potential for scarless antibiotic release in the bacterial periplasm of the corresponding strain.** The % yield (% scarless cleavage) in periplasmic extract may not reflect the extent of non-scarless cleavage or the amount of antibiotic released in the periplasm of the live bacterial cell. The best yield for the scarless antibiotic release of eperezolid-NH<sub>2</sub> (5) from conjugate 8 was observed in the extract for *E. coli* Δ*bamB*Δ*tolC*, in which the conjugate was most active. Eperezolid-NH<sub>2</sub> is an efflux substrate in Gram-negative bacteria, which may rationalize the low correlations of cleavage and activity for 8 in wild-type strains.

## Oxazolidinones As Efflux Substrates in Gram-Negative Bacteria, and Eperezolid-OH Permeates the OM, Unlike Eperezolid-NH<sub>2</sub>



**Figure S1B.** Comparison of the cleaved components resulting from proteolysis of eperezolid amide conjugate **8** and hydrolysis of eperezolid ester conjugate **S1**.

**Discussion of Figure S1.** Although some Gram-positive antibiotics are not active in *E. coli* because they cannot penetrate the outer membrane, many are small enough to pass through porins but are prone to efflux as evidenced by their activity against efflux-pump knockouts.<sup>S1</sup> Indeed, many oxazolidinones, like the alcohol analog of **5**, eperezolid-OH (**S2**), are known efflux substrates in Gram-negative pathogens,<sup>S2</sup> and are only active in efflux-knockouts of *E. coli* (Table S1B). Eperezolid-NH<sub>2</sub> (**5**) is also an efflux substrate as evidenced by the exclusive activity of conjugate **8** in *E. coli*  $\Delta$ *bamB* $\Delta$ *tolC*. Conjugate **S1** required rapid assay evaluation following its synthesis due to its tendency to hydrolyze in solution.

## Siderophore-Mediated Transport Is Supported by MIC Data at Variable Concentrations of Dipyridyl (DP)

Minimum Inhibitory Concentration ( $\mu\text{M}$ )	<i>E. coli</i> $\Delta\text{bamB}\Delta\text{tolC}$ no DP	<i>E. coli</i> $\Delta\text{bamB}\Delta\text{tolC}$ 129 $\mu\text{M}$ DP	<i>E. coli</i> $\Delta\text{bamB}\Delta\text{tolC}$ 200 $\mu\text{M}$ DP
<b>SACs (Depend On Fe-concentration)</b>			
L-Linker Eperezolid Amide Conjugate ( <b>8</b> )	19	5	1
Eperezolid Ester Conjugate ( <b>S1</b> )	ND <sup>a</sup>	5	0.6
Conjugate With Inactive Enantiomer ( <b>15</b> )	>38	ND <sup>a</sup>	9
<b>Controls (not dependent on Fe-conc.)</b>			
polymyxin B	0.4	0.7	0.4
Eperezolid-OH	5	5	5
Ent-Eperezolid-OH	>170	>170	>170
Eperezolid-NH <sub>2</sub> ( <b>5</b> )	>171	>171	>171
Ent-Eperezolid-NH <sub>2</sub>	>171	>171	>171



**Table S3A.** <sup>a</sup>ND = Not Determined. The MICs of siderophore conjugates **8**, **15**, and **S1** at different concentrations of DP are consistent with siderophore-mediated transport. A concentration of 200  $\mu\text{M}$  DP proved optimal for siderophore transport of all three conjugates. Although not shown in this Table, 600  $\mu\text{M}$  DP was determined to be optimal for siderophore uptake in *P. aeruginosa*; other species did not grow at this concentration. Each MIC is the result of at least two biological replicates, and each replicate was evaluated in triplicate. abbreviations: DP=dipyridyl (iron scavenger), Ent-eperezolid = enantiomer of eperezolid (inactive molecule).

**Discussion of Table S3A:** The MICs of the controls do not vary significantly with DP concentration, while the siderophore conjugates become more active with increasing levels of DP. This phenomenon can be explained by the enhanced expression of outer-membrane transport proteins for siderophore uptake in iron-deficient media.

Upon hydrolysis, the ester conjugate **S1** releases eperezolid-OH (**S2**). The amide conjugate **8** releases eperezolid-NH<sub>2</sub> (**5**) following proteolysis. The similar MICs obtained for both ester conjugate **S1** and amide conjugate **8** in *E. coli*  $\Delta\text{bamB}\Delta\text{tolC}$  suggest that they may proceed by a similar mechanism of action *via* conjugate uptake and proteolytic cleavage. For consistent replicates, the MICs of conjugate **S1** required immediate evaluation after solubilizing in DMSO due to its hydrolytic instability.

Solithromycin Conjugates and Controls	<i>A. nosocomialis</i> pathogenic	<i>S. typhi</i> pathogenic	<i>S. enterica</i> pathogenic	<i>E. aerogenes</i> pathogenic	<i>K. pneumoniae</i> pathogenic	<i>E. coli</i> K12 wild type		
	<b>200 <math>\mu</math>M Dipyriddy</b>							
<i>L</i> -Linker Solithromycin Conjugate ( <b>9</b> )	7	7	7	7	13	3		
<i>D</i> -Linker Solithromycin Conjugate ( <b>16</b> )	>27	>27	>27	>27	>27	3		
Solithromycin ( <b>6</b> )	5	1	1	9	9	5		
<b>0 <math>\mu</math>M Dipyriddy</b>								
<i>L</i> -Linker Solithromycin Conjugate ( <b>9</b> )	>27	>27	>27	>27	>27	>27		
Solithromycin ( <b>6</b> )	5	1	1	19	28	5		
MIC Color Scale								
	1	3	5	11	19	23	48	>48

**Table S3B.** To determine that premature cleavage was not largely responsible for the activity of solithromycin conjugate **9** in pathogenic *A. nosocomialis*, *S. typhi*, *S. enterica*, *E. aerogenes*, and *K. pneumoniae*, we tested the MIC of the *L*-linker conjugate **9** at 0  $\mu$ M DP to prevent siderophore transport, which revealed that the conjugate was not active at this DP concentration. This result confirmed that premature cleavage was not largely responsible for the activity of conjugate **9**, as release of solithromycin in the extracellular medium would result in some activity. The equal activities of the *L*- and *D*-linker solithromycin conjugates **9** and **16** in *E. coli* suggest the possibility of bacterial-growth inhibition without cleavage. To test this hypothesis, we showed that conjugate **9** and its *D*-linker analogue **16** inhibit in-vitro translation of the *E. coli* ribosome (Table 5 in manuscript). The *L*- and *D*-linker solithromycin conjugates **9** and **16** may also inhibit translation in the five pathogenic strains if they had equal access to the target. This possibility is supported by solithromycin's ability to inhibit the growth of these pathogenic strains. In the five pathogenic strains, periplasmic cleavage of **9** may be responsible for its activity for the following reasons: 1) Since the *D*-linker solithromycin conjugate **16** is not active in the pathogenic strains, it may not be reaching its ribosomal target in the cytoplasm. 2) Proteolytic cleavage is responsible for the activity in these strains based on large-activity differences between the *D*- and *L*-linker conjugates. 3) The *D*- and *L*-linker conjugates **9** and **16** may not passively diffuse through the inner membrane due to low activity in *E. coli*  $\Delta$ *surA* and *S. aureus* Newman (Table S1B). There may be a cooperative effect between DP and solithromycin (**6**) in *E. aerogenes* (~2-fold activity increase at 200  $\mu$ M DP) and *K. pneumoniae* (~3-fold activity increase at 200  $\mu$ M DP).

SACs (Depend On Fe-concentration)	<i>E. coli</i> K12 MG1655 wild type 0 $\mu$ M DP	<i>E. coli</i> K12 MG1655 wild type 10 $\mu$ M DP	<i>E. coli</i> K12 MG1655 wild type 20 $\mu$ M DP	<i>E. coli</i> K12 MG1655 wild type 40 $\mu$ M DP	<i>E. coli</i> K12 MG1655 wild type 80 $\mu$ M DP	<i>E. coli</i> K12 MG1655 wild type 120 $\mu$ M DP	<i>E. coli</i> K12 MG1655 wild type 160 $\mu$ M DP	<i>E. coli</i> K12 MG1655 wild type 200 $\mu$ M DP	<i>E. coli</i> K12 MG1655 wild type 250 $\mu$ M DP
<i>L</i> -Linker Solithromycin Conjugate ( <b>9</b> )	>27	>27	>27	>27	>27	>27	2	0.8	0.8
<i>D</i> -Linker Solithromycin Conjugate ( <b>16</b> )	27	27	27	27	13	13	0.8	0.8	0.8
<b>Controls (not dependent on Fe-conc.)</b>									
solithromycin	5	10	5	5	5	5	5	5	5
MIC Color Scale									
	1	3	5	11	19	23	48	>48	

**Table S3C.** Siderophore transport is activated between 120-160  $\mu$ M DP in *E. coli* K12. MICs at various DP concentrations show no cooperativity between solithromycin and DP in this strain. An optimal concentration of DP for siderophore uptake was determined to be 200  $\mu$ M for the solithromycin conjugate. The equal activities of **9** and **16** in *E. coli* suggest that the conjugate is inhibiting translation without cleavage. However, we cannot rule-out the possibility of cleavage in *E. coli* despite the ability of the whole conjugate to inhibit translation (Table 5 of manuscript), and the proteolysis of **9** is required for its activity in several pathogenic strains (see **Table S3B**).

## Oxazolidinone Conjugate Activities in *E. coli* $\Delta bamB\Delta tolC$ May be Explained by Linker Proteolysis in the Cytoplasm

MIC ( $\mu$ M)	<i>E. coli</i> $\Delta bamB\Delta tolC$ BW25113 efflux-knockout	<i>E. coli</i> $\Delta surA$ BW2113 outer-membrane knockout	<i>S. aureus</i> Newman Gram-positive
<b>Conjugate Components</b>			
Siderophore-Linker-OH ( <b>11</b> )	48	>48	>48
Eperezolid-OH ( <b>S2</b> )	5	3	5
Eperezolid-NH <sub>2</sub> ( <b>5</b> )	>163	41	41
<b>MIC Color Scale</b>	1	3	5

MIC ( $\mu$ M)	<i>E. coli</i> $\Delta bamB\Delta tolC$ BW25113 efflux-knockout
<b>Conjugates</b>	
L-Linker Eperezolid Ester Conjugate ( <b>S1</b> )	0.6
L-Linker Eperezolid Amide Conjugate ( <b>8</b> )	1

MIC ( $\mu$ M)	<i>E. coli</i> $\Delta bamB\Delta tolC$ BW25113 efflux-knockout
<b>Combination of the Conjugate Components for S34 in Equal Concentrations</b>	
Siderophore-Linker-OH ( <b>11</b> ) + Eperezolid-OH ( <b>S2</b> )	2

MIC ( $\mu$ M)	11	19	23	48	>48			
<b>MIC Color Scale</b>	1	3	5	11	19	23	48	>48

**Table S4.** The combination of cytoplasmic cleavage and synergy or coalism may explain the activity of oxazolidinone conjugates in *E. coli*  $\Delta bamB\Delta tolC$ .

**Discussion of Table S4.** Synergy or coalism between the cleaved conjugate components following cytoplasmic transport and intracellular hydrolysis may explain the enhanced activity of ester conjugate **S1**, which is significantly more active than its cleaved components (eperezolid-OH (**S2**) and Siderophore-Linker-OH **11**, >8-fold and 80-fold, respectively) in *E. coli*  $\Delta bamB\Delta tolC$ . To investigate the possibility of synergy or coalism, we combined eperezolid-OH and the siderophore-linker component **11** in equimolar quantities, which led to a 2-fold improvement in activity (MIC = 2  $\mu$ M). However, the improvement remains >3-fold less active than conjugate **S1** (0.6  $\mu$ M vs. 2  $\mu$ M), and the activity is similar to that of eperezolid-OH (**S2**) in an outer-membrane knockout of *E. coli* (MIC = 3  $\mu$ M), suggesting that cytoplasmic transport and proteolysis may be responsible for the enhanced activity of **S1** (MIC = 0.6  $\mu$ M). Indeed, cytoplasmic uptake would enable eperezolid-OH to bypass the outer- and inner-membrane barriers, which may explain why the combination of component **11** and eperezolid-OH (**S2**) does not reach the full potential of conjugate **S1**.

Conjugate transport of the related analogue **8** to the cytoplasm is also a likely possibility in *E. coli*  $\Delta bamB\Delta tolC$ . In the event of periplasmic cleavage of **8**, the passive diffusion of the cleaved eperezolid-NH<sub>2</sub> (**5**) through the *E. coli* cytoplasmic membrane may result in low activities because eperezolid-NH<sub>2</sub> (**5**) is not active in a mutant of *E. coli* with a compromised outer membrane (*E. coli*  $\Delta surA$  BW2113) or in Gram-positive strains that lack an outer membrane (MIC = 41  $\mu$ M). Therefore, proteolysis in the cytoplasm may be responsible for the high activity of conjugate **8** (MIC = 1  $\mu$ M).

Proteolysis of conjugate **8** would be necessary to inhibit bacterial growth based on its inability to inhibit translation in *E. coli* at 38  $\mu$ M (Figure 4 of manuscript).

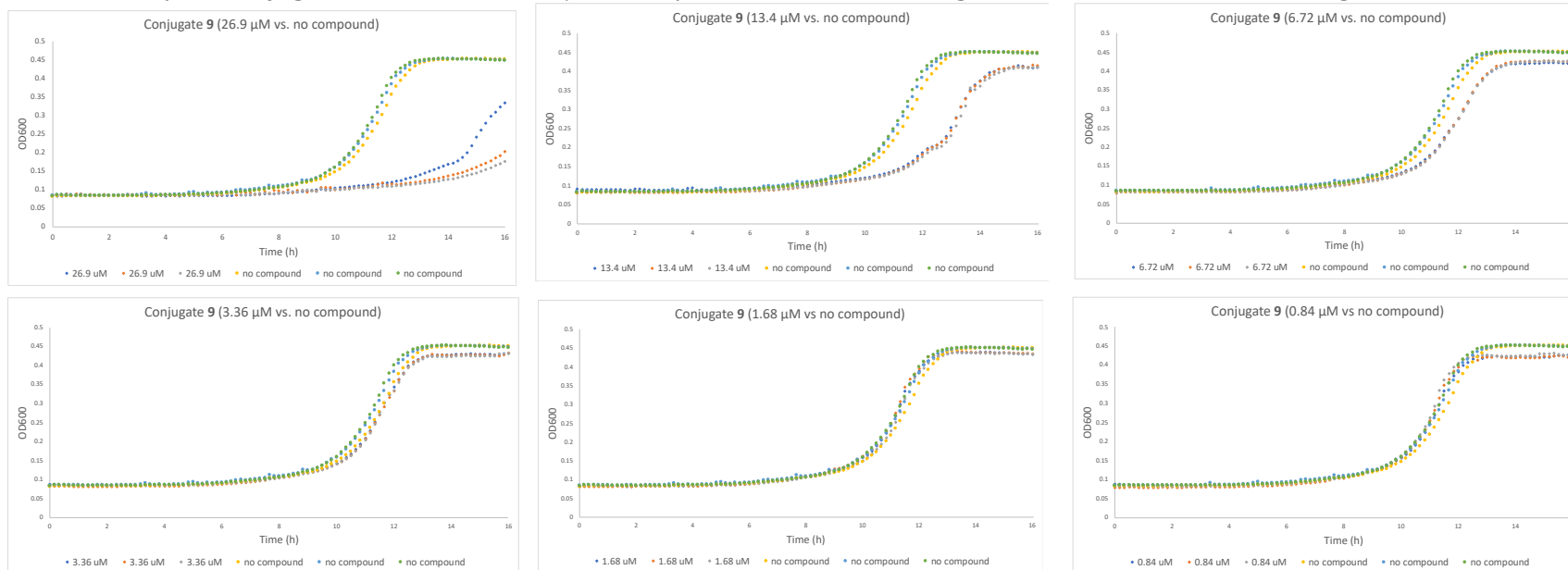


### Cleavage-Site Analysis For Enriched Sequences Identified from Substrate Phage Display

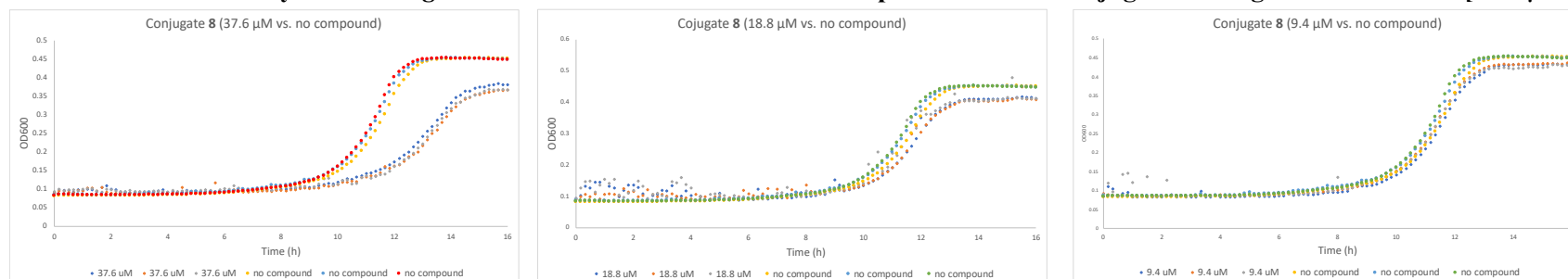
Synthesized Sequence	P-Side (Major)	P-Side (Minor)	P' side (Major)	P' side (Minor)	Uncleaved Parent Peptide	Major Fragment
WSKNQSLGG	W	ND <sup>a</sup>	SKNQSLGG	ND <sup>a</sup>	50%	ND <sup>a</sup>
WSGSDSSVG	W	ND <sup>a</sup>	SGSDSSVG	ND <sup>a</sup>	50%	ND <sup>a</sup>
WSNHADVHG	NH2-WSNHA	ND <sup>a</sup>	SNHADVHG	ND <sup>a</sup>	30%	SNHADVHG
WSKSEMLSG	ND <sup>a</sup>	ND <sup>a</sup>	MLSG	SKSEMLSG	20%	MLSG-OH
WSWCKWASG	WSWC	ND <sup>a</sup>	KWASG	ND <sup>a</sup>	3%	WSWC and KWASG
WSPKYMRFG	ND <sup>a</sup>	WSPKYM	RFG	YMRFG and MRFG	30%	RFG

**Table S5.** Cleavage-site analysis of sequences from substrate phage display. The synthesized sequences (25  $\mu$ M) were incubated with *E. coli* K12 periplasmic extract (Total Protein: 100  $\mu$ g/mL) at 37 °C for 18 h. Determination of the cleavage site was revealed by LC/MS analysis. The % uncleaved parent peptide was based on the relative areas under the curve for uncleaved peptide (remaining starting material) and product fragments. <sup>a</sup>ND=not determined

## Solithromycin Conjugate 9 [at 13.4 and 26.9 $\mu\text{M}$ ] Delays the Growth of *P. aeruginosa* ATCC 10145, a Multi-Drug Resistant Strain



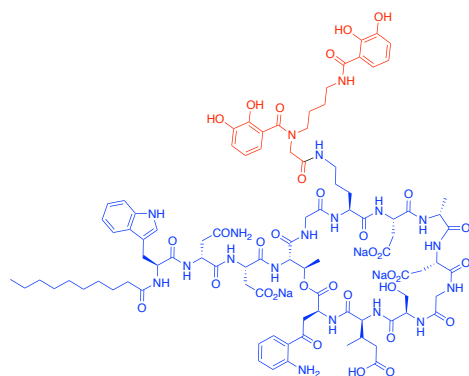
## A Similar Growth Delay for *P. aeruginosa* ATCC 10145 is Observed with Eperezolid-NH<sub>2</sub> Conjugate 8 at high concentrations [37.6 $\mu\text{M}$ ]



**Figure S2.** Growth curves of *P. aeruginosa* ATCC 10145 with conjugates 8 and 9. Three rationales may explain the delayed growth caused by 8 and 9 at 64  $\mu\text{g}/\text{mL}$  ([8, 37.2  $\mu\text{M}$ ], [9, 26.9  $\mu\text{M}$ ]): 1) an iron-withholding effect at higher concentrations, 2) siderophore-conjugate out-competition with endogenous siderophores (this strain is known to produce the pyoverdine siderophore), or 3) through expression of orthogonal outer-membrane-protein receptors for siderophore-mediated transport. All growth curves were conducted in sterilized 96-well plates and monitored at OD<sub>600</sub> over 16 h in 50% reduced MH-II broth containing 600  $\mu\text{M}$  DP. Each conjugate was evaluated at the following concentrations: 64, 32, 16, 8, 4, 2, and 1  $\mu\text{g}/\text{mL}$ ; the concentrations in  $\mu\text{g}/\text{mL}$  were then converted into  $\mu\text{M}$  for comparison. Solithromycin (6) has an MIC of 19  $\mu\text{M}$  in this strain, which would result in 100% growth inhibition at 26.9  $\mu\text{M}$ . Eperezolid-NH<sub>2</sub> (5) has an MIC of >171  $\mu\text{M}$ , thus minimal growth inhibition should be observed at 37.6  $\mu\text{M}$ .

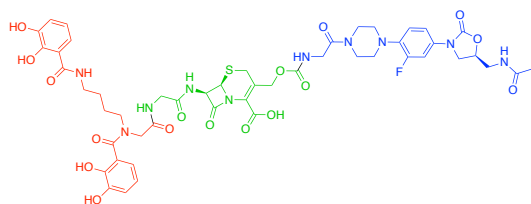
## Comparison of the Conjugates in This Work to Similar Conjugates Previously Reported

### Conjugates Reported by Miller



**HMM siderophore-daptomycin conjugate**

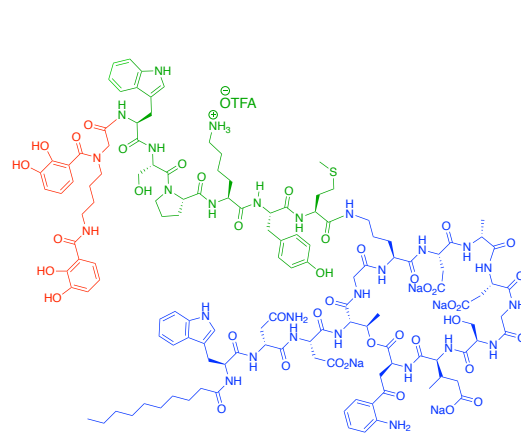
Miller and coworkers, *ACS Infect. Dis.* 2018, 4, 1529



**HMM siderophore-cephalosporin-oxazolidinone conjugate**

Miller and coworkers, *J. Med. Chem.* 2018, 61, 3845

### Conjugates Reported Herein

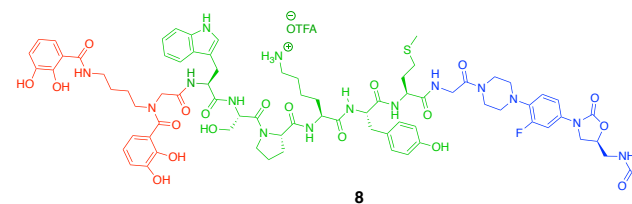


**HMM Siderophore**

**WSPKYM**

**daptomycin**

7

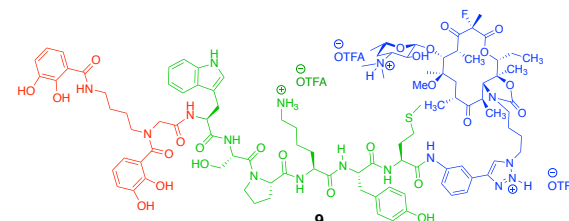


**HMM Siderophore**

**WSPKYM**

**eperezolid**

8



**HMM Siderophore**

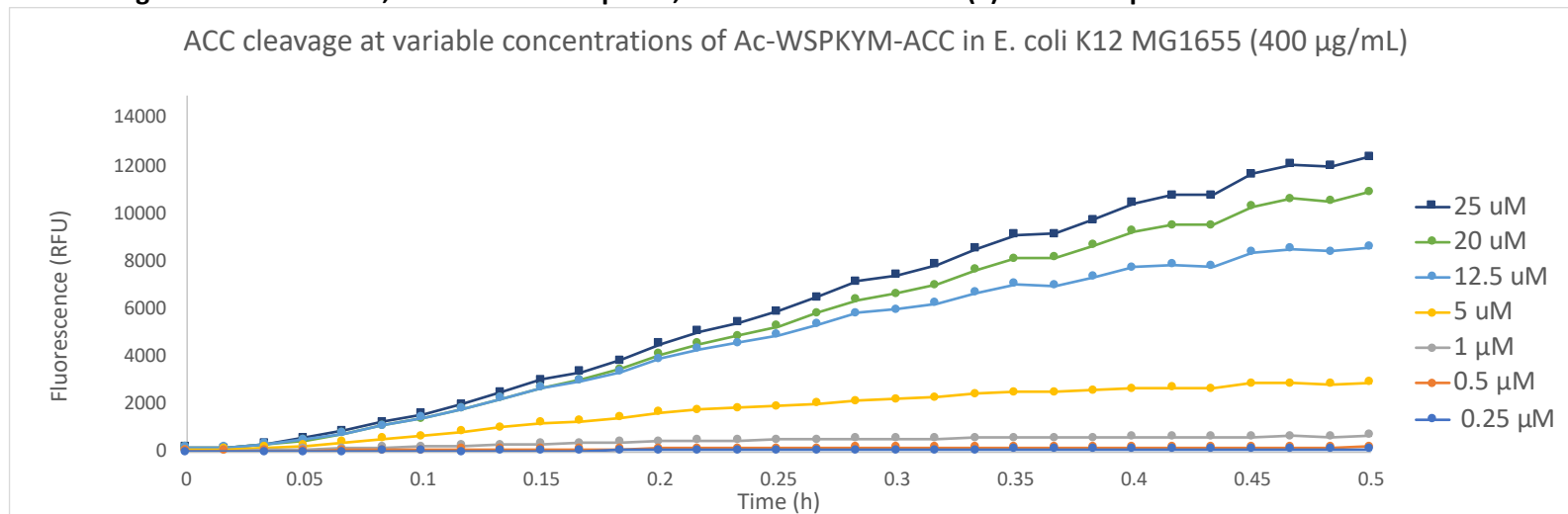
**WSPKYM**

**solithromycin**

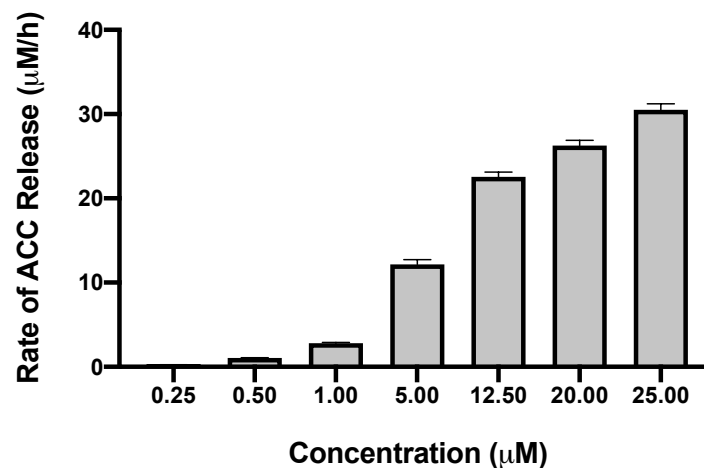
9

**Figure S3.** Conjugates reported by Miller and coworkers compared to conjugates 7-9 described in this work. The activities of conjugates 7-9 cannot be compared to previously reported conjugates as the specific linker contributes differently to the activity of each conjugate. Therefore, in this work, we limit our comparisons of the cleavable conjugates 7-9 to their closely related non-cleavable D-linker variants 13, 14, and 16. In this study, the linker serves to deactivate daptomycin (Table 5), eperezolid-NH<sub>2</sub> (Figure 4), and solithromycin (in pathogenic strains, see Table 3). With the exception of solithromycin conjugate 9 in non-pathogenic *E. coli*, we show that cleavable conjugates are more active than their non-cleavable variants. The activities we achieve are comparable or improved relative to previously reported cleavable linkers.

### Monitoring the Release of ACC, a Turn-On Fluorophore, from Ac-WSPKYM-ACC (1) in the Periplasmic Extract of *E. coli* K12 MG1655

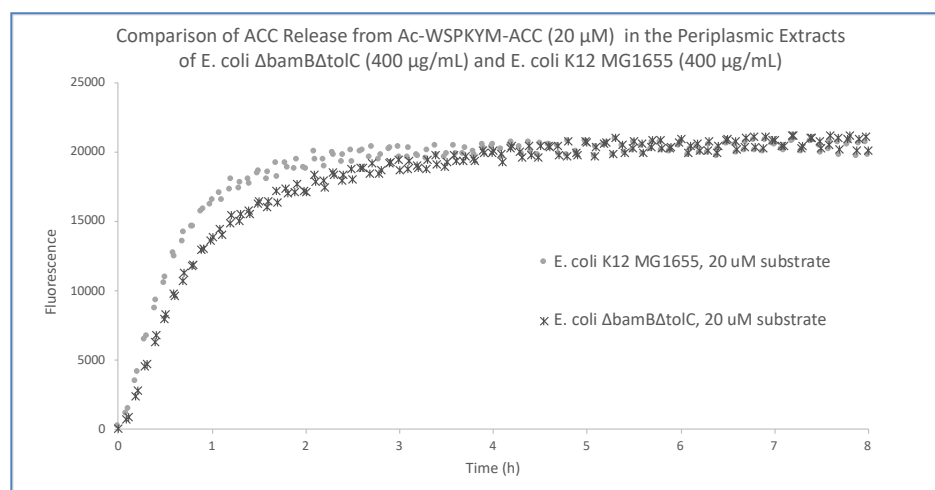
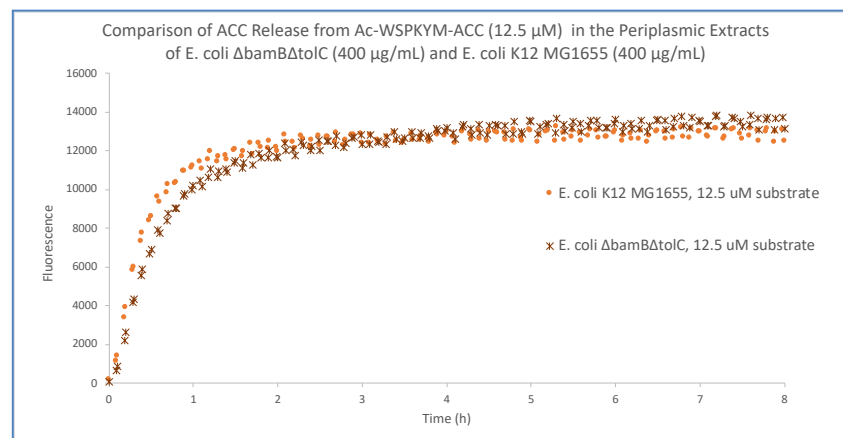
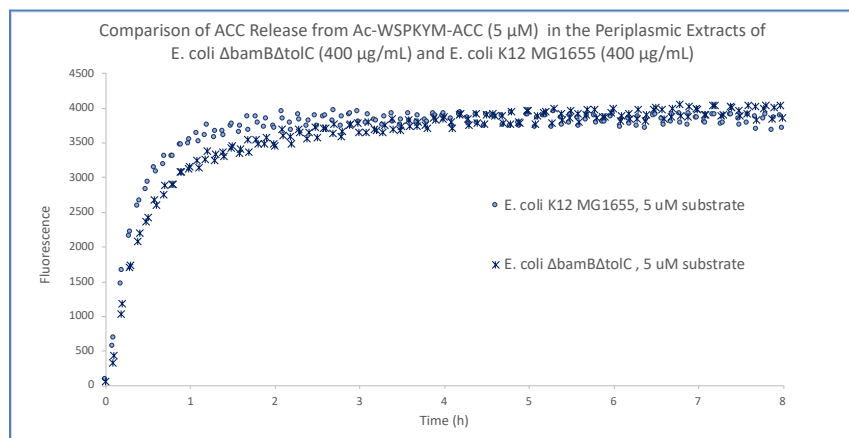


### Rate of ACC Release at Variable Concentrations of Ac-WSPKYM-ACC



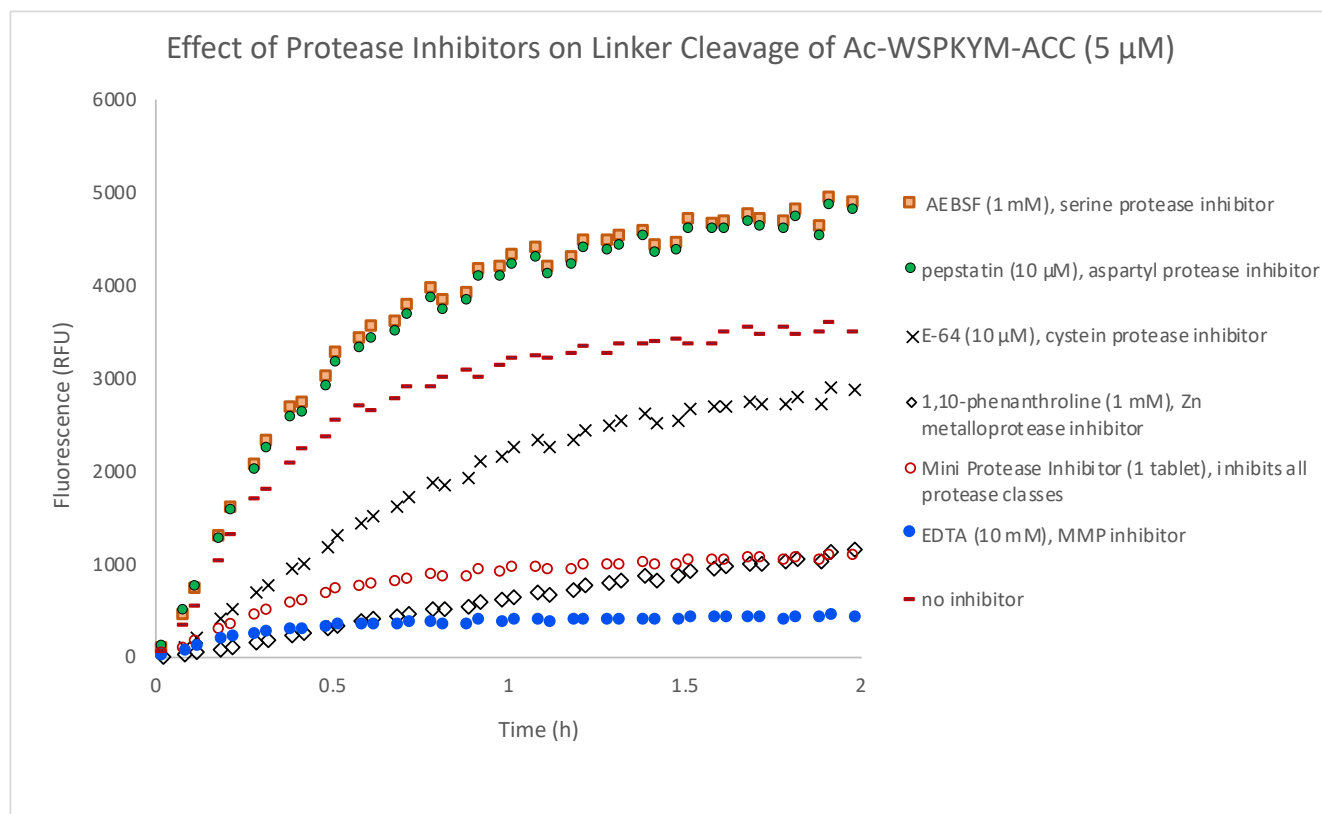
**Figure S4.** Evaluation of ACC release at seven concentrations of Ac-WSPKYM-ACC (0.25  $\mu\text{M}$ , 0.5  $\mu\text{M}$ , 1  $\mu\text{M}$ , 5  $\mu\text{M}$ , 12.5  $\mu\text{M}$ , 20  $\mu\text{M}$ , 25  $\mu\text{M}$ ) in a periplasmic extract of *E. coli* K12 MG1655 (400  $\mu\text{g}/\text{mL}$ ) over the course of 8 h. The fluorescence at  $t=8$  h (Figure 3) was used as an approximation of the maximal amount of ACC that can be released from peptide **1** (Ac-WSPKYM-ACC) over the reaction time course. Refer to page S76 for additional details on reaction set-up. ACC=7-amino-4-carbamoylmethylcoumarin, a turn-on fluorescent coumarin.

**Periplasmic Extract from *E. coli* K12 MG1655 and *E. coli*  $\Delta$ bamB $\Delta$ tolC BW25113 Cleave ACC from Ac-WSPKYM-ACC (1) at Similar Rates**



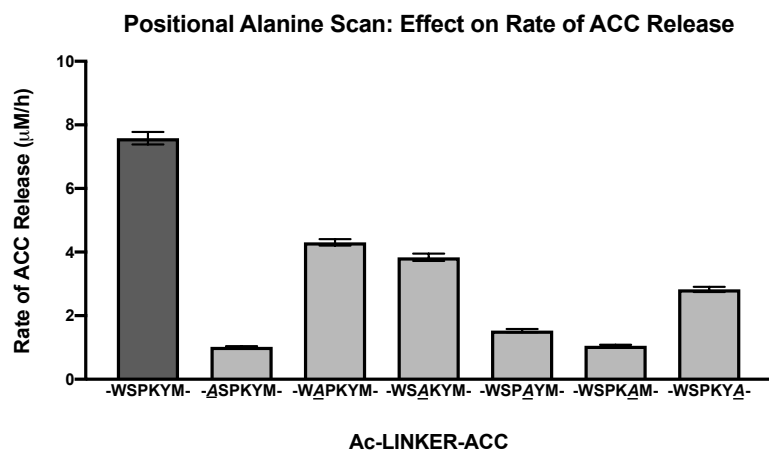
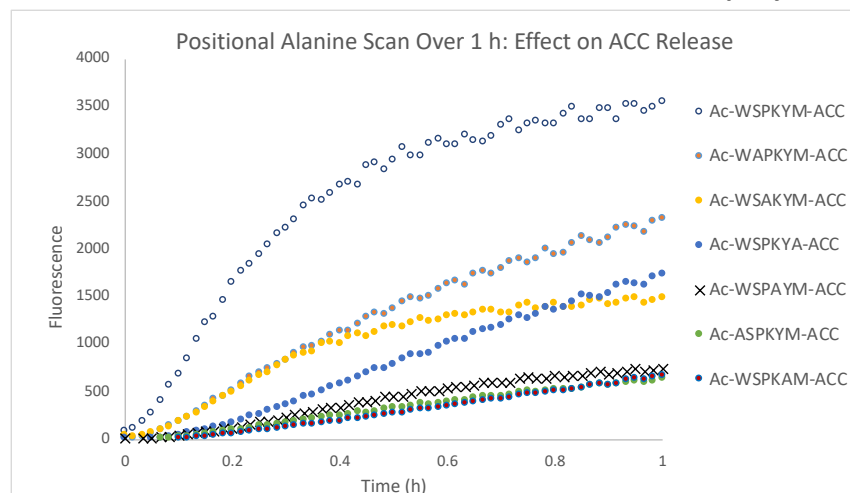
**Figure S5.** ACC release from Ac-WSPKYM-ACC (shown for 5, 12.5, and 20  $\mu$ M) in the periplasmic extract of *E. coli*  $\Delta$ bamB $\Delta$ tolC BW25113 (400  $\mu$ g/mL) was similar to that in the extract of *E. coli* K12 MG1655 (400  $\mu$ g/mL). Refer to page S76 for additional details on reaction set-up. ACC=7-amino-4-carbamoylmethylcoumarin, a turn-on fluorescent coumarin.

## Protease Inhibitors Suggest that Metalloproteases are Responsible for Cleavage of Ac-WSPKYM-ACC



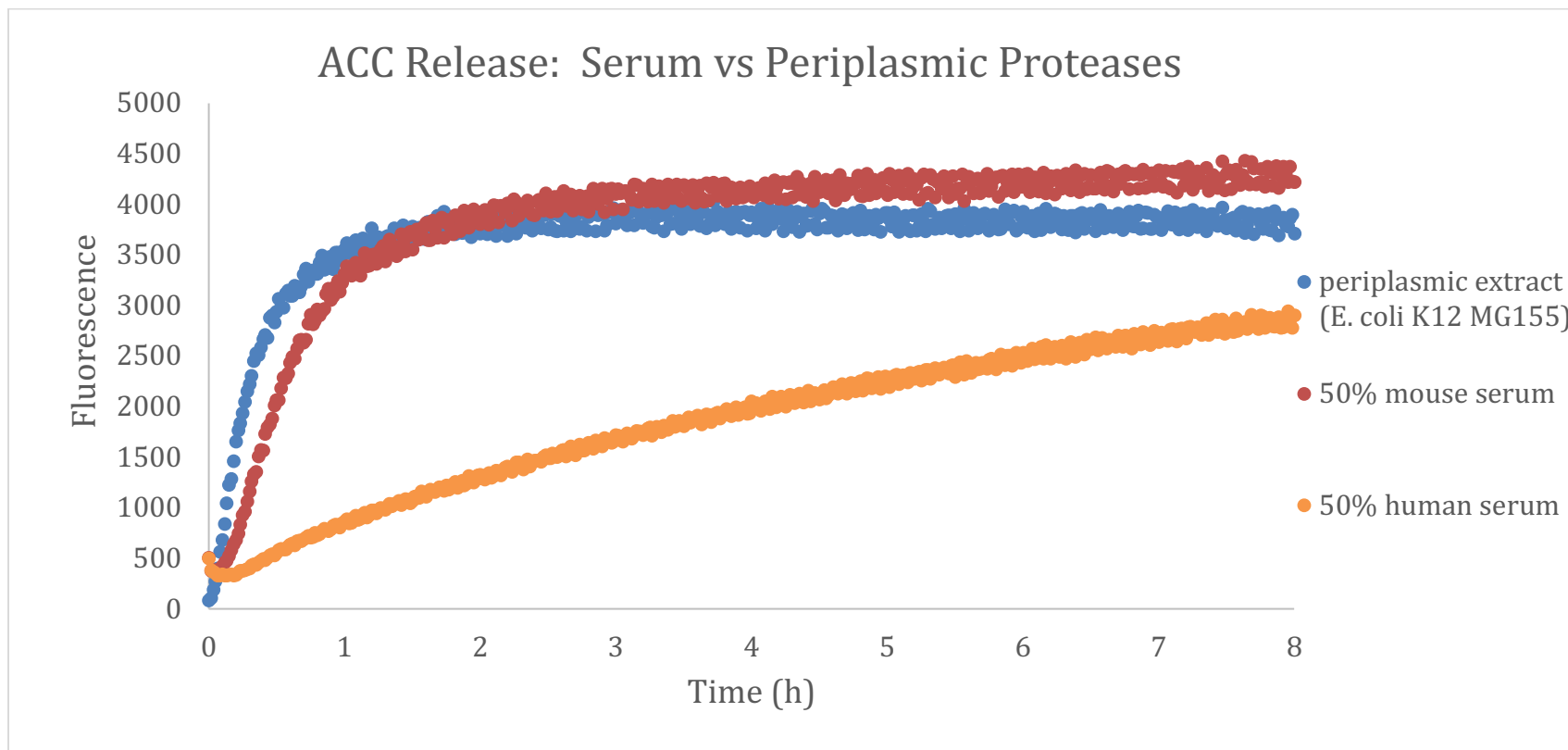
**Figure S6.** The effect of protease inhibitors on ACC release from Ac-WSPKYM-ACC (5  $\mu$ M) in a periplasmic extract of *E. coli*  $\Delta$ bamB $\Delta$ tolC BW25113 (400  $\mu$ g/mL) was evaluated to determine the type of proteases that may be responsible for the activity of conjugate 8 in *E. coli*  $\Delta$ bamB $\Delta$ tolC BW23115. These protease inhibitors included pepstatin (10  $\mu$ M), AEBSF (1 mM), E-64 (10  $\mu$ M), 1,10-phenanthroline (1 mM), EDTA (10 mM), and a multi-protease inhibiting tablet as a negative control. Protease inhibitors were pre-incubated with periplasmic extract for 5-10 min prior to adding substrate. A control without periplasmic extract was also evaluated for baseline correction. The enzymes of interest likely include a metalloprotease or a calcium dependent protease, although we are unable to rule out that the divalent ion site is playing a structural rather than catalytic role from these data alone. Refer to page S76 for additional details on reaction set-up. ACC=7-amino-4-carbamoylmethylcoumarin, a turn-on fluorescent coumarin.

## A Positional Alanine Scan Revealed Ac-WSPKYM-ACC Released ACC More Rapidly Than All Other Analogues



**Figure S7.** Alanine positional scanning was used to determine which amino acids of peptide **1** may contribute to its rate of ACC release in a periplasmic extract of *E. coli* K12 MG1655 (400 μg/mL). Ac-WSPKYA-ACC (**S28**), Ac-WSPKAM-ACC (**S29**), Ac-WSPAYM-ACC (**S30**), Ac-WAPKYM-ACC (**S31**), Ac-WSPKYM-ACC (**S32**), and Ac-ASP KYM-ACC (**S33**) were cleaved at a concentration of 5 μM. The peptide Ac-WSPKYM-ACC, discovered from substrate phage display, cleaved at the fastest rate. The Tyr (P2), Trp (P6), and Lys (P3) residues appear to play a dominant role in achieving high rates of ACC release, while the Met (P1), Pro (P4), and Ser (P5) residues contribute less significantly. In calculating the rates (μM/h), the fluorescence of peptide **1** at time t=8 h (Figure 3) was used as an approximation of the maximum amount of ACC that can be released from each alanine analogue over the reaction time course. See page S76 for additional details on reaction set-up. ACC=7-amino-4-carbamoylmethylcoumarin, a turn-on fluorescent coumarin. Each alanine-containing compound is characterized on S69-S72.

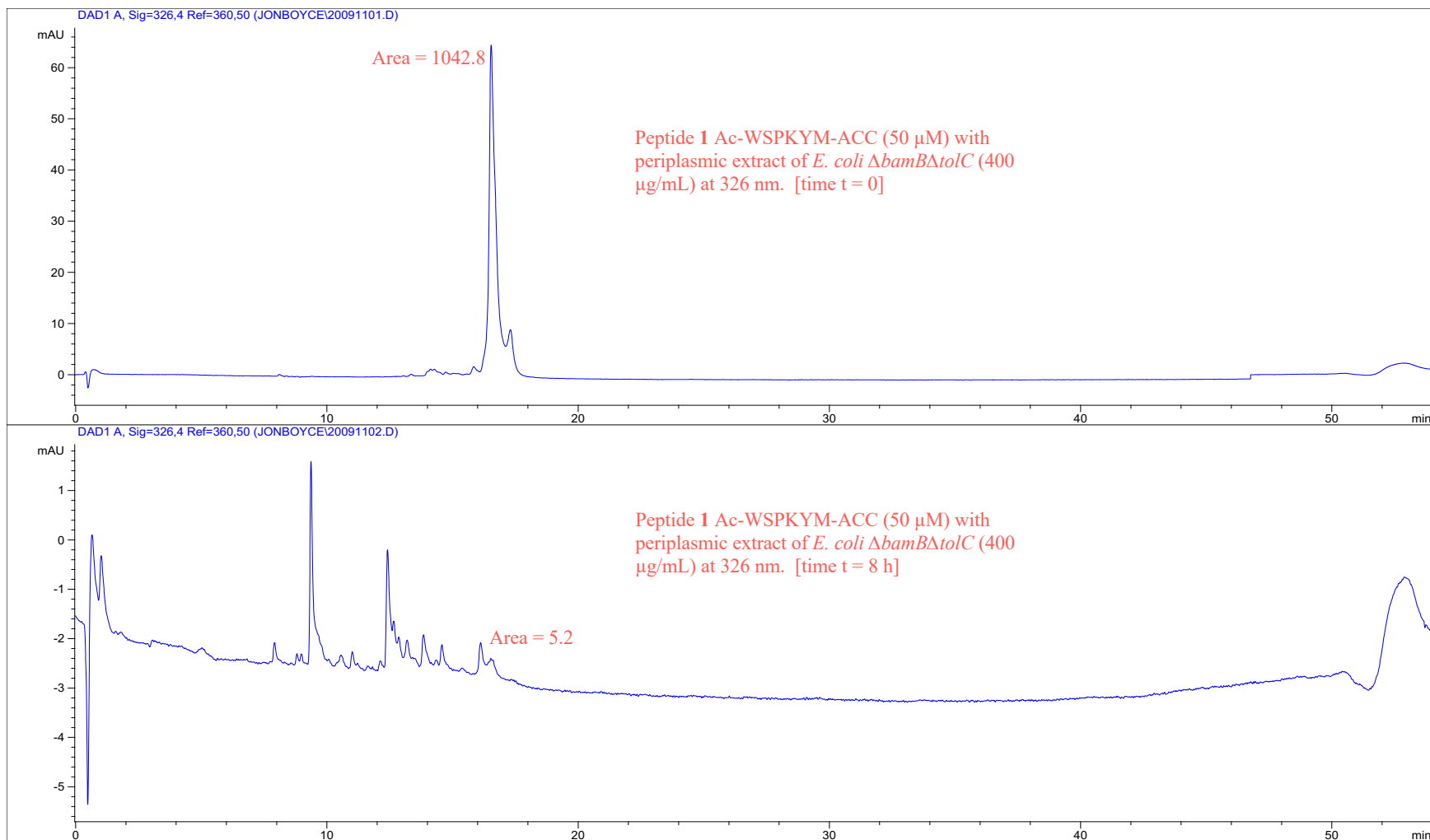
ACC Cleavage from Ac-WSPKYM-ACC was Observed in Both Periplasmic Extract and Serum (Mouse and Human)



**Figure S8.** ACC cleavage from Ac-WSPKYM-ACC (**1**, 5  $\mu$ M) in a periplasmic extract of *E. coli* K12 MG1655 (400  $\mu$ g/mL), 50% mouse serum, and 50% human serum. Mouse serum and periplasmic extract release ACC from **1** at comparable rates, with an average half-life of  $0.37 \pm 0.02$  h. Human serum released ACC less rapidly, with a half-life of  $4.05 \pm 0.02$  h. ACC=7-amino-4-carbamoylmethylcoumarin, a turn-on fluorescent coumarin.



### Cleavage of Ac-WSPKYM-ACC (1) in a Periplasmic Extract of *E. coli* at Time t=0 and t=8 h



**Figure S9.** Cleavage of Ac-WSPKYM-ACC (**1**, 50 μM) in a periplasmic extract of *E. coli* ΔbamBΔtolC BW21153 (400 μg/mL) at time t=0 h and time t=8 h (determined by HPLC at 326 nm). The area of Ac-WSPKYM-ACC (**1**, 50 μM) at t=0 h was 1042.8, and at t=8 h the area was reduced to 5.2, suggesting >99% cleavage of **1** over the time course of the reaction.

## II. General Information

### A. Instrumentation and Methods

All reactions were carried out in oven-dried glassware under an argon atmosphere unless otherwise noted. Proton nuclear magnetic resonance ( $^1\text{H}$  NMR) spectra and carbon nuclear magnetic resonance ( $^{13}\text{C}$  NMR) spectra were recorded on 400 MHz Bruker Avance III HD 2-channel instrument NMR spectrometers at 23 °C or 50 °C. Proton chemical shifts are expressed in parts per million (ppm,  $\delta$  scale) and are referenced to residual protium in the NMR solvent ( $\text{CHCl}_3$ :  $\delta$  7.26).  $^{13}\text{C}$  NMR were recorded at 100 MHz at ambient temperature with the same solvents unless otherwise stated. Chemical shifts are reported in parts per million relative to  $\text{CDCl}_3$  ( $^1\text{H}$ ,  $\delta$  7.26;  $^{13}\text{C}$ ,  $\delta$  77.0) or  $\text{CD}_3\text{OD}$  ( $^1\text{H}$ ,  $\delta$  3.31, 4.78;  $^{13}\text{C}$ ,  $\delta$  49.3). All  $^{13}\text{C}$  NMR spectra were recorded with complete proton decoupling. Analytical thin-layer chromatography (TLC) was performed using glass plates pre-coated with silica gel (0.25-mm, 60-Å pore size, 230–400 mesh, SILICYCLE INC) impregnated with a fluorescent indicator (254 nm). TLC plates were visualized by exposure to ultraviolet light (UV), and then were stained by submersion in a basic aqueous solution of potassium permanganate or with an acidic ethanolic solution of anisaldehyde, followed by brief heating. An Acquity UPLC BEH C18 1.7  $\mu\text{m}$  column was used for analytical UPLC-MS. Preparative HPLC purification was performed on a Waters Prep HPLC or Varian ProStar HPLC using a C4 or C18 column. Analytical reverse-phase HPLC analyses were performed on an Agilent 1100 series HPLC system using a Phenomenex Kinetex 2.6  $\mu\text{m}$  C18, 50  $\times$  2.1 mm column. Flow rates were controlled at 0.35 mL/min. Peptide detection was based on UV absorption at 220 nm, while conjugate detection (following siderophore attachment) was based on UV absorption at 254nm. Mass spectrometry data for conjugates and peptides were obtained using Shimadzu AXIMA Performance MALDI-TOF spectrometer in a reflectron mode with  $\alpha$ -Cyano-4-hydroxycinnamic acid as the matrix. Liquid chromatography/MS experiments were conducted on a Shimadzu HPLC with an Applied Biosystems 3200 QTRAP LC/MS/MS system. High resolution mass spectra were obtained using a Waters Acquity UPLC (Ultra Performance Liquid Chromatography) with a binary solvent manager, SQ mass spectrometer, Waters 2996 PDA (Photo-Diode Array) detector, and Evaporative Light Scattering Detector (ELSD).

## B. Reagents and Solvents

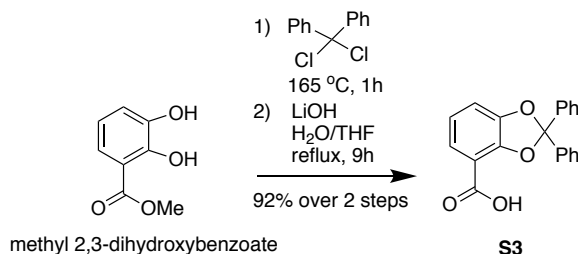
CH<sub>2</sub>Cl<sub>2</sub>, DMF, THF, ethyl ether, and acetonitrile to be used in anhydrous reaction mixtures were dried by passage through activated alumina columns immediately prior to use. Hexanes used were  $\geq 85\%$  *n*-hexane. Other commercial solvents and reagents were used as received, unless otherwise noted. Fmoc-protected amino acids were purchased from GL Biochem. 2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate (HCTU), trifluoroacetic acid (TFA) were purchased from Chem-Impex International and Aldrich. 4-Methylpiperidine was purchased from Acros Organics. Rink Amide-ChemMatrix resin (0.5 mmol/g loading) was purchased from Biotage. The oxazolidinones eperzolid-NH<sub>2</sub> (**5**) and eperzolid-OH (**S2**) were prepared by the protocols of Miller and Rafai Far.<sup>S3,S4</sup> All other reagents and relevant catalysts were purchased from Sigma-Aldrich, Acros, Alfa Aesar, and Strem Chemicals.

## Solid-phase peptide synthesis (SPPS)

All peptides were synthesized on 3.7 mmol scale using a CHEMGLASS® glass 500-mL medium-porosity sintered glass solid phase peptide synthesis vessel GL-32 or at 0.15 mmol scale using a Biotage peptide synthesizer. 2-Chlorotrityl resin (Chem-Impex, 1.0-2.0 mmol/g loading) or rink amide-ChemMatrix® resin (Biotage, 0.5 mmol/g loading) were used for the synthesis. A typical SPPS reaction cycle includes Fmoc deprotection, washing, coupling, and post-coupling washing steps. On the peptide synthesizer, the deprotection was carried-out for 5 minutes at 23 °C with 20% 4-methylpiperidine in dimethylformamide (DMF). On the peptide synthesizer, a standard double coupling was accomplished in 8 min at 23 °C with 5 equivalents of Fmoc-protected amino acids, 4.98 equivalents of HCTU, and 10 equivalents of DIEA (relative to the amino groups on resin) in DMF at a final concentration of 0.125 M amino acids. Chromatographic separations for 8-mer peptide syntheses were obtained using a linear gradient of 1-61% acetonitrile (with 0.08% TFA) in water (with 0.1% TFA) over 10 min, 1-41% acetonitrile (with 0.08% TFA) in water (with 0.1% TFA) over 20 min, or 1-41% acetonitrile (with 0.08% TFA) in water (with 0.1% TFA) over 40 min with column at room temperature. The synthesis on larger scales (3.7 mmol) are described herein (Section III).

### III. Experimental Procedures and Compound Characterization

#### A. Siderophore Synthesis



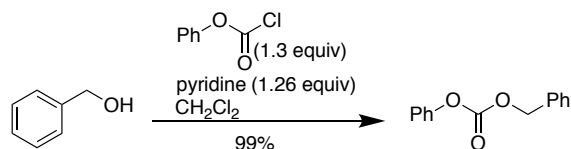
**S3: Method A (purification by column chromatography):** To a flame-dried 100-mL round-bottom flask containing methyl 2,3-dihydroxybenzoate (500 mg, 2.97 mmol, 1.0 equiv) under argon was added dichlorodiphenylmethane (0.86 mL, 4.46 mmol, 1.5 equiv). The mixture was then heated to  $165\text{ }^\circ\text{C}$  for 1h. After cooling to  $23\text{ }^\circ\text{C}$ , the mixture was diluted with EtOAc (8 mL). The organic layers were washed with  $\text{NaHCO}_3$  (5 mL),  $\text{H}_2\text{O}$  (5 mL), brine (5 mL), dried over  $\text{MgSO}_4$ , and concentrated under reduced pressure to provide crude product (767 mg), which was used directly in the next reaction without further purification. The crude product (767 mg, 2.31 mmol) was dissolved in THF/ $\text{H}_2\text{O}$  (1:1, 60 mL). Lithium hydroxide monohydrate (1.32 g, 31.5 mmol, 13.6 equiv) was added in a single portion, and the mixture was heated to reflux for 24 h. After cooling to  $23\text{ }^\circ\text{C}$ , the reaction was neutralized with 10% AcOH/ $\text{H}_2\text{O}$  (50mL) until the solution reached pH 4. The solution was extracted into EtOAc (3 x 100 mL), and the combined organic layers were dried over  $\text{MgSO}_4$ , filtered, and concentrated under reduced pressure. The crude solid was purified by column chromatography (10 - 30% EtOAc/hexanes, then 95% EtOAc/AcOH) to provide 867.6 mg (92% over 2 steps) of **S3** as a white solid. Spectroscopic data for **S3** were found to be identical with those reported in the literature.<sup>S5,S6</sup>

**S3: Method B (purification by trituration, no column chromatography):** To a 100-mL round-bottom flask containing methyl-2,3-dihydroxybenzoate (8.0 g, 47.6 mmol, 1.0 equiv) under argon was added dichlorodiphenylmethane (14 mL, 71.4 mmol, 1.5 equiv). The mixture was then heated to  $165\text{ }^\circ\text{C}$  for 1h, cooled to  $23\text{ }^\circ\text{C}$ , and diluted with EtOAc (400 mL). The solution was washed with  $\text{NaHCO}_3$  (1x120 mL),  $\text{H}_2\text{O}$  (1x120 mL), and brine (1x120 mL). The organic layer was

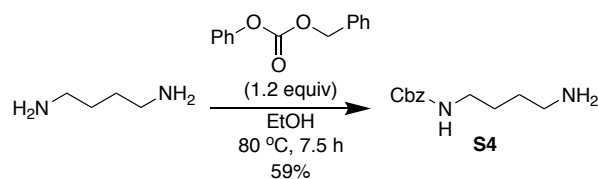
concentrated under reduced pressure [note: did not dry over MgSO<sub>4</sub> or filter] to provide a crude residue (15.8 g, 47.6 mmol). In a 1-L round-bottom flask containing the crude residue was added THF/H<sub>2</sub>O (1:1, 400 mL). Lithium hydroxide monohydrate (21.0 g, 500 mmol, 10.5 equiv) was added. The mixture was heated to 100 °C for 9 h, cooled to r.t., and concentrated under reduced pressure. To the aqueous mixture was added 1M HCl (400 mL) and EtOAc (600 mL). The layers were separated, and the aqueous layer was extracted with EtOAc (2 x 400 mL). The combined organic layers were washed with H<sub>2</sub>O (1x200 mL) and brine (1x200 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated under reduced pressure. The crude material was transferred to a 125-mL round-bottom flask in CH<sub>2</sub>Cl<sub>2</sub> and concentrated to a volume of ~50 mL. A white solid began to precipitate in the flask and then hexanes (10 mL) was added. The mother liquor was carefully decanted into a 250-mL round-bottom flask so as not to disturb the solid, and the product was washed with 1:1 hexanes/CH<sub>2</sub>Cl<sub>2</sub> (1x25 mL). The wash was carefully decanted into the mother liquor so as to avoid decantation of solid product. The first crop of crystals provided 6.88 g (45 % over 2 steps) of product **S3** as a white crystalline solid. [Note: the pure product is best solvated in a 1:1 mixture of CH<sub>2</sub>Cl<sub>2</sub>/methanol; it is less soluble in CH<sub>2</sub>Cl<sub>2</sub>, EtOAc, or methanol]. A second crop was afforded by concentrating the mother liquor under reduced pressure to ~5 mL. Spectroscopic data for **S3** were found to be identical with those reported in the literature.<sup>S5,S6</sup>

**S3: Method C (purification by potassium salt formation, no column chromatography):** To a 100-mL round-bottom flask containing methyl-2,3-dihydroxybenzoate (3.30 g, 19.6 mmol, 1.0 equiv) under argon was added dichlorodiphenylmethane (5.50 mL, 28.6 mmol, 1.5 equiv). The mixture was then heated to 165 °C for 1h. After cooling to 23 °C, the mixture was diluted with EtOAc (100 mL), washed with NaHCO<sub>3</sub> (1x30 mL), H<sub>2</sub>O (1x30 mL), brine (1x30 mL), dried over MgSO<sub>4</sub>, and concentrated under reduced pressure to provide crude product (6.52 g), which was used directly in the next reaction. The crude product **S3** (6.52 g) was dissolved in THF/H<sub>2</sub>O (1:1, 610 mL), lithium hydroxide monohydrate (13.5 g) was added, and the mixture was heated to reflux for 24 h. After cooling to 23 °C, the mixture was neutralized with 10% AcOH/H<sub>2</sub>O (733 mL) to pH=4 and extracted into EtOAc (3 x 1 L). The combined organic layers were dried over MgSO<sub>4</sub>, filtered, and concentrated under reduced pressure. The crude product was dissolved in Et<sub>2</sub>O (1000 mL), and pure product (226.9 mg) was collected as a solid precipitate. The Et<sub>2</sub>O solution was extracted with aqueous saturated K<sub>2</sub>CO<sub>3</sub> (200 mL), which led to the formation of a potassium salt

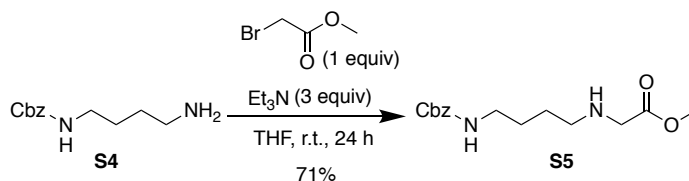
precipitate. The Et<sub>2</sub>O layer was concentrated under reduced pressure to a volume of 200 mL, and the white solid K-salt was stirred for 24h, filtered, and washed with Et<sub>2</sub>O. The aqueous layer was extracted with EtOAc (3x250 mL). The combined organic layers were dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated, and the resulting K-salt was combined with the filtered K-salt. To a suspension of the product potassium salt in EtOAc (250 mL) was added 1M HCl (250 mL), and the aqueous layer was extracted with EtOAc (2x250 mL). The combined organic layers were washed with H<sub>2</sub>O (250 mL) and brine (250 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated under reduced pressure to provide 4.25 g (67% over 2 steps) of product **S3** as the pure free-acid. Spectroscopic data for **S3** were found to be identical with those reported in the literature.<sup>S5,S6</sup> **S3**:  $R_f = 0.28$  (40 % EtOAc/hexanes); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  11.9 (br s, 1H), 7.74 – 7.61 (m, 4H), 7.52 (dd,  $J = 8.2, 1.2$  Hz, 1H), 7.46 – 7.36 (m, 6H), 7.10 (dd,  $J = 7.7, 1.2$  Hz, 1H), 6.91 (t,  $J = 8.0$  Hz, 1H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  170.4, 149.0, 148.5, 139.7, 129.5, 128.5, 126.5, 123.4, 121.4, 118.4, 113.3, 112.2. **HR-MS**:  $m/z$  Calcd. for C<sub>20</sub>H<sub>14</sub>O<sub>4</sub> [M+H<sup>+</sup>]:319.0892, Found 319.1050.



**Benzyl Phenyl Carbonate:** To a 1-L flame-dried, round-bottom flask containing a solution of benzyl alcohol (15.5 mL, 150 mmol, 1.0 equiv) in CH<sub>2</sub>Cl<sub>2</sub> (210 mL) under argon was added pyridine (15.0 mL, 189 mmol, 1.26 equiv). The flask was immersed in an ice-water bath and stirred for 10 min. A solution of phenyl chloroformate (24.5 mL, 195 mmol, 1.3 equiv) in CH<sub>2</sub>Cl<sub>2</sub> (115 mL) was prepared in a 250-mL flame-dried, round-bottom flask, which was transferred *via* syringe in a dropwise manner to the solution of benzyl alcohol. The mixture was then warmed to 23 °C, and monitored by TLC (2.5% EtOAc/pentane). The reaction was complete after 4 h. The mixture was transferred to a 2-L separatory funnel, diluted with CH<sub>2</sub>Cl<sub>2</sub> (500 mL), and washed with water (300 mL), 5% NaOH (300 mL), 1M HCl (300 mL), and brine (300 mL). The organic layer was then dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated under reduced pressure. Purification by column chromatography (silica gel, 2.5% EtOAc/pentane) afforded 33.9 g (99%) of benzyl phenyl carbonate as a clear oil. Spectroscopic data for benzyl phenyl carbonate were found to be identical with those reported in the literature.<sup>S7</sup>

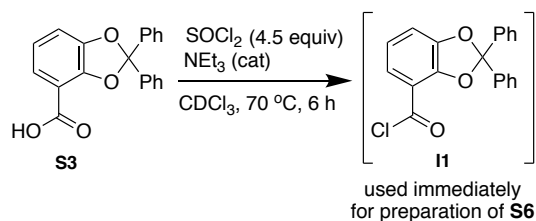


**S4:** To a 1-L round-bottom flask containing a solution of 1,4-diaminobutane (6.50 mL, 65.0 mmol, 1.0 equiv) in EtOH (54.0 mL) under argon was added a solution of benzyl phenyl carbonate (15.0 mL, 78.0 mmol, 1.2 equiv) in ethanol (18.0 mL) via syringe over 20 minutes. Following the addition of benzyl phenyl carbonate, a white solid precipitated after 5 minutes. The mixture was then heated to 80 °C for 7.5 h. After cooling to 23 °C, additional white solid precipitated. The white solid was filtered, washed with EtOH (2x60 mL), and discarded. [Note: The white solid is bis-Cbz-protected-diaminobutane]. The EtOH filtrate was concentrated under reduced pressure, diluted in CH<sub>2</sub>Cl<sub>2</sub> (300 mL), and washed with 1 M HCl (300 mL, note: a white colloidal dispersion formed). The aqueous layer was extracted with CH<sub>2</sub>Cl<sub>2</sub> (5x100 mL). The combined CH<sub>2</sub>Cl<sub>2</sub> layers (a white colloidal dispersion) were discarded. The aqueous layer was then basified with 15% NaOH until pH 14, extracted with CH<sub>2</sub>Cl<sub>2</sub> (2x300 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated under reduced pressure to provide 8.53 g (59%) of the primary amine **S4** as an oil (suspended with white crystalline solid). *R<sub>f</sub>* = 0.34 (20% MeOH/CH<sub>2</sub>Cl<sub>2</sub> with 3% NH<sub>4</sub>OH); <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD) δ 7.46 – 7.23 (m, 5H), 5.06 (s, 2H), 3.12 (t, J = 6.6 Hz, 2H), 2.62 (t, J = 6.7 Hz, 2H), 1.64 – 1.35 (m, 4H). <sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD) δ 157.5, 137.1, 128.0, 127.5, 127.4, 65.9, 40.8, 40.2, 29.6, 26.9. **HR-MS:** *m/z* Calcd. for C<sub>12</sub>H<sub>18</sub>N<sub>2</sub>O<sub>2</sub> [M+H<sup>+</sup>]:223.1368, Found 223.1530.



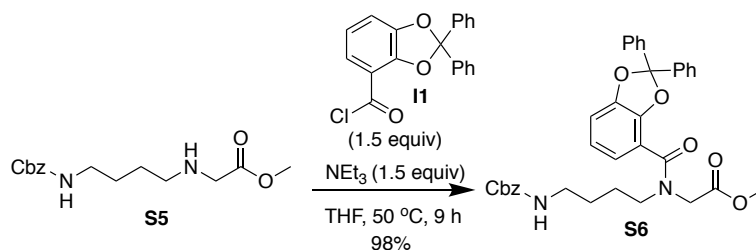
**S5:** To a 500-mL flame-dried, round-bottom flask under argon containing compound **S4** (3.65 g, 16.4 mmol, 1.0 equiv) was added anhydrous THF (55 mL) and triethylamine (6.8 mL, 49.3 mmol, 3.0 equiv), followed by dropwise addition of methyl bromoacetate over 10 minutes. The reaction was monitored by TLC (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). After 24 h, the reaction was complete, and the triethylammonium salt was filtered. The filtrate was concentrated under reduced pressure to

provide the crude product as a clear oil. Upon transferring to a 50-mL round-bottom flask in  $\text{CH}_2\text{Cl}_2$ , a white emulsion formed. Purification by column chromatography (2.5-5%  $\text{MeOH}/\text{CH}_2\text{Cl}_2$ ) provided 3.42 g (71%) of methyl ester **S5**. **S5**:  $R_f = 0.31$  (5%  $\text{MeOH}/\text{CH}_2\text{Cl}_2$ );  $^1\text{H NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.39 – 7.26 (m, 5H), 5.19 – 4.99 (m, 3H), 3.73 (s, 3H), 3.46 (s, 2H), 3.26 – 3.16 (m, 2H), 2.78 – 2.63 (m, 2H), 2.47 (br s, 2H), 1.67 – 1.50 (m, 4H).  $^{13}\text{C NMR}$  (100 MHz,  $\text{CDCl}_3$ )  $\delta$  171.8, 156.5, 136.6, 128.5, 128.1, 66.6, 52.0, 50.0, 48.8, 40.7, 27.5, 26.5. **HR-MS**:  $m/z$  Calcd. for  $\text{C}_{15}\text{H}_{22}\text{N}_2\text{O}_4$   $[\text{M}+\text{H}^+]$ : 295.1580, Found 295.1765.



**Intermediate I1 (for preparation of compound S6 and S7):** Acid **S3** (1.38 g, 4.35 mmol, 1.0 equiv) was added to a 500-mL flame-dried, round-bottom flask. The flask was purged with argon, and acid **S3** was suspended in  $\text{CDCl}_3$  (22 mL). Triethylamine (21  $\mu\text{L}$ , 3.5 mol%) was then added, which resulted in a red solution. The temperature of an oil bath was stabilized between 45-50  $^\circ\text{C}$ , and the mixture was stirred for 10 minutes at this temperature. Thionyl chloride (1.43 mL, 19.6 mmol, 4.5 equiv) was added dropwise over the course of 30 min. The mixture was then heated to 70  $^\circ\text{C}$  and stirred for 6 h. The reaction was monitored by  $^1\text{H-NMR}$ . Upon completion, the mixture was cooled to 23  $^\circ\text{C}$ , and concentrated under reduced pressure using a high vacuum manifold. A water bath was used to warm the flask to ensure that all solvent had evaporated to provide a yellow oil. After backfilling with argon, the crude acid chloride **I1** was used immediately for the preparation of **S6** (*vide infra*) without further purification.





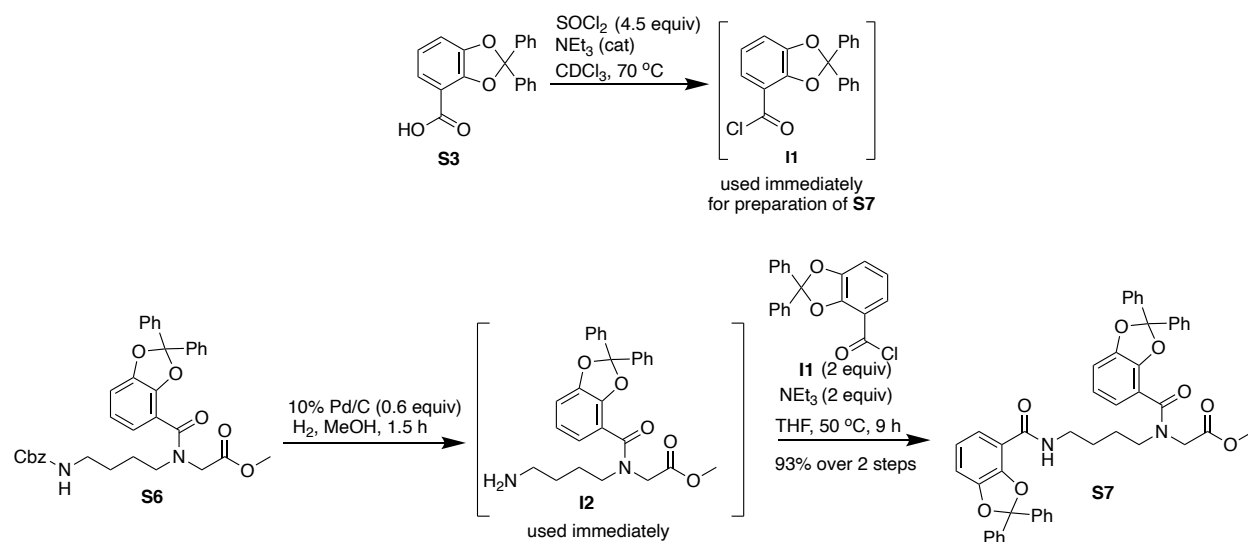
**S6:** To a 250-mL flame-dried, round-bottom flask containing a solution of amine **S5** (839 mg, 2.85 mmol, 1.0 equiv) in dry THF (12.2 mL) under argon was added triethylamine (0.61 mL, 4.36 mmol, 1.53 equiv). Acid chloride **II** (1.46 g, 4.35 mmol, 1.53 equiv) was then added in three portions as a solution in THF (24.4 mL; portion 1: 15 mL, rinse portion 2: 5 mL, and rinse portion 3: 4.4 mL). Following the addition of the **II**, the reaction began to smoke and a white milky suspension formed. The mixture was then heated to 50 °C. After 9 h, triethylamine (0.1 mL, 0.7 mmol, 0.25 equiv) was added, and the reaction was stirred for an additional 30 min. The mixture was then cooled to 23 °C, and CH<sub>2</sub>Cl<sub>2</sub> (20 mL) and 1M HCl (20 mL) were added. The layers were separated, and the aqueous layer was extracted with CH<sub>2</sub>Cl<sub>2</sub> (2x20 mL). The combined organic layers were washed with H<sub>2</sub>O (1x30 mL) [note: Brine was avoided to prevent bad emulsion.], dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 1-5% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) to provide 1.66 g (98%) of amide **S6**. [note: During the purification, impurities were eluted in pure CH<sub>2</sub>Cl<sub>2</sub>, and the product was eluted in 2 % MeOH/CH<sub>2</sub>Cl<sub>2</sub>]. **S6:** *R<sub>f</sub>* = 0.32 (4% MeOH/CH<sub>2</sub>Cl<sub>2</sub>); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>; ~1.8:1 mixture of rotamers as determined by <sup>1</sup>H NMR analysis) δ 7.61 – 7.49 (m, 4H), 7.41 – 7.27 (m, 11H), 6.96 – 6.81 (m, 3H), 5.07 (d, *J* = 8.0 Hz, 2H), 4.92 (t, *J* = 6.1, 5.6 Hz, 0.4H)\*\*, 4.47 (t, *J* = 6.0 Hz, 0.6H)\*, 4.22 (s, 1.23H)\*, 3.97 (s, 0.77H)\*\*, 3.77 (s, 1.9H)\*, 3.58 (t, *J* = 7.0 Hz, 1H), 3.46 (s, 1.1H)\*\*, 3.30 – 3.18 (m, 2H), 2.81 (q, *J* = 6.6 Hz, 1H), 1.75 – 1.55 (m, 2H), 1.47 – 1.22 (m, 2H), 1.05 (p, *J* = 7.1 Hz, 1H).

\*denotes major rotamer

\*\* denotes minor rotamer

<sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>; ~1.8:1 mixture of rotamers as determined by <sup>1</sup>H NMR analysis) δ 169.5, 169.5, 167.8, 156.5, 156.3, 147.2, 143.2, 143.1, 139.7, 139.6, 136.6, 136.6, 129.3, 129.3, 128.5, 128.5, 128.3, 128.3, 128.2, 128.1, 128.1, 126.6, 126.4, 122.3, 122.2, 121.0, 120.9, 117.6,

117.5, 117.4, 109.9, 109.8, 66.6, 52.2, 52.1, 50.4, 49.6, 46.9, 46.2, 40.7, 40.2, 29.7, 27.1, 26.7, 25.4, 24.3. **HR-MS:**  $m/z$  Calcd. for  $C_{35}H_{34}N_2O_7$   $[M+H^+]$ :595.2366, Found 595.2504.



**Intermediate I2 (for preparation of compound S7):** To a 500-mL flame-dried, round-bottom flask containing a solution of compound **S6** (1.62 g, 2.72 mmol) in anhydrous MeOH (27 mL) was added 10% Pd/C (174 mg, 1.63 mmol). The flask was fitted with a 3-way valve-adaptor to evacuate and purge the system with  $H_2$  gas. After purging the system with  $H_2$  (3x), the mixture was stirred at  $23^\circ\text{C}$  for 1.5 h, at which point the reaction was complete as determined by TLC analysis (50% EtOAc/hexanes). The mixture was filtered through celite, washed with MeOH, and concentrated under reduced pressure to provide 1.24 g of the crude intermediate **I2**, which was used immediately without further purification in the next reaction. **I2:** **HR-MS:**  $m/z$  Calcd. for  $C_{27}H_{28}N_2O_5$   $[M+H^+]$ :461.1998, Found 461.2154.

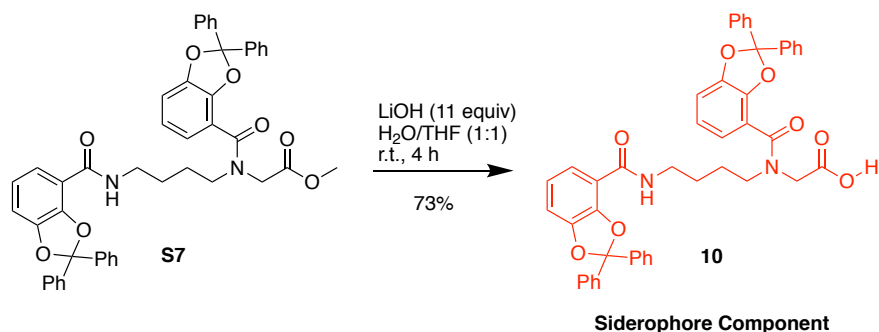
**S7:** To a 250-mL flame-dried, round-bottom flask containing a solution of the crude intermediate **I2** (1.02 g, 2.22 mmol, 1.0 equiv) in THF (9.5 mL) under argon was added TEA (0.61 mL). To this was added the acid chloride **I1** (1.46 g, 4.35 mmol, 1.96 equiv, prepared as described above) in three portions as a solution in THF (19 mL; portion 1: 11.7 mL, rinse portion 2: 3.9 mL, and rinse portion 3: 3.4 mL). The reaction was placed in a preheated oil bath at  $50^\circ\text{C}$  and stirred for 9 h at this temperature. The mixture was then cooled to  $23^\circ\text{C}$ , and TLC analysis (55% EtOAc/hexanes) suggested that the reaction was complete. The reaction was then diluted with  $CH_2Cl_2$  (20 mL) and 1 M HCl (20 mL), and the layers were separated. The aqueous layer

was extracted with CH<sub>2</sub>Cl<sub>2</sub> (2 x 20 mL). The combined organic layers were washed with H<sub>2</sub>O (1x20 mL) and brine (1 x 20 mL), dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated under reduced pressure. Purification by column chromatography (silica gel, 20-60% EtOAc/hexanes; note: The product began eluting at 50% EtOAc/hexanes, and the product was fully-eluted at 60% EtOAc/hexanes) provided 771 mg (46% over 2 steps) of compound **S7** and an additional 805 mg of crude **S7**. [note: Further purification of the recovered 805 mg of crude **S7** was not necessary, which allowed for saponification (described below) and subsequent purification to provide the ketal-protected siderophore **10** as a pure product]. **S7**:  $R_f$  = 0.25 (55% EtOAc/hexanes); <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD, ~2:1 mixture of rotamers as determined by <sup>1</sup>H NMR analysis) δ 7.72 – 6.34 (m, 26H), 4.22 (s, 1.2H)\*, 4.00 (m, 0.5H)\*\*, 3.94 (s, 0.2H)\*\*, 3.72 – 3.48 (m, 0.94H), 3.65 (s, 2.1H)\*, 3.39 (s, 0.9H)\*\*, 3.06 (t,  $J$  = 6.7 Hz, 1.44H), 1.85 – 1.70 (m, 1.1H), 1.55 – 1.33 (m, 1.5H), 1.31 – 1.08 (m, 1.6H).

\*denotes major rotamer

\*\* denotes minor rotamer

<sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD; ~2:1 mixture of rotamers as determined by <sup>1</sup>H NMR analysis) δ 169.6, 169.4, 168.8, 168.6, 164.7, 164.5, 147.5, 147.3, 147.2, 144.9, 144.8, 143.0, 142.7, 139.7, 139.5, 139.3, 139.2, 129.4, 129.3, 129.2, 129.1, 129.0, 128.9, 128.2, 128.1, 128.0, 128.0, 128.0, 127.9, 127.7, 126.1, 126.0, 126.0, 125.9, 125.9, 125.8, 122.2, 122.1, 121.9, 121.8, 121.3, 120.3, 120.1, 118.2, 117.5, 117.3, 117.1, 115.9, 115.8, 111.4, 111.4, 109.7, 109.6, 51.2, 51.2, 50.0, 49.9, 46.3, 39.0, 38.5, 26.6, 26.0, 25.3, 24.2. **HR-MS**:  $m/z$  Calcd. for C<sub>47</sub>H<sub>40</sub>N<sub>2</sub>O<sub>8</sub> [M+H<sup>+</sup>]:761.2785, Found 761.2534.



**Siderophore Component 10:** To a 20-mL scintillation vial containing a solution of methyl ester **S7** in THF/H<sub>2</sub>O (1:1, 11.6 mL) was added lithium hydroxide monohydrate (261 mg, 6.21 mmol, 11 equiv). The mixture was stirred at 23 °C for 4 h. The reaction was complete as determined by TLC analysis (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>). The mixture was then acidified with 1 M HCl (4 mL), stirred for 2 min, and extracted with CH<sub>2</sub>Cl<sub>2</sub> (3x4 mL). The combined organic layers were dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated under reduced pressure. Purification by column chromatography (silica gel, 55% EtOAc/hexanes (to remove impurity); then 2-10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) provided 320 mg (73%) of diphenylketal-protected siderophore **10**. **Siderophore Component 10:**  $R_f = 0.52$  (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>); <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD, ~1.9:1 mixture of rotamers as determined by <sup>1</sup>H NMR analysis)  $\delta$  7.87 – 6.58 (m, 26H), 4.18 (s, 1.3H)\*, 3.94 (s, 0.7H)\*\*, 3.61 (t,  $J = 6.0$  Hz, 0.7H), 3.47 (q,  $J = 6.1$  Hz, 0.7H), 3.23 (t,  $J = 7.6$  Hz, 1.3H), 3.00 (q,  $J = 6.5$  Hz, 1.3H), 1.71 (t,  $J = 3.0$  Hz, 1.4H), 1.42 (p,  $J = 8.2$  Hz, 1.3H), 1.10 (p,  $J = 7.2$  Hz, 1.3H).

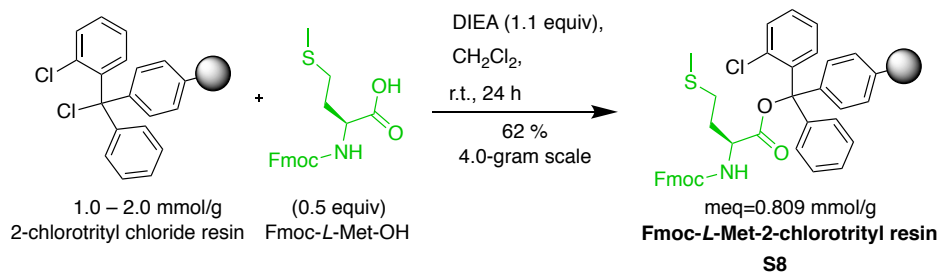
\*denotes major rotamer

\*\* denotes minor rotamer

<sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD; ~1.9:1 mixture of rotamers as determined by <sup>1</sup>H NMR analysis)  $\delta$  170.7, 170.6, 168.8, 168.5, 164.6, 164.4, 164.4, 147.5, 147.5, 147.3, 147.2, 145.0, 144.8, 143.0, 142.8, 139.7, 139.5, 139.3, 139.2, 129.5, 129.3, 129.1, 129.0, 128.3, 128.2, 128.1, 126.1, 126.0, 126.0, 125.9, 122.2, 122.2, 122.0, 121.9, 121.5, 120.3, 120.2, 118.2, 117.6, 117.5, 117.3, 116.0, 115.9, 111.5, 111.4, 109.7, 109.6, 53.5, 50.1, 49.9, 46.8, 46.2, 39.2, 38.8, 38.7, 26.7, 26.1, 25.4, 24.3. **HR-MS:**  $m/z$  Calcd. for C<sub>46</sub>H<sub>38</sub>N<sub>2</sub>O<sub>8</sub> [M+H<sup>+</sup>]:747.2628, Found 747.2713.

## B. Linker Synthesis [amino acid sequences: WSPKYM and WSWC]

**Resin Loading onto 2-Chlorotrityl Resin:** For large-scale peptide synthesis (>3 mmol), the amino acids were manually loaded onto 2-chlorotrityl chloride resin (1-2 mmol/g, Chem-Impex). For small-scale peptide synthesis (<0.1 mmol), the P1 amino acid was purchased preloaded onto 2-chlorotrityl chloride resin. For manual loading, 2-chlorotrityl chloride resin (>4.2 g) and amino acid (>3.0 mmol) were dried on high vacuum overnight before use in 20-mL scintillation vials. Only anhydrous reagents were used.



### General Resin-Loading Procedure (Gram-Scale):

**Fmoc-*L*-methionine-2-chlorotrityl resin S8:** To a 500-mL flame-dried, round-bottom flask was added dry Fmoc-*L*-methionine (1.11 g, 3.0 mmol). The flask was purged with argon, and the amino acid was then suspended in anhydrous CH<sub>2</sub>Cl<sub>2</sub> (40 mL). The flask was placed in a sonicator for 5 min to assist with solubilizing the amino acid. (note: A uniform solution did not form until DIEA was added). Dry 2-chlorotrityl chloride resin (4.0 g, ~6.0 mmol, 1.0 – 2.0 mmol/g) was added in a single portion, and the sides of the flask were rinsed with additional anhydrous CH<sub>2</sub>Cl<sub>2</sub> (20 mL). The addition of anhydrous DIEA (1.2 mL, 6.90 mmol) resulted in a dark uniform solution, and the mixture was mixed vigorously on a Mistral Multi-Shaker® for 24 h. The resin was then filtered through a 50-mL solid phase peptide synthesis vessel and washed with CH<sub>2</sub>Cl<sub>2</sub> (3x20 mL). Resin capping was achieved by adding a 2.5% solution of MeOH (0.8 mL) and DIEA (0.8 mL) in CH<sub>2</sub>Cl<sub>2</sub> (30.4 mL), and the solid phase peptide synthesis vessel was rotated for 30 min on a Fisherbrand™ nutator. The resin was then washed with CH<sub>2</sub>Cl<sub>2</sub> (3x20 mL), dried on high vacuum to provide 4.61 g (3.73 mmol, 0.809 mmol/g) of **S8** as a violet-colored resin in 62% yield.

### General Procedure for Quantification of Resin Loading:

To three, 20-mL scintillation vials containing Fmoc-*L*-methionine-2-chlorotrityl resin **S8** (10 mg) was added a solution of 30% piperidine in DMF (0.5 mL). The vials were capped, mixed, and allowed to stand for 30 min. Absolute ethanol (19.5 mL) was added to each of the three vials, which were then shaken, and the resin was allowed to settle for 5 min. The absorbance was measured at 300nm using a Thermo Scientific NanoDrop® ND-1000 spectrophotometer. The following equation was used to calculate the resin loading from absorbance:

$$\text{Resin Loading (mmol/g)} = [3.05 \times (\text{absorbance average})/10 \text{ mg}] \times 10$$

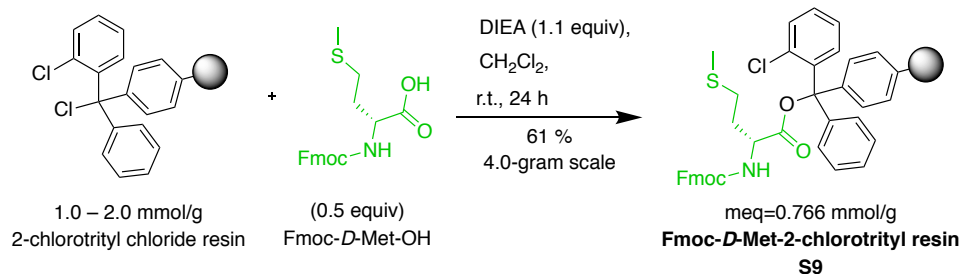
The x10 factor is due to the small path length of the nanodrop; 3.05 was calculated on a 1.0 cm quartz cuvette.

Vial 1:  $\text{abs}_{300\text{nm}}$ : 0.292 nm, 0.285 nm, 0.285 nm, 0.298 nm, 0.291 nm  $\text{abs}_{\text{avg}}$ : 0.290 nm  
 $3.05 \times 0.290 / 10.1 \text{ mg} \times 10 = 0.876 \text{ mmol/g}$ .

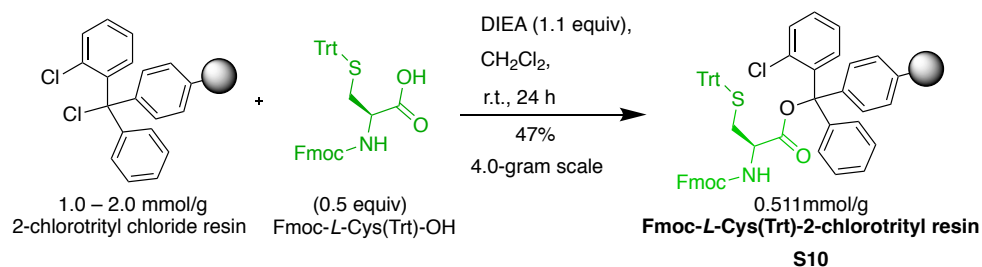
Vial 2:  $\text{abs}_{300\text{nm}}$ : 0.227 nm, 0.234 nm, 0.238 nm, 0.224 nm, 0.221 nm  $\text{abs}_{\text{avg}}$ : 0.229 nm  
 $3.05 \times 0.229 / 10 \text{ mg} \times 10 = 0.698 \text{ mmol/g}$ .

Vial 3:  $\text{abs}_{300\text{nm}}$ : 0.290 nm, 0.272 nm, 0.273 nm, 0.282 nm, 0.281 nm  $\text{abs}_{\text{avg}}$ : 0.280 nm  
 $3.05 \times 0.280 / 10 \text{ mg} \times 10 = 0.854 \text{ mmol/g}$ .

**Resin loading for Fmoc-*L*-methionine-2-chlorotrityl resin = 0.809 mmol/g (Loading Average)**



**Fmoc-*D*-methionine-2-chlorotrityl resin S9** (4.76 g, 3.64 mmol, 0.766 mmol/g) was provided in 61% yield in the same manner described above for the preparation of Fmoc-*L*-methionine-2-chlorotrityl resin **S8** from Fmoc-*D*-methionine (1.11 g, 3.00 mmol), 2-chlorotrityl chloride resin (4.0 g, ~6.00 mmol, 1 – 2 mmol/g), DIEA (1.2 mL, 6.9 mmol), and CH<sub>2</sub>Cl<sub>2</sub> (60 mL).

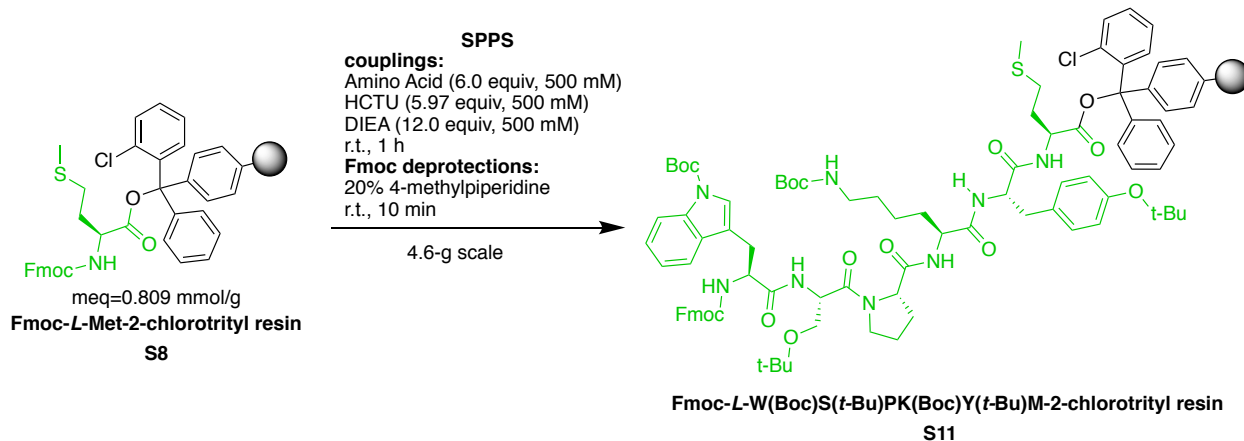


**Fmoc-L-Cys(Trt)-2-chlorotrityl resin S10** (5.48 g, 2.8 mmol, 0.511 mmol/g) was provided in 47% yield in the same manner described above for the preparation of Fmoc-L-methionine-2-chlorotrityl resin **S8** from Fmoc-L-Cys(Trt)-OH (1.8 g, 3.0 mmol), 2-chlorotrityl chloride resin (4.0 g, ~6.00 mmol, 1 – 2 mmol/g), DIEA (1.2 mL, 6.9 mmol), and CH<sub>2</sub>Cl<sub>2</sub> (60 mL).

### General Procedure for Linker Synthesis on 2-Chlorotrityl Resin (Gram-Scale):



Nitrogen flow was used to mix the beads (see picture above). When vacuum was applied to remove solvent from the vessel, the system was purged with argon using an argon balloon and a 24/40 septum that was fitted on top of the vessel to prevent undesired oxidation. For the following coupling reactions and Fmoc-deprotections described below, all stock solutions of 4-methylpiperidine, amino acids, HCTU, and DIEA were prepared in DMF directly before use.



**Fmoc-L-(W(Boc)S(t-Bu)PK(Boc)Y(t-Bu)M)-2-chlorotrityl resin S11:** To a CHEMGLASS® 500-mL medium frit solid phase peptide synthesis vessel (see photo) was added Fmoc-L-methionine-2-chlorotrityl resin **S8** (4.62 g, 3.73 mmol, 0.809 mmol/g). The resin was swelled for 5 min in CH<sub>2</sub>Cl<sub>2</sub> (100 mL). For each Fmoc-deprotection, 20% 4-methylpiperidine was added, followed by nitrogen mixing for 10 min (2x100 mL). After each Fmoc-deprotection, the resin was washed with DMF (4x100 mL, mixed with nitrogen for 3 min/wash) and CH<sub>2</sub>Cl<sub>2</sub> (3 x 100 mL, allowed to sit for 3 min/wash without mixing). The coupling of each amino acid was accomplished by sequentially adding the following 500 mM stock solutions in DMF: amino acid (44.8 mL, 22.4 mmol, 6.0 equiv), HCTU (44.7 mL, 22.3 mmol, 5.98 equiv), followed by DIEA (89.6 mL, 44.8 mmol, 12.0 equiv). The reagents were mixed by applying steady nitrogen pressure for 1 h. In the case of serine coupling to proline's secondary amine, the coupling procedure was repeated. [note: Analytical HPLC and LC/MS analysis of Fmoc-L-S(t-Bu)PK(Boc)Y(t-Bu)M-OH revealed that the coupling of Fmoc-L-S(t-Bu)-OH to proline's secondary amine of the peptide PK(Boc)Y(t-Bu)M-2-chlorotrityl resin was incomplete after a single coupling step]. After each coupling was complete, the solvent was removed and the resin was washed with DMF (3x100 mL) and CH<sub>2</sub>Cl<sub>2</sub> (3x100 mL). The reaction progress was evaluated after each step of the synthesis by removing a small aliquot of the resin (~20 mg) and mixing with AcOH/TFE/CH<sub>2</sub>Cl<sub>2</sub> (1:1:3) with 2.5% 1,2-ethanedithiol (EDT) for 15 minutes on a nutator. The filtrate was collected and concentrated under reduced pressure, followed by <sup>1</sup>H-NMR and analytical HPLC analysis. The final coupling reaction provided 7.04 g of Fmoc-L-W(Boc)S(t-Bu)PK(Boc)Y(t-Bu)M-2-chlorotrityl resin **S11**, which can be stored for more than a year at -20 °C without decomposition or methionine oxidation.

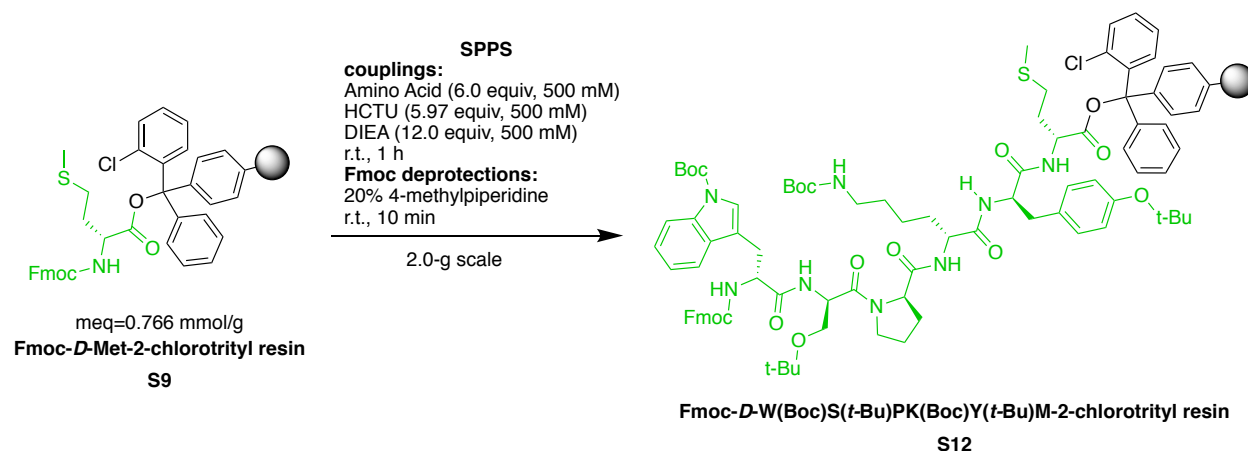


### Amino Acid Stocks (500 mM):

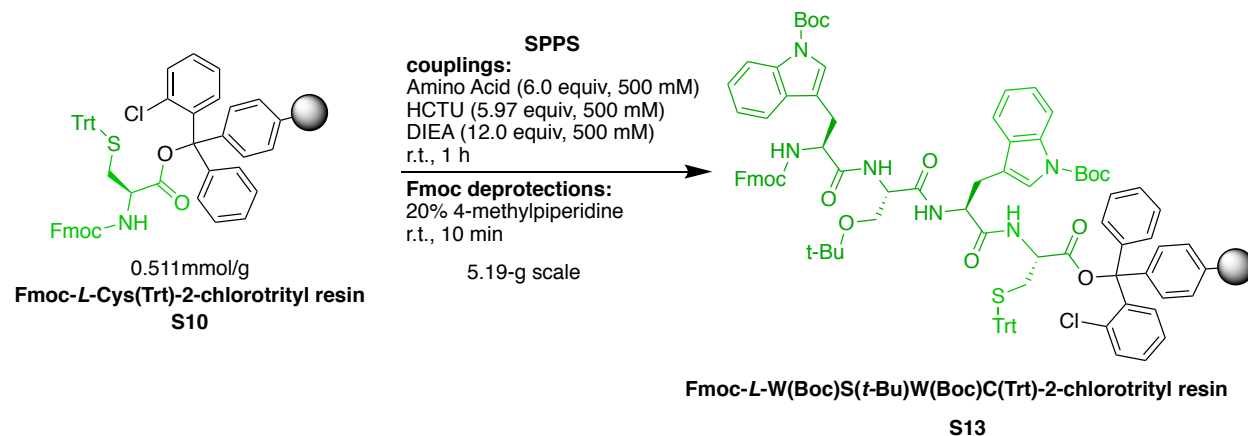
Fmoc-Tyr(*t*-Bu)-OH (11.3 g) in DMF (49.3 mL, 500 mM), Fmoc-Lys(Boc)-OH (11.5 g) in DMF (49.3 mL, 500 mM), Fmoc-Pro-OH (8.31 g) in DMF (49.3 mL, 500 mM), Fmoc-Ser(*t*Bu)-OH (9.45 g) in DMF (49.3 mL, 500 mM), Fmoc-Trp(Boc)-OH (13.0 g) in DMF (49.3 mL, 500 mM),

**HCTU Stock (500 mM):** HCTU (10.2 g) in DMF (49.1 mL, 500 mM)

**DIEA Stock (500 mM):** DIEA (8.6 mL) in DMF (90 mL)



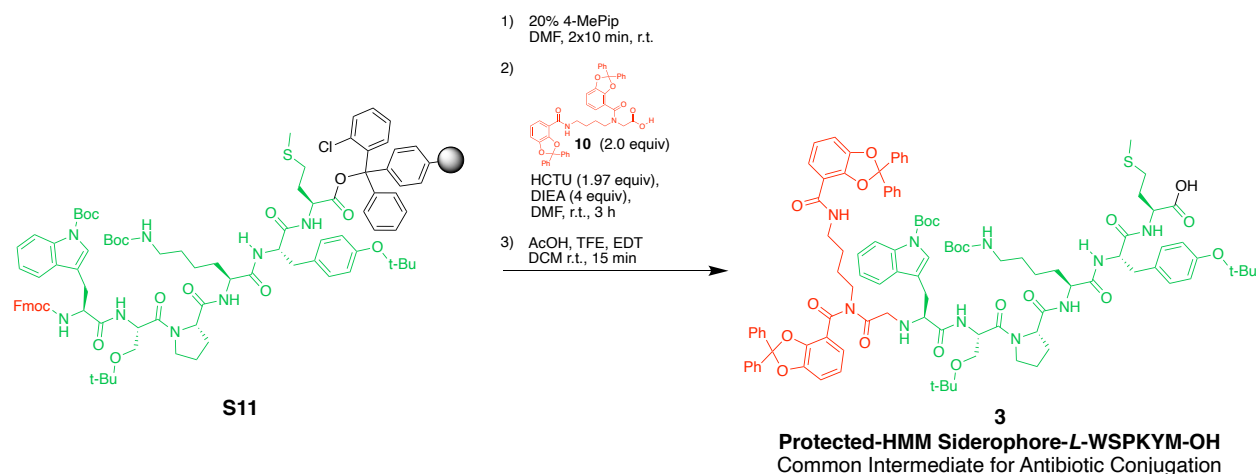
**Fmoc-*D*-W(Boc)S(*t*-Bu)PK(Boc)Y(*t*-Bu)M-2-chlorotrityl resin S12** (2.93 g) was afforded from Fmoc-*D*-methionine-2-chlorotrityl resin **S9** (2.0 g, 1.53 mmol, 0.766 mmol/g) in the same manner described above for the preparation of Fmoc-*L*-W(Boc)S(*t*-Bu)PK(Boc)Y(*t*-Bu)M-2-chlorotrityl resin **S11**.



**Fmoc-*L*-W(Boc)S(*t*-Bu)W(Boc)C(Trt)-2-chlorotrityl resin S13** (5.53 g) was afforded from Fmoc-*L*-Cys(Trt)-2-chlorotrityl resin **S10** (5.19 g, 4.68 mmol, 0.511 mmol/g) in the same manner described above for the preparation of Fmoc-*L*-W(Boc)S(*t*-Bu)PK(Boc)Y(*t*-Bu)M-2-chlorotrityl resin **S11**.

### C. Conjugate Synthesis (On Solid Phase):

#### General Procedure for Siderophore Coupling and Resin Cleavage (detailed for compound 3):



#### Synthesis of Protected-HMM Siderophore-*L*-WSPKYM-OH, compound 3:

**Step 1 (Fmoc-Deprotection):** Fmoc-*L*-W(Boc)S(*t*-Bu)PK(Boc)Y(*t*-Bu)M-2-chlorotrityl resin **S11** (470 mg) was Fmoc-deprotected by the procedure described in **Section IIIB** [General Procedure for Linker Synthesis on 2-Chlorotrityl Resin] to provide *L*-W(Boc)S(*t*-Bu)PK(Boc)Y(*t*-Bu)M-2-chlorotrityl resin (448.2 mg). The product resin was washed with DMF and swelled in CH<sub>2</sub>Cl<sub>2</sub>, dried under high vacuum, and immediately used in Step 2 (*vide infra*).

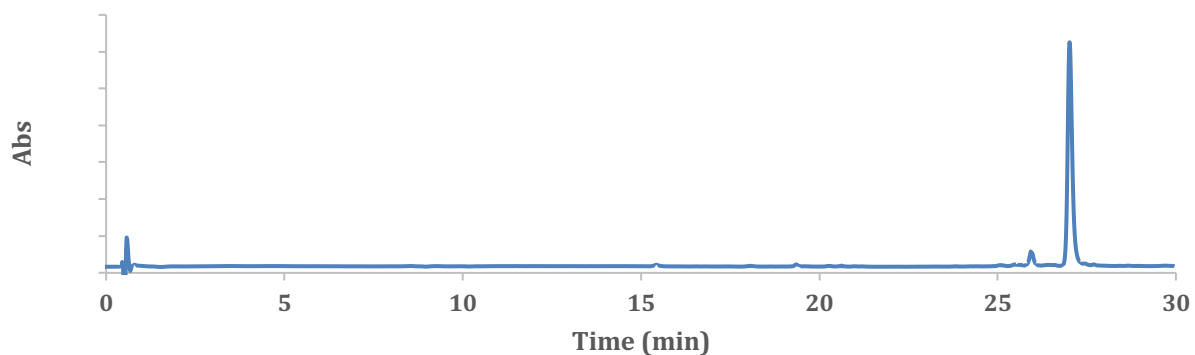
**Step 2 (Siderophore Coupling):** To a 1-dram (3.7 mL) scintillation vial containing *L*-W(Boc)S(*t*-Bu)PK(Boc)Y(*t*-Bu)M-2-chlorotrityl resin (99 mg, 0.080 mmol, 0.809 mmol/g) was added the following solutions in DMF: siderophore component **10** (256 uL, 0.128 mmol, 500 mM in DMF), HCTU (253 uL, 0.126 mmol, 500 mM in DMF), and DIEA (512 uL, 0.256 mmol, 500 mM in DMF). The vial was capped, and the mixture was shaken vigorously on a Mistral Multi-Shaker® for 3 h. The resin was transferred *via* pipet (with DMF) to a 50-mL solid phase peptide synthesis vessel. The product resin was washed with DMF (3x6 mL) and CH<sub>2</sub>Cl<sub>2</sub> (3x6 mL) and immediately used in the next step.

**Step 3 (Resin Cleavage, Synthesis of Protected-HMM Siderophore-*L*-WSPKYM-OH, Compound 3):** To the 50-mL solid phase peptide synthesis vessel containing the protected-HMM Siderophore-*L*-WSPKYM-2-chlorotrityl resin (0.080 mmol, isolated in Step 2) was added the following cocktail: AcOH/TFE/CH<sub>2</sub>Cl<sub>2</sub>/EDT (2 mL/2 mL/6 mL/300 μL). The reaction vessel was then capped and mixed on a nutator for 15 min. The mixture was filtered, and the filtrate was

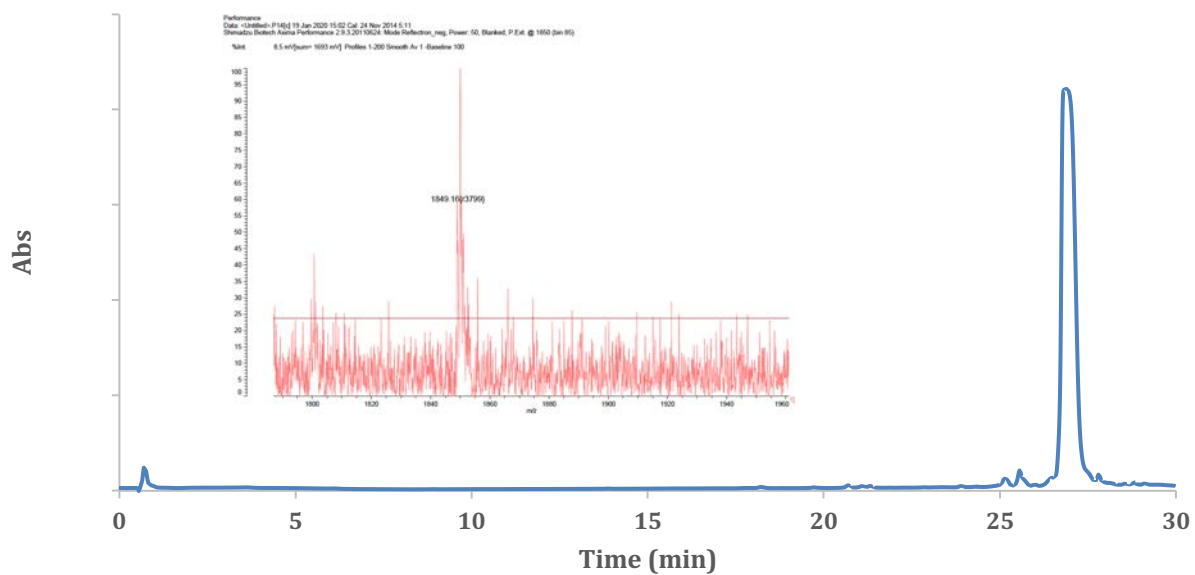
concentrated under reduced pressure. The product was azeotroped with benzene (3x10 mL), sonicating for 10 seconds after adding benzene. [notes: Sonication of the product as a solution in benzene led to the rapid formation of a dry, white solid upon evaporation. Azeotroping with benzene was necessary to remove residual AcOH, which accelerates the oxidation of sulfides in air.<sup>S8</sup> Exposure to oxygen was minimized by backfilling the rotary evaporator with argon when releasing the vacuum. [note: Compound **3** oxidizes slowly in air and will oxidize more rapidly in the presence of AcOH.] Compound **3** was afforded as a white solid in 50% yield over 13 steps (74.8 mg, 40.4  $\mu$ mol). The product was sufficiently pure by analytical HPLC and NMR analysis, and was used in subsequent coupling reactions without further purification.

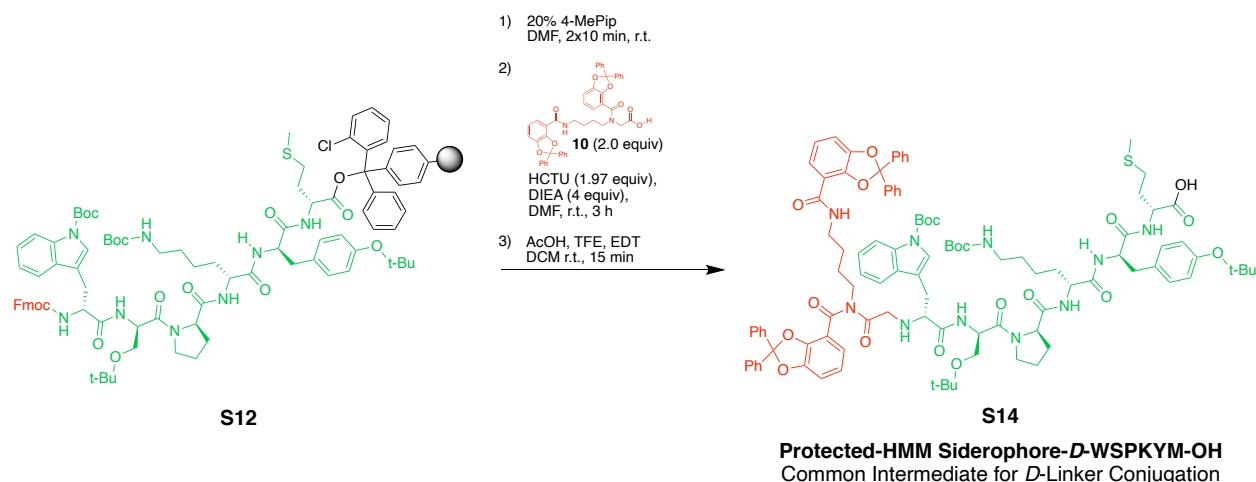
If further purification is needed in the event of oxidation, then purification by column chromatography (silica gel, 2-16% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) provided 62.1 mg (42% over 13 steps) of product **3** as a white solid. [note: The use of AcOH for column chromatography was avoided to minimize further oxidation of product. However, this resulted in significant product dragging with some product remaining on the column. In the case of acids **3** and **S14-S16** it was preferred to take the crude material forward without further purification. Removal of any residual oxidized product could be purified following coupling to the antibiotic (vide infra).] **Protected-HMM Siderophore-L-WSPKYM-OH 3:**  $R_f = 0.27$  (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>); <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD)  $\delta$  8.12 – 7.74 (m, 1H), 7.70 – 6.45 (m, 35H), 4.73 – 3.78 (m, 7H), 3.78 – 3.35 (m, 5H), 3.27 – 2.04 (m, 11H), 2.03 – 1.76 (m, 5H), 1.76 – 1.46 (m, 12H), 1.37 (s, 13H), 1.29 – 1.12 (m, 12H), 1.12 – 0.97 (m, 12H). <sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD)  $\delta$  168.3, 164.4, 156.9, 153.9, 149.5, 147.5, 147.3, 144.8, 142.9, 139.6, 139.3, 139.2, 135.3, 130.4, 129.7, 129.4, 129.2, 129.1, 128.2, 128.2, 128.1, 126.1, 126.0, 125.8, 123.9, 122.0, 121.5, 120.3, 118.7, 118.1, 117.6, 117.3, 115.8, 115.0, 111.5, 109.6, 83.6, 78.4, 78.0, 73.8, 53.5, 27.9, 27.5, 27.1, 26.5. **MALDI-TOF (reflectron mode):**  $m/z$  Calcd. for C<sub>103</sub>H<sub>122</sub>N<sub>10</sub>NaO<sub>20</sub>S [M-H<sup>+</sup>]:1849.86, Found 1849.16.

Protected-HMM Siderophore-*L*-WSPKYM-OH; **3**  
(crude product)

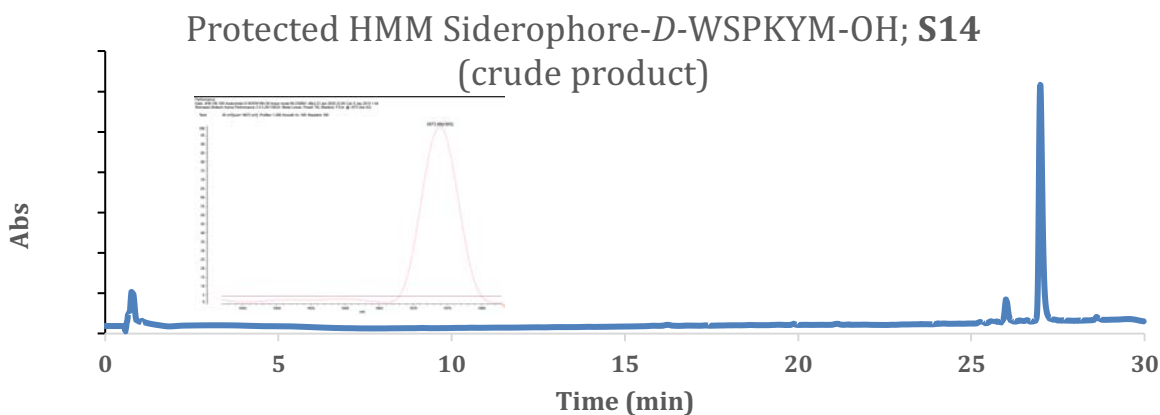


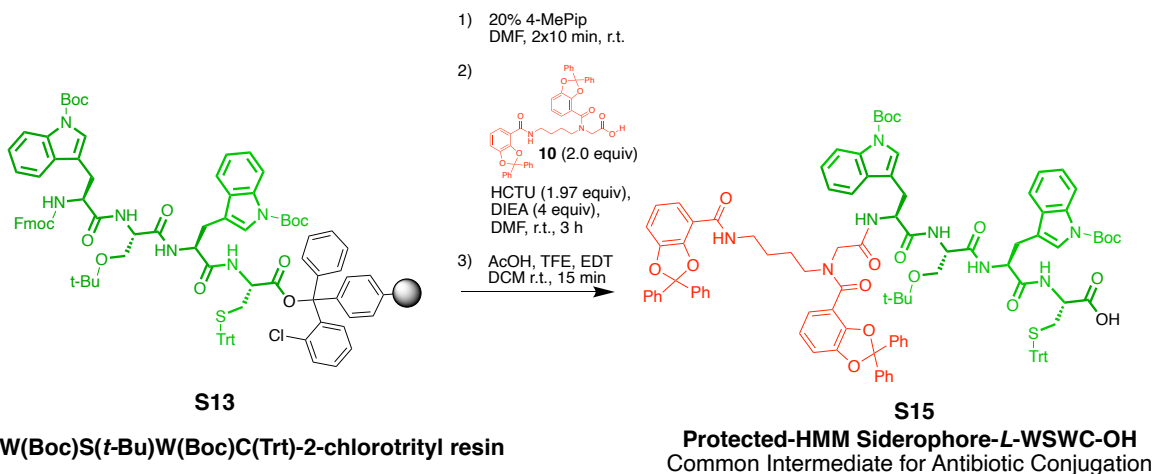
Protected-HMM Siderophore-*L*-WSPKYM-OH; **3**  
(purified product)





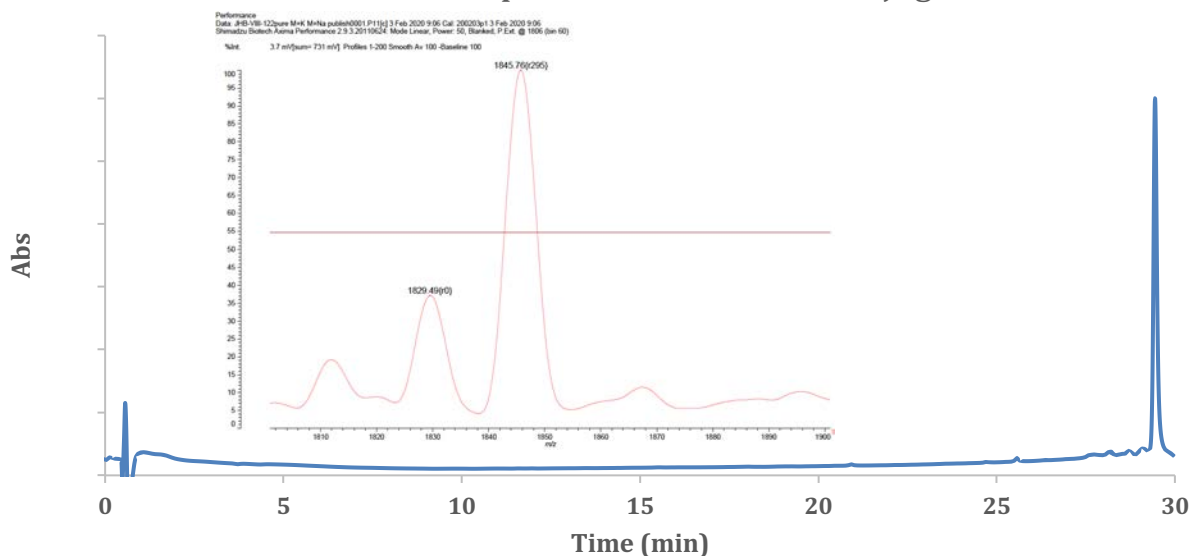
**Protected Siderophore-*D*-WSPKYM-OH, compound S14** (61.2 mg, 47% over 13 steps) was provided by Fmoc-deprotection of **S12** (0.070 mmol), coupling to the siderophore component **10**, and cleavage from resin as described above in the *General Procedure for Siderophore Coupling and Resin Cleavage*. **S14**:  $R_f = 0.44$  (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>); <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD) δ 8.39 – 6.47 (m, 35H), 4.72 – 4.49 (m, 3H), 4.44 – 3.74 (m, 3H), 3.73 – 3.39 (m, 5H), 3.27 – 2.75 (m, 9H), 2.71 – 2.39 (m, 2H), 2.33 – 1.77 (m, 9H), 1.77 – 1.50 (m, 12H), 1.50 – 1.20 (m, 26H), 1.16 – 0.88 (m, 10H). <sup>13</sup>C NMR (100 MHz, MeOD) δ 173.3, 173.2, 172.8, 172.7, 172.6, 172.0, 171.8, 170.1, 168.9, 168.5, 164.3, 164.3, 157.0, 153.8, 149.6, 147.5, 147.2, 147.1, 145.0, 144.8, 142.9, 139.7, 139.3, 139.2, 129.6, 129.5, 129.2, 129.1, 128.3, 128.2, 128.0, 127.9, 126.1, 126.0, 125.8, 124.1, 123.8, 122.3, 122.0, 121.5, 120.3, 118.2, 117.6, 117.3, 115.8, 114.8, 111.5, 109.6, 83.6, 78.4, 78.0, 73.7, 62.3, 61.4, 60.6, 54.5, 54.5, 54.4, 54.1, 53.0, 51.4, 50.0, 48.6, 41.9, 39.8, 39.1, 38.7, 36.6, 31.4, 31.2, 30.9, 30.6, 29.8, 29.4, 29.2, 27.9, 27.5, 27.1, 26.7, 26.4, 26.3, 26.1, 25.3, 24.7, 24.1, 23.1, 13.9. **MALDI-TOF (linear mode)**:  $m/z$  Calcd. for C<sub>103</sub>H<sub>122</sub>N<sub>10</sub>NaO<sub>20</sub>S [M+Na<sup>+</sup>]:1873.85, Found 1873.88.

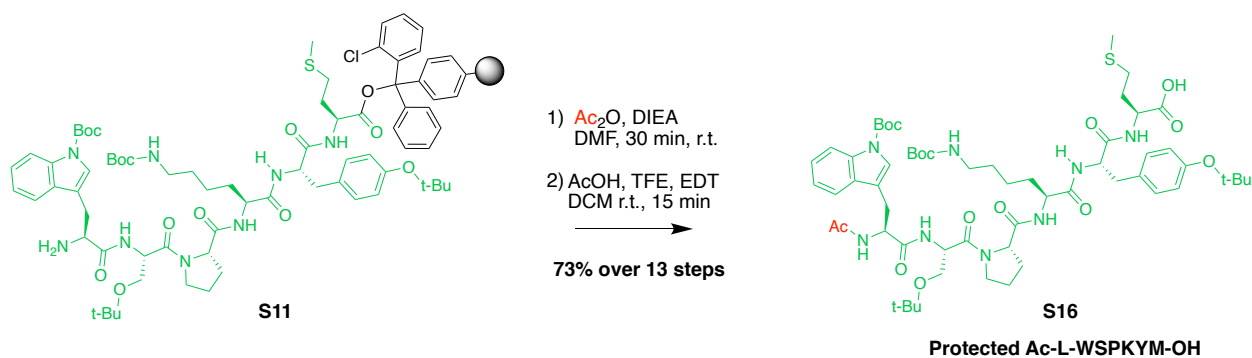




**Protected-HMM Siderophore-L-WSWC-OH, compound S15** (26.4 mg, 36% over 9 steps) was provided by Fmoc deprotection of **S13** (0.041 mmol), coupling to the siderophore component **10**, and cleavage from resin as described above in the *General Procedure for Siderophore Coupling and Resin Cleavage*. **S15**:  $R_f = 0.11$  (5% MeOH/CH<sub>2</sub>Cl<sub>2</sub>); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.38 – 6.40 (m, 50H), 5.20 – 1.95 (m, 18H), 1.80 – 0.65 (m, 31H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 149.6, 149.5, 149.5, 147.2, 146.9, 145.3, 144.7, 144.4, 144.3, 144.0, 143.0, 139.5, 139.0, 138.9, 135.5, 129.7, 129.5, 128.5, 128.3, 128.0, 127.9, 127.8, 127.7, 127.3, 126.8, 126.5, 126.4, 124.5, 122.7, 122.5, 122.2, 115.2, 115.1, 110.0, 100.0, 83.4, 74.0, 29.7, 28.2, 27.3, 27.1, 27.1, 27.1. **MALDI-TOF (linear mode)**:  $m/z$  Calcd. for C<sub>107</sub>H<sub>106</sub>N<sub>8</sub>NaO<sub>17</sub>S [M+Na<sup>+</sup>]:1829.73, Found 1829.49;  $m/z$  Calcd. for C<sub>107</sub>H<sub>106</sub>KN<sub>8</sub>O<sub>17</sub>S [M+K<sup>+</sup>]:1845.70, Found 1845.76.

### Protected-HMM Siderophore-L-WSWC-OH; conjugate **S15**





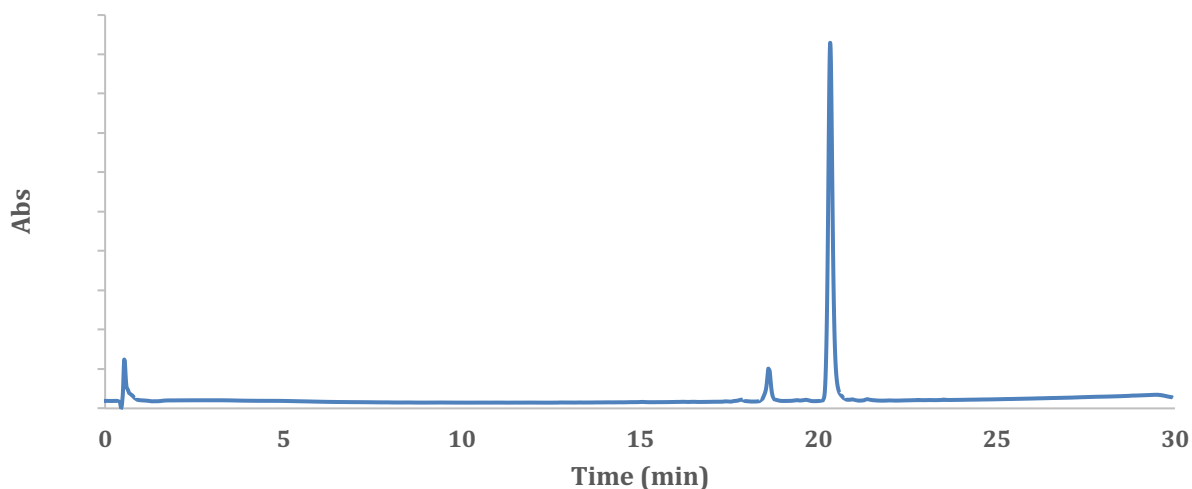
### Protected Ac-L-WSPKYM-OH, compound **S16**:

To a 50-mL solid phase peptide synthesis vessel containing the Fmoc-deprotected resin **S11** (484 mg, 0.391 mmol, 0.809 mmol/g) was added  $\text{CH}_2\text{Cl}_2$  (6 mL) to swell the resin for 2 min. The solvent removed, and DMF (11 mL),  $\text{Ac}_2\text{O}$  (2.3 mL), and DIEA (2.3 mL) were added. The vessel was capped, and the mixture was rotated on a nutator for 15 min. The contents were drained, and the resin was resubjected to the reaction conditions and rotated for an additional 15 min. Following solvent removal, the resin was washed with DMF (3X6 mL),  $\text{CH}_2\text{Cl}_2$  (1x6 mL), MeOH (3x6 mL), and  $\text{CH}_2\text{Cl}_2$  (3x6 mL). The resin was dried under high vacuum and immediately used in the next step (resin cleavage). Cleavage from resin wash achieved as described above for Step 3 in the *General Procedure for Siderophore Coupling and Resin Cleavage*. After drying on high vacuum, the white solid was washed with hexane to remove residual ethanedithiol providing 335 mg (287  $\mu\text{mol}$ , 73% yield) of the *N*-acetylated compound **S16**. The crude product was sufficiently pure by  $^1\text{H}$  NMR and LC analysis. [Use of the crude sample in subsequent steps showed no reduction in yield when compared to that of the purified sample (see below).]

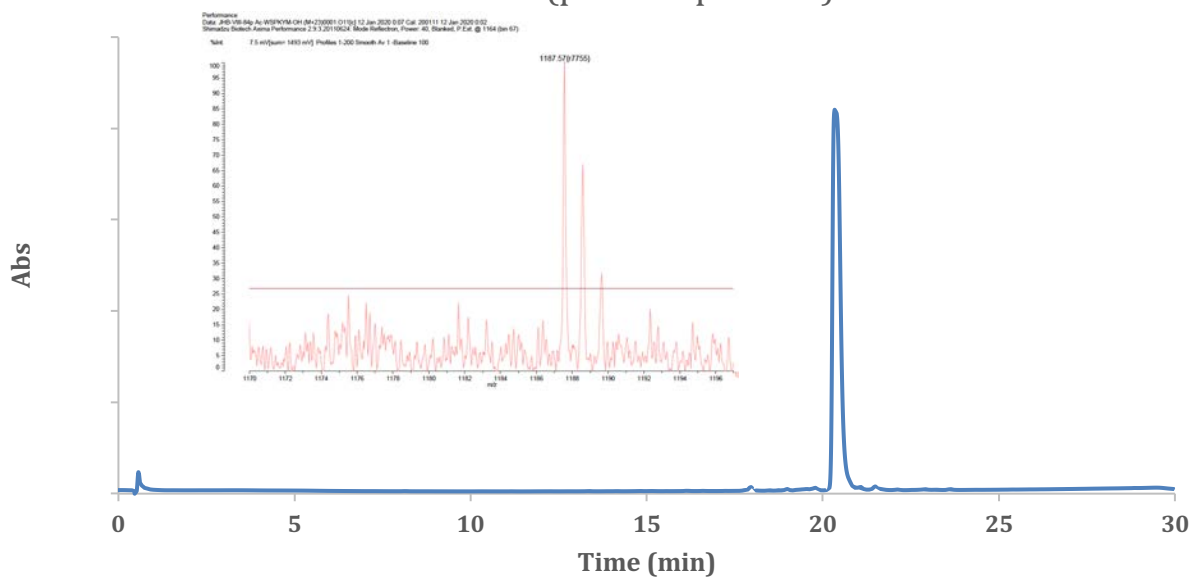
If oxidation product was present as determined by LC/MS analysis, the product was further purified by column chromatography (silica gel, 2-14% MeOH/ $\text{CH}_2\text{Cl}_2$ ; 20% MeOH/ $\text{CH}_2\text{Cl}_2$ ) to provide 200 mg (44%). [note: The use of AcOH for column chromatography was avoided to minimize further oxidation of product. However, this resulted in significant product dragging with some product remaining on the column, which may explain the reduced yield upon chromatographic purification. In the case of acids **3** and **S14-S16**, it was preferred to take the crude material forward without further purification. Any undesired oxidized product could be removed following coupling to the antibiotic (vide infra).] **S16**:  $R_f$  = 0.35 (10% MeOH/ $\text{CH}_2\text{Cl}_2$ );  $^1\text{H}$  NMR (400 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  8.42 – 6.79 (m, 11H), 4.80 – 4.04 (m, 5H), 3.74 – 2.76 (m, 10H), 2.61 – 2.39 (m, 2H), 2.32 – 0.96 (m, 53H).  $^{13}\text{C}$  NMR (100 MHz, MeOD)  $\delta$  174.4, 173.0, 173.0,

172.7, 172.3, 172.2, 171.8, 171.7, 171.5, 171.1, 170.1, 156.9, 153.9, 149.6, 135.3, 132.2, 132.1, 130.5, 130.4, 129.7, 129.6, 124.2, 124.1, 123.9, 123.8, 122.3, 118.8, 116.2, 116.2, 116.0, 114.8, 83.6, 83.4, 79.2, 78.4, 78.0, 73.7, 73.5, 62.4, 61.4, 60.9, 60.4, 54.5, 54.1, 53.7, 53.5, 53.1, 53.0, 52.1, 51.7, 46.8, 39.9, 36.6, 31.2, 29.8, 29.2, 29.0, 28.0, 27.6, 27.2, 26.4, 26.4, 24.8, 23.2, 23.0, 22.2, 21.4, 14.0. **MALDI-TOF (Reflectron mode):**  $m/z$  Calcd. for  $C_{59}H_{88}N_8NaO_{14}S$   $[M+Na^+]$ :1187.60, Found 1187.57.

Protected Ac-L-WSPKYM-OH; **S16**  
(crude product)

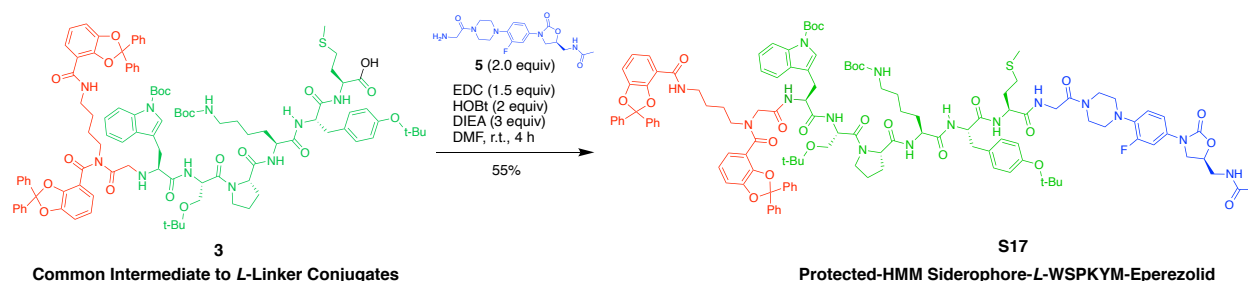


Protected Ac-L-WSPKYM-OH; **S16**  
(purified product)



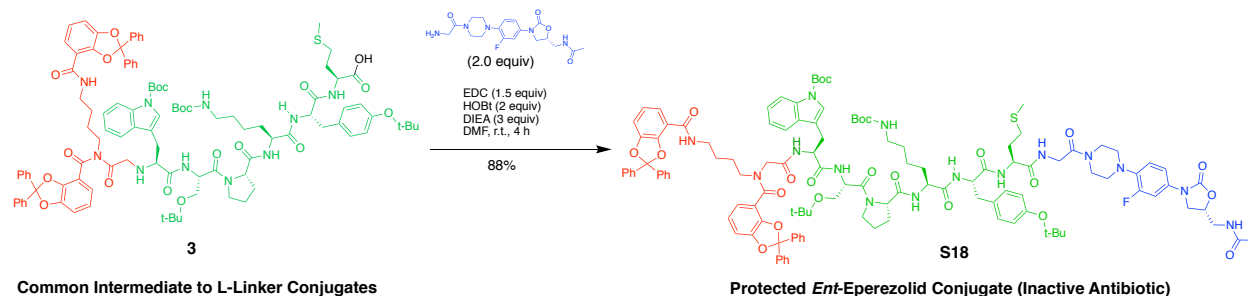


## D. Conjugate Synthesis (Coupling to Antibiotics):



### General Procedure for Eperezolid-NH<sub>2</sub> Coupling:

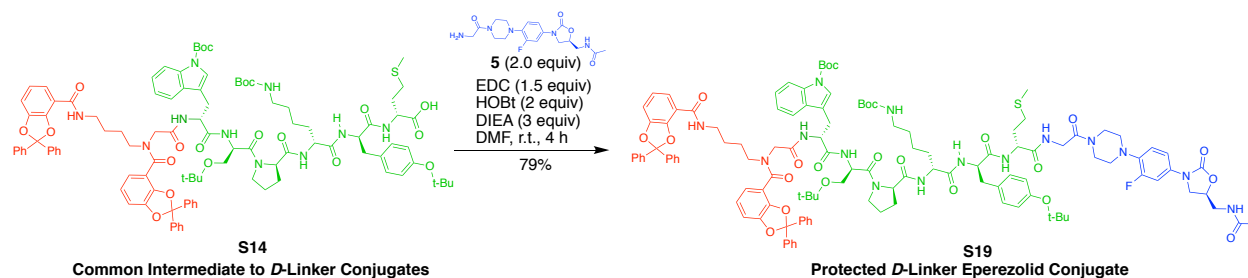
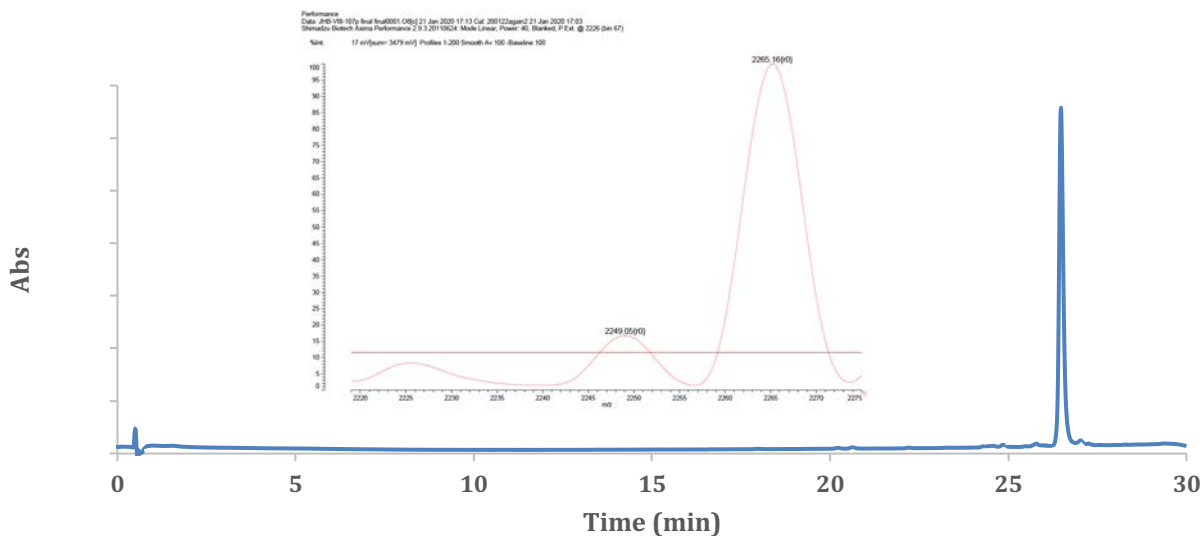
**Protected-HMM Siderophore-L-WSPKYM-Eperezolid, compound S17:** To a 5-mL round-bottom flask was added the protected-HMM Siderophore-L-WSPKYM-OH **3** (20 mg, 0.011 mmol, 1.0 equiv), eperezolid-NH<sub>2</sub> **5** (8.5 mg, 0.022 mmol, 2.0 equiv), EDC (3.1 mg, 0.016 mmol, 1.45 equiv), and HOBt (4.1 mg, 0.022 mmol, 80 wt% in H<sub>2</sub>O, 2.0 equiv). DMF (0.11 mL) was then added, followed by DIEA (5.6  $\mu$ L, 0.032 mmol, 2.91 equiv). The mixture was sonicated until all of the solid dissolved and then was stirred for 4 h. The mixture was concentrated under reduced pressure, and purification by column chromatography (silica gel, 1-10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) provided 13.3 mg (55%) of product **S17** as a white solid. **S17**:  $R_f$  = 0.29 (5% MeOH/CH<sub>2</sub>Cl<sub>2</sub>); <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD)  $\delta$  8.22 – 6.34 (m, 38H), 4.79 – 3.39 (m, 24H), 3.21 – 2.86 (m, 11H), 2.26 – 1.84 (m, 11H), 1.73 – 1.52 (m, 11H), 1.49 – 0.97 (m, 37H). **LC/MS**:  $m/z$  Calcd. for C<sub>121</sub>H<sub>146</sub>FN<sub>15</sub>O<sub>23</sub>S<sup>2+</sup> [M+2/2<sup>+</sup>]:1114.5, Found 1114.6.



**Protected-HMM Siderophore-L-WSPKYM-*ent*-Eperezolid, compound S18** (20.2 mg, 88% yield) was provided from the coupling of the common intermediate **3** (19.0 mg, 10.3  $\mu$ mol, 1.0 equiv) and *ent*-eperezolid-NH<sub>2</sub> (8.07 mg, 20.5  $\mu$ mol, 2.0 equiv) using the procedure described above for the preparation of compound **S17**. **S18**:  $R_f$  = 0.15 (5% MeOH/CH<sub>2</sub>Cl<sub>2</sub>); <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD)  $\delta$  8.13 – 6.38 (m, 38H), 4.83 – 3.39 (m, 24H), 3.25 – 2.82 (m, 12H), 2.63 – 1.51 (m, 27H), 1.51 – 0.98 (m, 34H). <sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD)  $\delta$  172.9, 172.8, 172.6, 172.1, 172.1,

171.7, 167.2, 157.0, 155.1, 154.2, 151.8, 149.6, 147.5, 147.3, 147.1, 144.8, 139.9, 139.6, 139.3, 139.2, 129.6, 129.4, 129.2, 129.1, 128.2, 128.2, 128.0, 126.1, 126.0, 125.8, 124.2, 123.9, 122.3, 122.2, 122.0, 121.4, 119.4, 118.2, 117.6, 117.3, 115.8, 114.1, 111.5, 111.4, 109.6, 107.1, 106.9, 78.4, 78.1, 73.9, 73.7, 73.5, 72.1, 53.4, 53.0, 50.6, 50.2, 48.3, 44.6, 42.0, 41.9, 41.8, 40.7, 38.7, 36.5, 31.0, 29.7, 29.2, 27.9, 27.5, 27.1, 26.4, 26.3, 23.1, 21.1, 13.9. **MALDI-TOF (linear mode):**  $m/z$  Calcd. for  $C_{121}H_{144}FKN_{15}O_{23}S$   $[M+K^+]$ :2264.99, Found 2265.16;  $m/z$  Calcd. for  $C_{121}H_{144}FN_{15}NaO_{23}S$   $[M+Na^+]$ :2249.02, Found 2249.05.

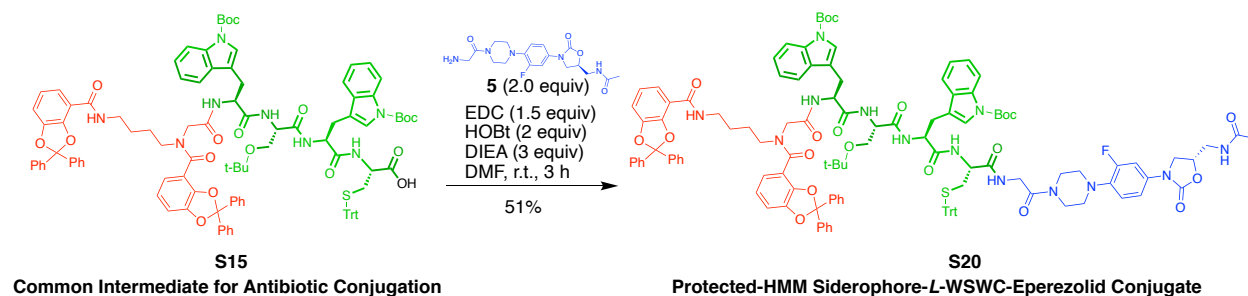
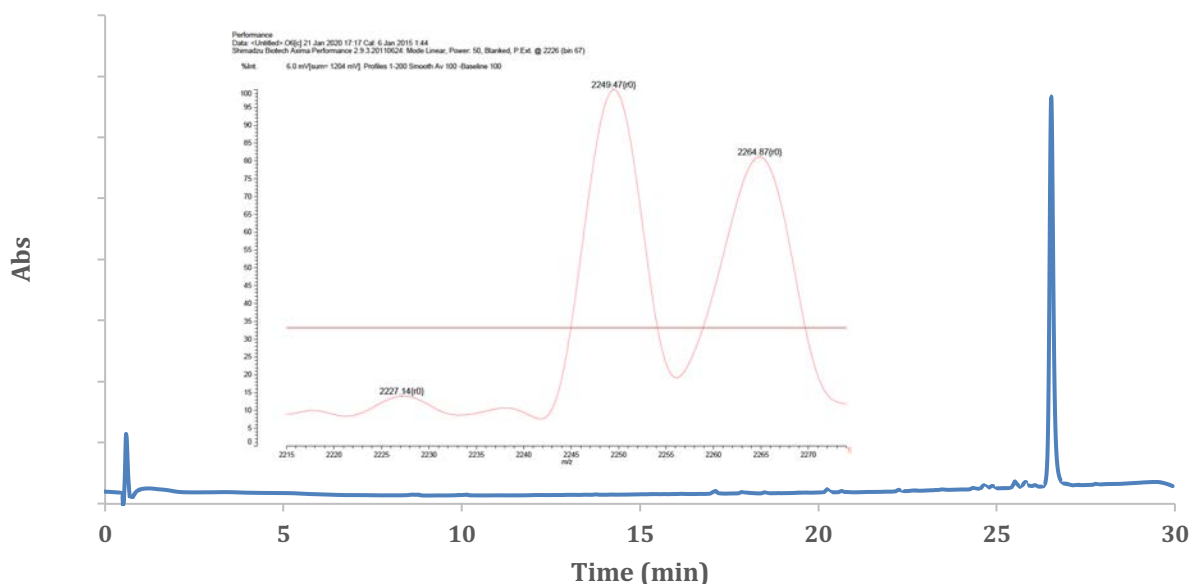
### Protected-HMM Siderophore-*L*-WSPKYM-*ent*-Eperezolid; **S18**



**Protected-HMM Siderophore-*D*-WSPKYM-Eperezolid, compound S19** (18.1 mg, 79% yield) was provided from the coupling of protected-HMM Siderophore-*D*-WSPKYM-OH **S14** (19.0 mg, 10.3  $\mu$ mol, 1.0 equiv) and eperezolid-NH<sub>2</sub> **5** (8.07 mg, 20.5  $\mu$ mol, 2.0 equiv) using the procedure described above for the preparation of protected conjugate **S17**. **S19**:  $R_f$  = 0.14 (5% MeOH/CH<sub>2</sub>Cl<sub>2</sub>); <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD)  $\delta$  8.10 – 6.37 (m, 38H), 4.80 – 4.70 (m, 1H), 4.64 – 4.50 (m, 3H), 4.42 – 3.37 (m, 20H), 3.24 – 2.80 (m, 12H), 2.68 – 2.34 (m, 2H), 2.22 – 1.78 (m, 12H), 1.73 – 1.49 (m, 13H), 1.49 – 1.19 (m, 25H), 1.15 – 0.97 (m, 9H). <sup>13</sup>C NMR (100 MHz,

MeOD)  $\delta$  191.7, 172.6, 167.2, 157.0, 156.6, 155.1, 154.2, 154.0, 147.5, 139.6, 139.2, 129.6, 129.4, 129.1, 128.2, 128.2, 128.0, 126.1, 126.0, 125.8, 123.9, 121.4, 119.5, 118.2, 117.6, 117.3, 115.8, 114.8, 114.0, 111.5, 109.6, 107.1, 83.6, 78.1, 73.7, 72.1, 63.0, 61.2, 54.3, 53.0, 50.6, 50.2, 41.8, 40.7, 40.0, 38.6, 36.5, 31.0, 29.7, 27.9, 27.5, 27.1, 26.4, 26.3, 21.1, 13.9. **MALDI-TOF (linear mode):**  $m/z$  Calcd. for  $C_{121}H_{144}FKN_{15}O_{23}S$   $[M+K^+]$ :2264.99, Found 2264.87;  $m/z$  Calcd. for  $C_{121}H_{144}FN_{15}NaO_{23}S$   $[M+Na^+]$ :2249.02, Found 2249.47.

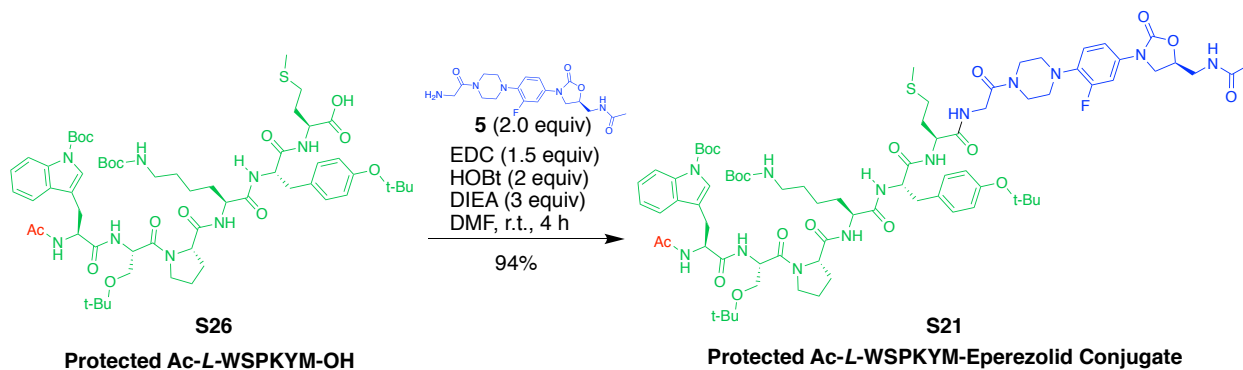
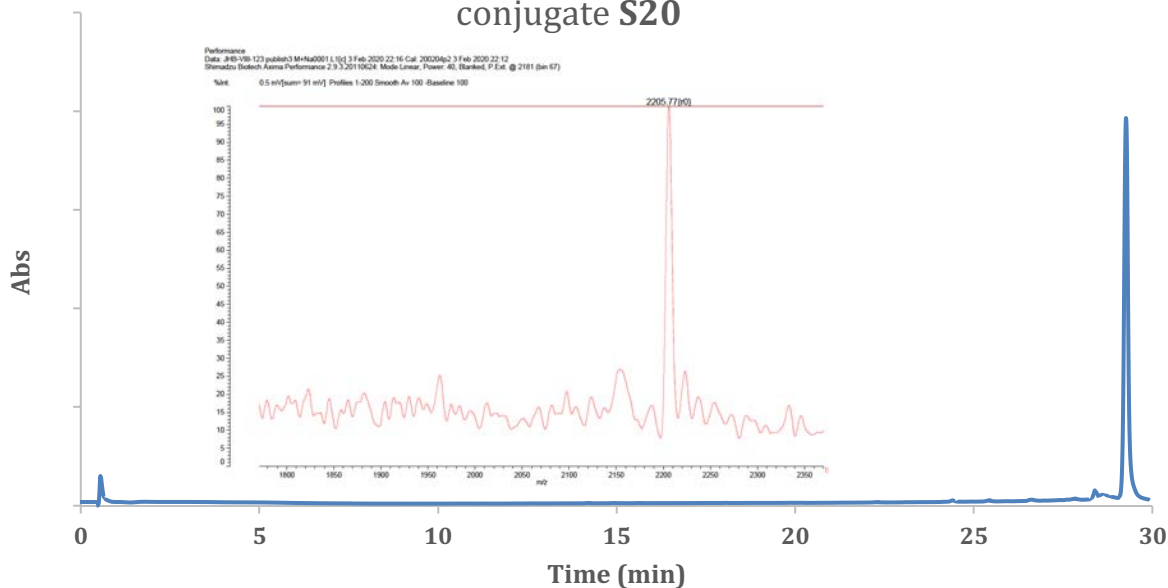
### Protected-HMM Siderophore-*D*-WSPKYM-Eperezolid; **S19**



**Protected-HMM Siderophore-*L*-WSWC-Eperezolid, compound S20** (11.7 mg, 51% yield) was prepared from **S15** (19.0 mg, 10.5  $\mu$ mol, 1.0 equiv) and eperezolid-NH<sub>2</sub> **5** (8.1 mg, 21  $\mu$ mol, 2.0 equiv) by the procedure described above for the preparation of compound **S17**. However, in this case, the reaction was only stirred for 3 h, and purification by column chromatography (silica gel, 0.5-5% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) required a slower gradient. **S20: R<sub>f</sub>** = 0.43 (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  8.13 – 7.27 (m, 31H), 7.25 – 6.54 (m, 19H), 5.25 – 2.54 (m, 33H), 2.04

– 0.67 (m, 34H). **MALDI-TOF (linear mode):**  $m/z$  Calcd. for  $C_{125}H_{128}FKN_{13}O_{20}S$   $[M+Na^+]$ :2264.99, Found 2264.87;  $m/z$  Calcd. for  $C_{121}H_{144}FN_{15}NaO_{23}S$   $[M+Na^+]$ :2204.90, Found 2205.77.

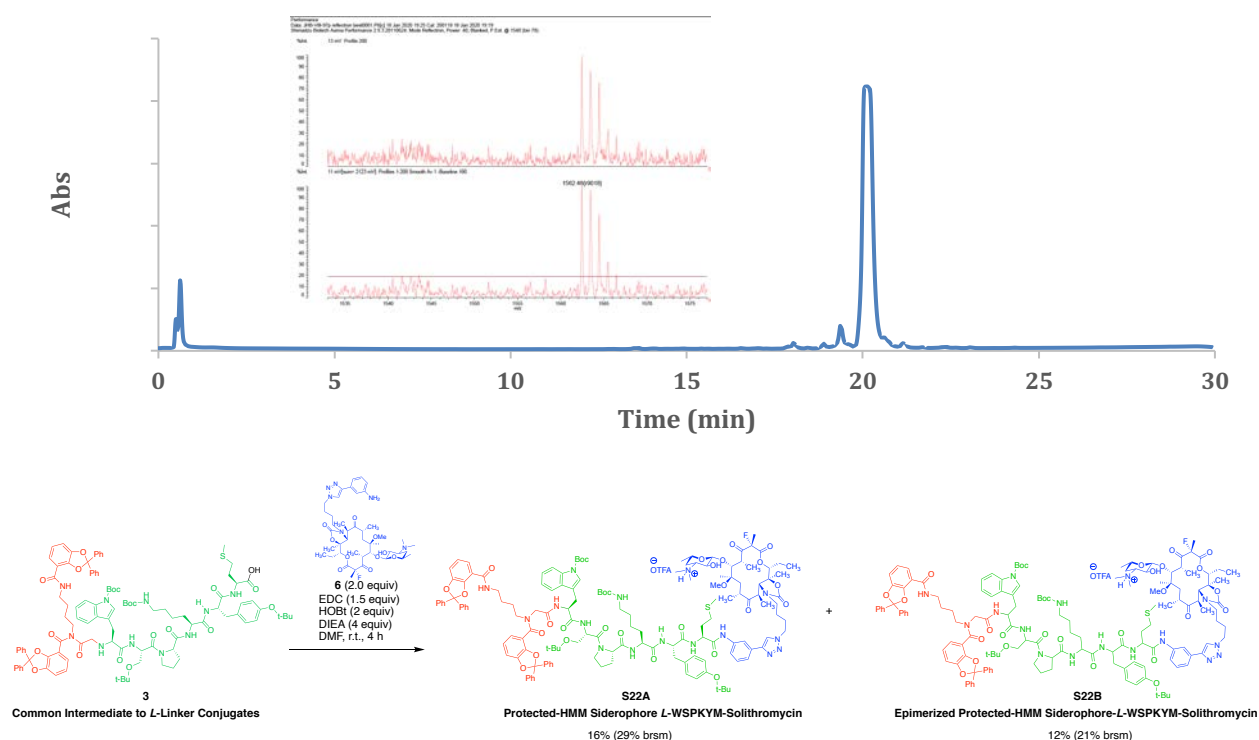
Protected-HMM Siderophore-*L*-WSWC-Eperezolid;  
conjugate **S20**



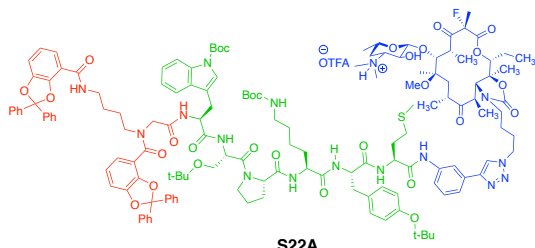
**Protected Ac-*L*-WSPKYM-Eperezolid, compound S21** (21.2 mg, 94% yield) was prepared from **S16** (17.0 mg, 14.6  $\mu$ mol, 1.0 equiv) and eperezolid-NH<sub>2</sub> **5** (11.5 mg, 29.2  $\mu$ mol, 2.0 equiv) by the procedure described above for the preparation of compound **S17**. **S21:  $R_f$**  = 0.41 (10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>); <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD)  $\delta$  8.47 – 7.73 (m, 5H), 7.74 – 6.73 (m, 12H), 4.85 – 3.96 (m, 10H), 3.83 – 3.45 (m, 11H), 3.29 – 2.86 (m, 10H), 2.21 – 1.78 (m, 15H), 1.77 – 1.24 (m, 33H), 1.17 (d,  $J$  = 2.7 Hz, 9H). <sup>13</sup>C NMR (100 MHz, CD<sub>3</sub>OD)  $\delta$  173.1, 173.0, 172.6, 172.1, 171.7, 170.1, 167.2, 157.0, 156.6, 155.1, 154.2, 154.0, 149.6, 149.6, 135.8, 135.8, 135.3, 133.9, 133.8, 132.0, 131.9, 130.4, 129.7, 129.6, 127.0, 124.2, 124.1, 123.9, 122.3, 119.5, 118.8, 116.0,

114.8, 114.0, 107.1, 106.9, 83.6, 78.4, 78.1, 73.7, 73.5, 72.1, 61.4, 60.9, 53.4, 53.1, 52.4, 50.6, 50.2, 44.6, 41.9, 41.8, 40.7, 39.8, 36.5, 31.1, 29.7, 29.2, 29.0, 27.9, 27.5, 27.1, 26.4, 26.3, 24.8, 22.9, 21.2, 21.1, 13.9. **MALDI-TOF (Reflectron mode):**  $m/z$  Calcd. for  $C_{77}H_{110}FN_{13}NaO_{17}S$   $[M+Na^+]$ :1562.77, Found 1562.48.

### Ac-*L*-WSPKYM-Eperezolid, conjugate **S21**



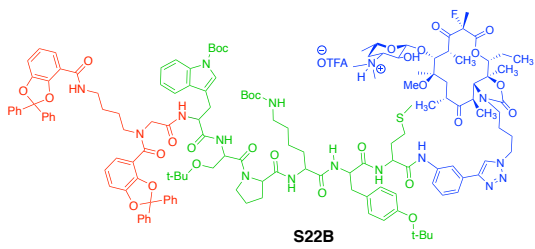
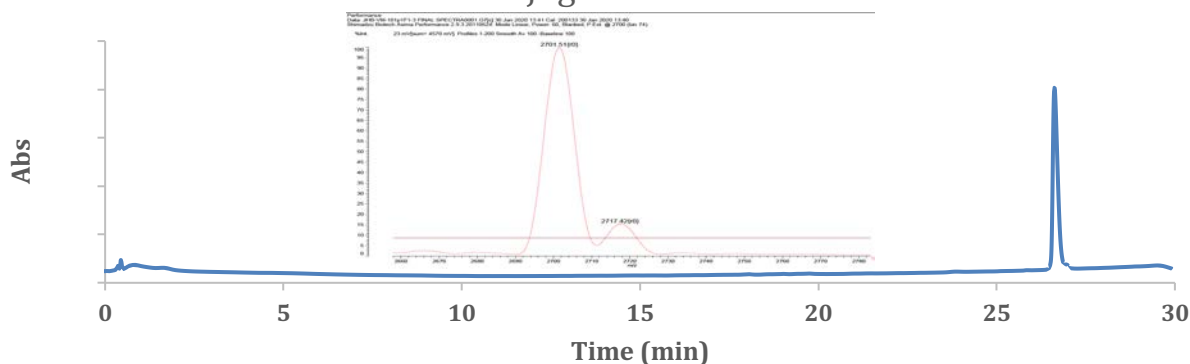
**Protected-HMM Siderophore-*L*-WSPKYM-Solithromycin, Compound **S22A**:** To a 5-mL round bottom flask containing compound **3** (26.9 mg, 15.0  $\mu$ mol, 1.0 equiv) was added solithromycin **6** (24.5 mg, 29.0  $\mu$ mol, 2.0 equiv), HOBt (5.6 mg, 29.0  $\mu$ mol, 2.0 equiv), EDC (4.2 mg, 22.0  $\mu$ mol, 1.50 equiv), DMF (0.15 mL), and DIEA (10.0  $\mu$ L, 58.0  $\mu$ mol, 3.0 equiv). The mixture was placed under argon and was stirred for 4 h. Dilution of the mixture with 70 %  $CH_3CN/H_2O$  (5 mL), followed by purification by prep HPLC (C18 column, 5-60%  $CH_3CN/H_2O$  with 0.1% TFA for 40 min, 60-80%  $CH_3CN/H_2O$  with 0.1% TFA for 30 min, and 80-95%  $CH_3CN/H_2O$  with 0.1% TFA for 5 min; Note: A C18 column is preferred over a C4 column for separation of these compounds.) afforded product **S22A** (6.5 mg, 16% yield, 29% brsm) as a white solid. [note: Product **S22A** elutes at ~70%  $CH_3CN/H_2O$  with 0.1% TFA]. The diastereomer **S22B** (4.8 mg, 12% yield, 21% brsm) was also isolated, which may have resulted from racemization during coupling, along with the recovered starting material **3** (12 mg, 45% yield).



**S22A**  
**Protected-HMM Siderophore *L*-WSPKYM-Solithromycin**  
 16% (29% brsm)

**S22A: MALDI-TOF (Linear mode):**  $m/z$  Calcd. for  $C_{146}H_{185}FN_{16}NaO_{29}S$   $[M+Na^+]$ :2701.31, Found 2701.51;  $m/z$  Calcd. for  $C_{146}H_{185}FKN_{16}O_{29}S$   $[M+K^+]$ :2717.29, Found 2717.42.

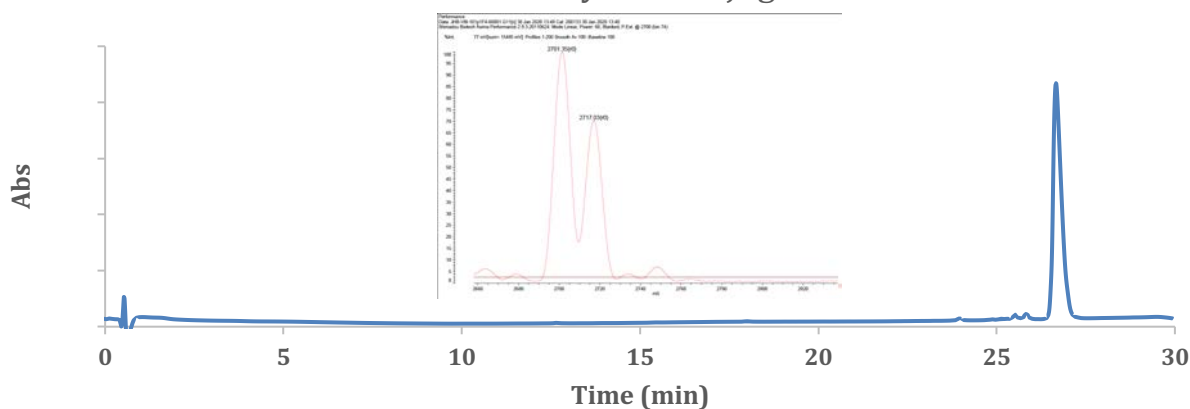
**Protected-HMM Siderophore-*L*-WSPKYM-Solithromycin, conjugate S22A**

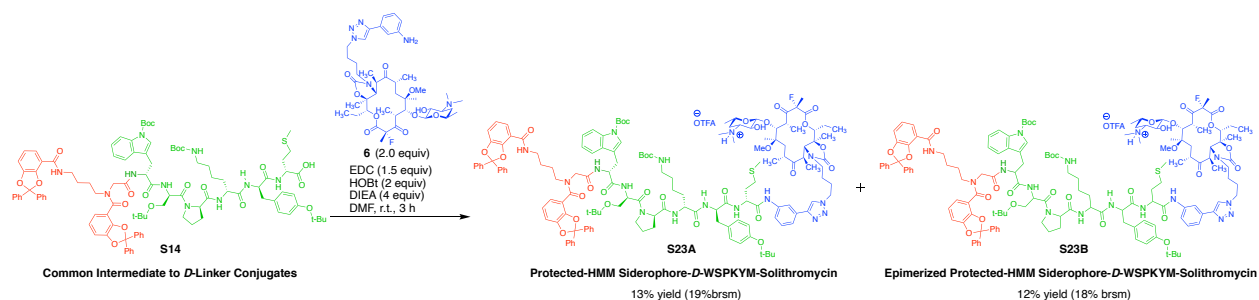


**S22B**  
**Epimerized Protected-HMM Siderophore-*L*-WSPKYM-Solithromycin**  
 12% (21% brsm)

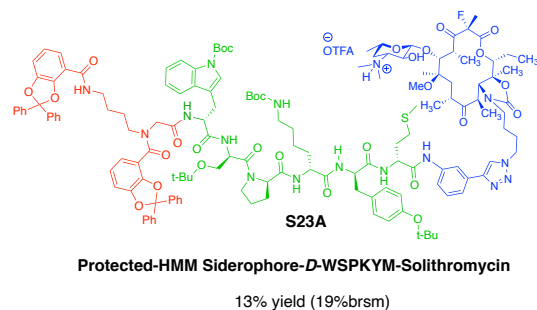
**S22B: MALDI-TOF (Linear mode):**  $m/z$  Calcd. for  $C_{146}H_{185}FN_{16}NaO_{29}S$   $[M+Na^+]$ :2701.31, Found 2701.35;  $m/z$  Calcd. for  $C_{146}H_{185}FKN_{16}O_{29}S$   $[M+K^+]$ :2717.29, Found 2717.03.

**Epimerized Protected-HMM Siderophore-*L*-WSPKYM-Solithromycin; Conjugate S22B**





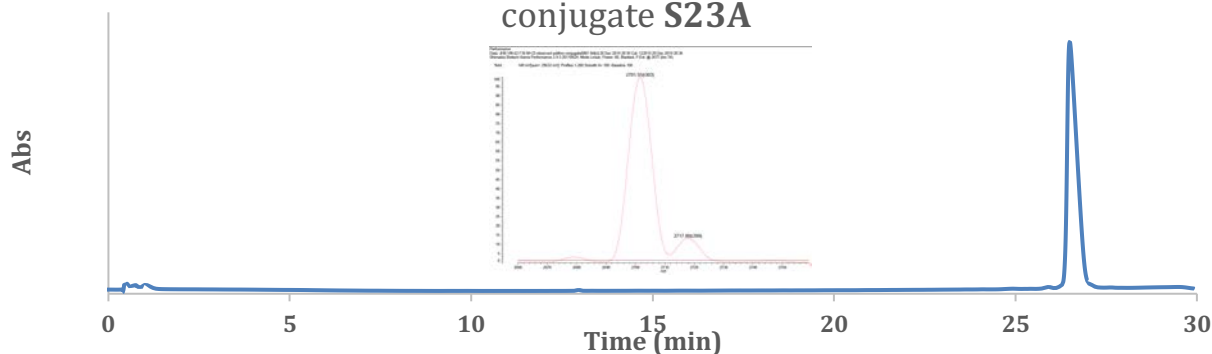
**Protected-HMM Siderophore-*D*-WSPKYM-Solothromycin, compound S23A** (5.2 mg, 13% yield, 19% brsm) was prepared from **S14** (27 mg, 14.6  $\mu\text{mol}$ , 1.0 equiv), solithromycin **6** (24.6 mg, 29.2  $\mu\text{mol}$ , 2.0 equiv), HOBt (5.6 mg, 29.2  $\mu\text{mol}$ , 2.0 equiv), EDC (4.2 mg, 21.9  $\mu\text{mol}$ , 1.5 equiv), DMF (0.15 mL), and DIEA (10.0  $\mu\text{L}$ , 58.3  $\mu\text{mol}$ , 4.0 equiv) as described above for the preparation of solithromycin *L*-linker conjugate **S22A**. After 3 h of reaction time, the mixture was concentrated under reduced pressure, and the residue was dissolved in 70%  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  and purified by prep HPLC (5 to 95%  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  with 0.1% TFA). [note: Product **S23A** elutes at  $\sim$ 70%  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ ]. Product **S23B** (4.8 mg, 12% yield, 18% brsm) was also isolated, which may have resulted from the racemization of **S23A**, along with recovered starting material **S24** (9.3 mg, 34% yield).

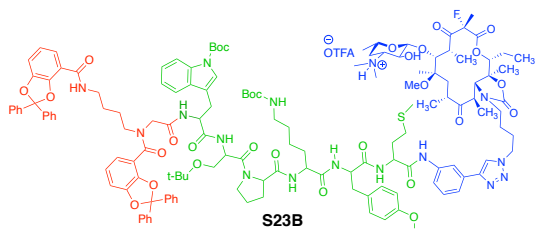


**S23A**:  $^1\text{H NMR}$  (400 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  9.70 – 9.47 (m, 1H), 8.65 – 6.56 (m, 45H), 4.71 – 3.38 (m, 24H), 3.22 – 2.72 (m, 15H), 2.68 – 2.30 (m, 5H), 2.29 – 0.59 (m, 89H). **MALDI-TOF (Linear mode)**:  $m/z$  Calcd. for  $\text{C}_{146}\text{H}_{185}\text{FN}_{16}\text{NaO}_{29}\text{S}$   $[\text{M}+\text{Na}^+]$ :2701.31, Found 2701.55;  $m/z$  Calcd. for  $\text{C}_{146}\text{H}_{185}\text{FKN}_{16}\text{O}_{29}\text{S}$

$[\text{M}+\text{K}^+]$ :2717.29, Found 2717.88.

### Protected-HMM Siderophore-*D*-WSPKYM-Solothromycin; conjugate **S23A**



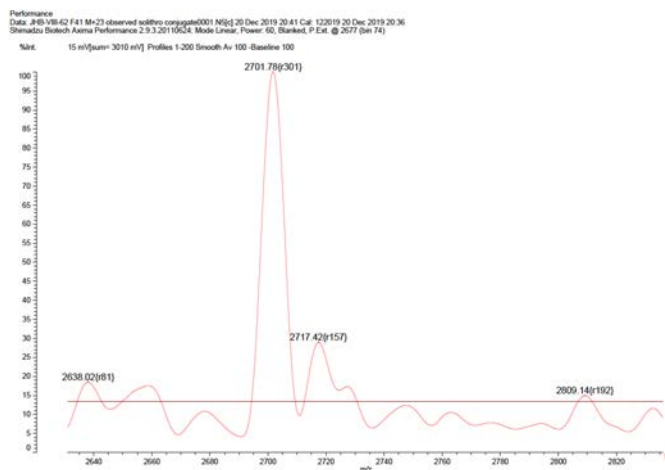


**Epimerized Protected-HMM Siderophore-*D*-WSPKYM-Solithromycin**

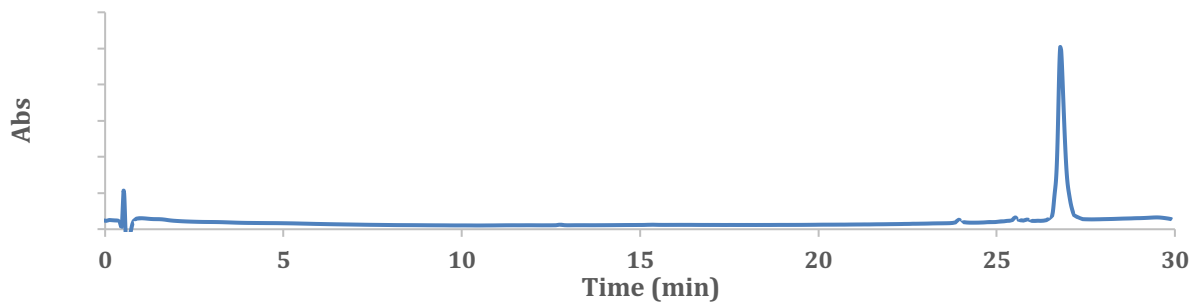
12% yield (18% brsm)

**S23B:**  $^1\text{H NMR}$  (400 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  8.45 – 6.64 (m, 46H), 4.71 – 3.83 (m, 4H), 3.78 – 3.33 (m, 7H), 3.23 – 1.79 (m, 32H), 1.79 – 0.65 (m, 89H).  
**MALDI-TOF (Linear mode):**  $m/z$  Calcd. for  $\text{C}_{146}\text{H}_{185}\text{FN}_{16}\text{NaO}_{29}\text{S}$   $[\text{M}+\text{Na}^+]$ :2701.31, Found 2701.78;  $m/z$  Calcd. for  $\text{C}_{146}\text{H}_{185}\text{FKN}_{16}\text{O}_{29}\text{S}$

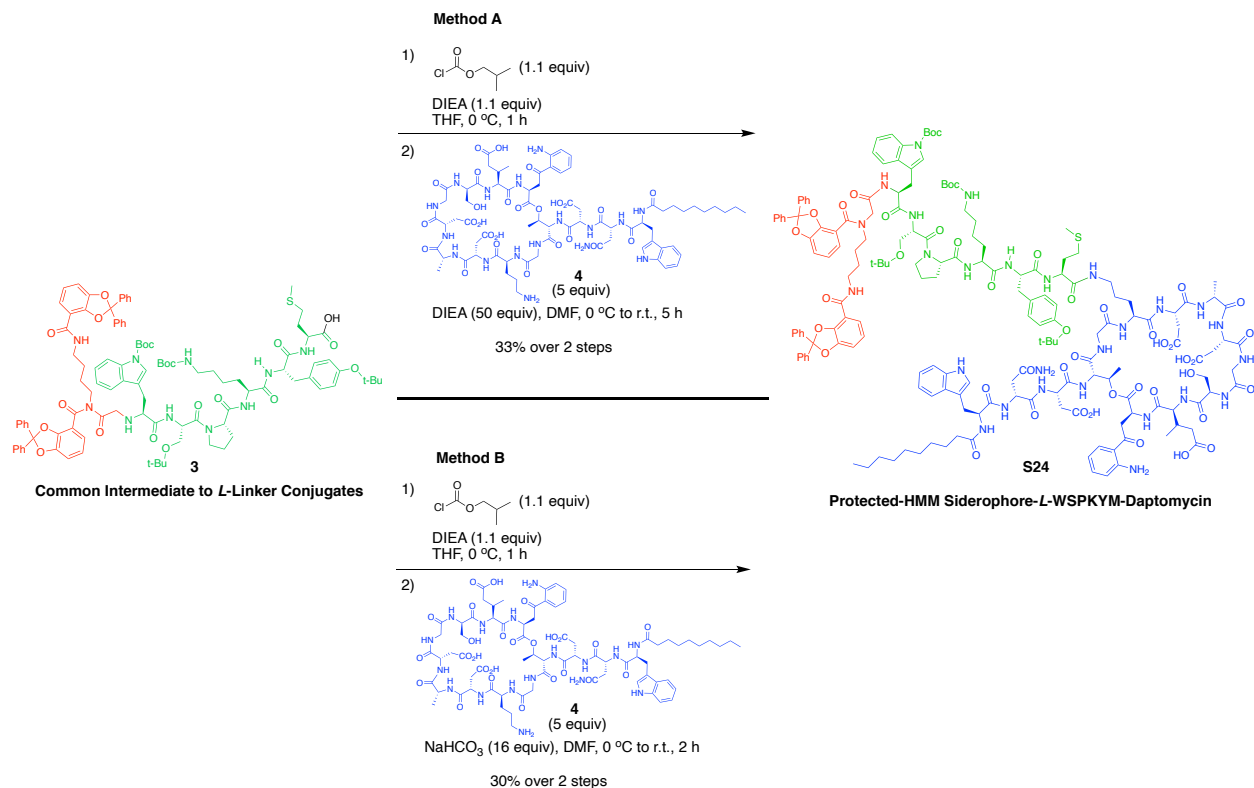
$[\text{M}+\text{K}^+]$ :2717.29, Found 2717.42.



Epimerized Protected-HMM Siderophore-*D*-WSPKYM-Solithromycin, conjugate **S23B**



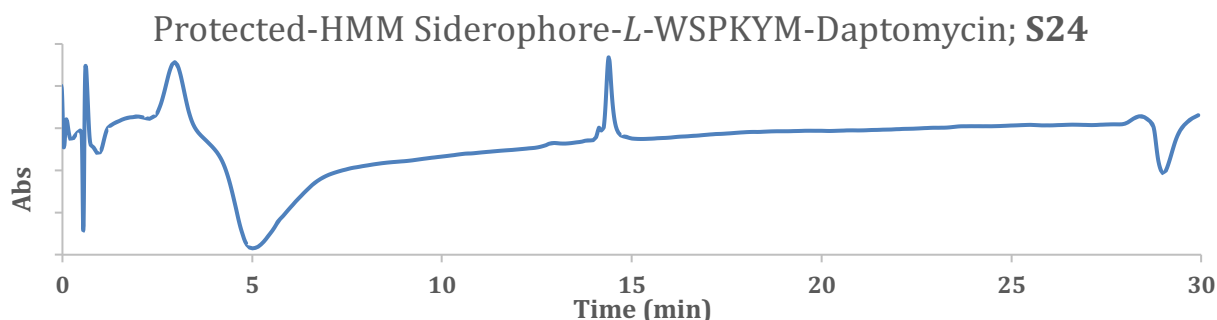
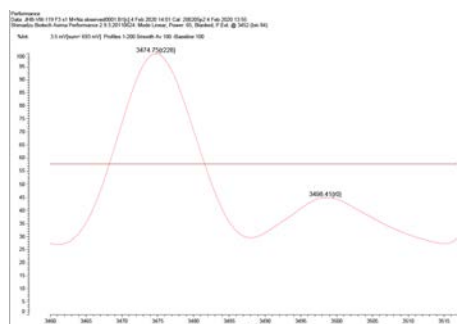


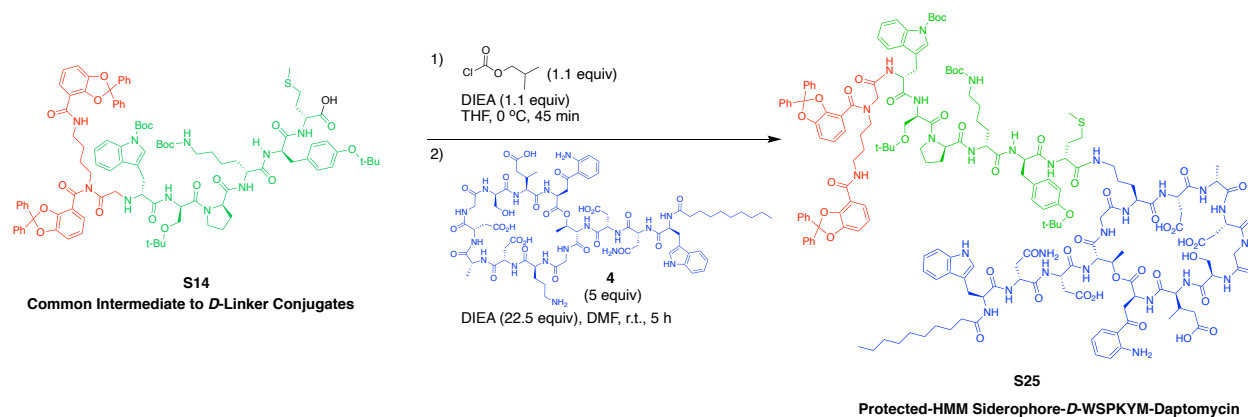


**Protected-HMM Siderophore-L-WSPKYM-Daptomycin, Compound S24, Method A:** To a flame-dried 5-mL round-bottom flask was added compound **3** (9.3 mg, 5.0  $\mu\text{mol}$ , 1.0 equiv). The flask was purged with argon, and THF (0.1 mL) was added. The solution was cooled to 0 °C, and a 1 M solution of DIEA in THF (6  $\mu\text{L}$ , 6  $\mu\text{mol}$ , 1.2 equiv) was added, followed by a 1 M solution of isobutyl chloroformate in THF (6  $\mu\text{L}$ , 6  $\mu\text{mol}$ , 1.2 equiv). The mixture was stirred at 0 °C for 1 h. The solution containing the resulting mixed anhydride product of compound **3** was transferred into a suspension of daptomycin (*vide infra*). To a 5-mL flame-dried, round-bottom flask was added daptomycin (40.7 mg, 25.1  $\mu\text{mol}$ , 5.02 equiv). The flask was purged with argon, and DIEA (44  $\mu\text{L}$ , 0.251 mmol, 50.2 equiv) was added, followed by DMF (0.1 mL) to form a suspension. The suspension was cooled to 0 °C in a dry ice/acetone bath. The solution of the **3**-derived mixed anhydride in THF was then added *via* syringe to the suspension of daptomycin over the course of 5 min. Additional DMF (0.2 mL) was used for transfer. The mixture went from suspension to clear solution 5 min after the mixed anhydride had been added. The mixture was stirred for 5 h and was warmed slowly to 23 °C in the ice-water bath. The mixture was diluted with 3:1 DMSO/H<sub>2</sub>O and purified by HPLC (5 to 95% CH<sub>3</sub>CN/H<sub>2</sub>O with 0.1% TFA). [note: The product eluted at ~80% CH<sub>3</sub>CN/H<sub>2</sub>O]. The product **S24** (5.7 mg, 33% yield) was provided as a white solid

following lyophilization. [note: The same yield was accomplished when allowing the mixture to stir for 18 h at 4 °C].

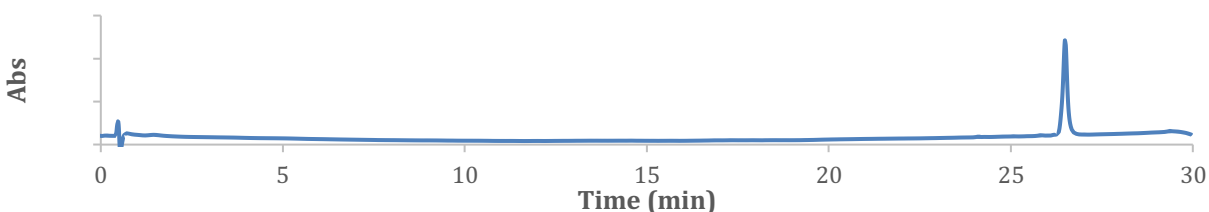
**Protected-HMM Siderophore-*L*-WSPKYM-Daptomycin, Compound S24, Method B:** The **3**-derived mixed anhydride (2.9 μmol) was prepared from compound **3** (5.4 mg, 2.9 μmol, 1 equiv), a 1 M solution of DIEA in THF (4.0 μL, 4.0 μmol, 1.38 equiv), and a 1 M solution of isobutylchloroformate in THF (4.0 μL, 4.0 μmol, 1.38 equiv) by the procedure described above in **Method A**. A 160 mM stock solution of sodium bicarbonate (39 mg) in MilliQ H<sub>2</sub>O (3 mL) was prepared. To a 5-mL round-bottom flask containing daptomycin **4** (14.2 mg, 8.76 μmol, 3 equiv) under argon was added an aqueous solution of NaHCO<sub>3</sub> (0.29 mL, 160 mM, 16 equiv). The solution was cooled to 0 °C, and the **S3**-derived mixed anhydride was then added dropwise as a solution in THF (0.1 mL) over the course of 2 min. Additional THF (0.2 mL) was used for the transfer. The ice bath was removed, and the reaction was stirred at 23 °C for 2 h. The mixture was concentrated under reduced pressure and purified by HPLC (5 to 95% CH<sub>3</sub>CN/H<sub>2</sub>O with 0.1% TFA). [note: The product eluted at 80% CH<sub>3</sub>CN/H<sub>2</sub>O with 0.1%TFA]. The protected conjugate **S24** (3.0 mg) was isolated in 30% yield. **S24: MALDI-TOF (Linear Mode):** *m/z* Calcd. for C<sub>175</sub>H<sub>221</sub>N<sub>27</sub>NaO<sub>45</sub>S<sup>+</sup> [M+Na<sup>+</sup>]:3475.55, Found 3474.75. **LC/MS (positive mode):** *m/z* Calcd. for C<sub>175</sub>H<sub>224</sub>N<sub>27</sub>O<sub>45</sub>S<sup>3+</sup> [M+3H<sup>+</sup>/3]:1152.2, Found 1152.8. **Analytical HPLC Conditions:** 2-50% CH<sub>3</sub>CN/H<sub>2</sub>O (2 min); 50-90% CH<sub>3</sub>CN/H<sub>2</sub>O (8 min); 90-95% CH<sub>3</sub>CN/H<sub>2</sub>O (10 min); 95% CH<sub>3</sub>CN/H<sub>2</sub>O (5 min); 2% CH<sub>3</sub>CN/H<sub>2</sub>O (5 min).





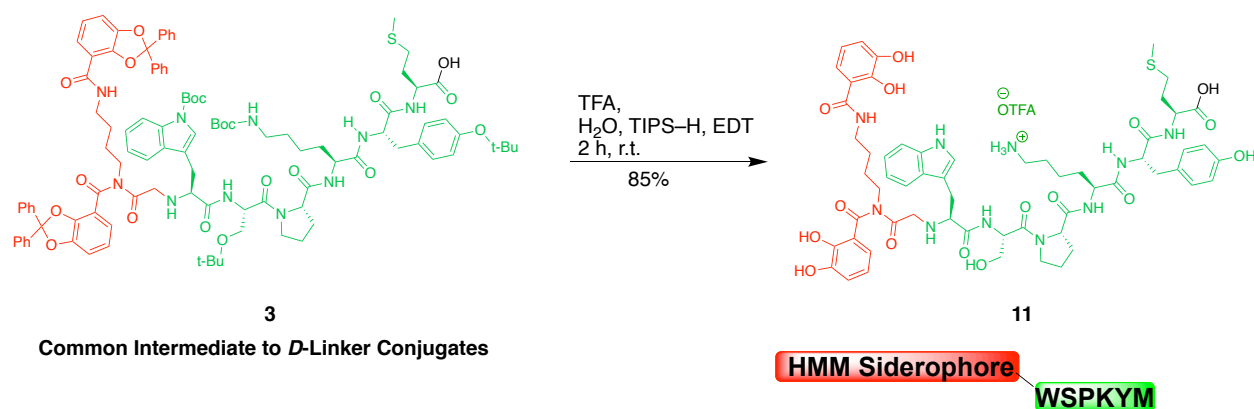
**Protected-HMM Siderophore-*D*-WSPKYM-Daptomycin, Compound S25:** To a flame-dried, 5-mL round-bottom flask containing common intermediate **S14** (12.0 mg, 6.48  $\mu\text{mol}$ , 1 equiv) under argon was added THF (0.13 mL). The solution was cooled to 0 °C and a 1 M stock solution of DIEA in THF (7.13  $\mu\text{L}$ , 7.13  $\mu\text{mol}$ , 1.1 equiv) was added, followed by the addition of a 1 M stock solution of isobutyl chloroformate in THF (7.13  $\mu\text{L}$ , 7.13  $\mu\text{mol}$ , 1.1 equiv). The mixture was stirred at 0 °C for 1 h. Before the mixed anhydride formation was complete, daptomycin **4** (54.0 mg, 33.3  $\mu\text{mol}$ , 5.14 equiv) was added to a separate flame-dried, 5-mL round-bottom flask. The flask was purged with argon, and DIEA (29  $\mu\text{L}$ , 0.166 mmol, 22.5 equiv) was added, followed by DMF (0.1 mL) to form a suspension. After the reaction was complete, the solution of the **S14**-derived mixed anhydride (6.48  $\mu\text{mol}$ ) was transferred in THF (0.1 mL) to the suspension of daptomycin in DMF *via* syringe over the course of 1 min. Additional THF (0.1 mL) was used to transfer remaining mixed anhydride. The mixture went from suspension to clear solution over the course of 3h. The mixture was stirred for a total of 5 h at 23 °C before purification by HPLC (5 to 95%CH<sub>3</sub>CN/H<sub>2</sub>O with 0.1%TFA) [Note: The product elutes at ~80% CH<sub>3</sub>CN/H<sub>2</sub>O]. The product **S25** was isolated in 17% yield (3.9 mg) as a white solid following lyophilization. **S25:** <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD)  $\delta$  8.21 – 6.36 (m, 60H), 4.78 – 3.38 (m, 37H), 3.20 – 2.28 (m, 33H), 2.28 – 0.50 (m, 92H). **HRMS:** *m/z* Calcd. for C<sub>175</sub>H<sub>224</sub>N<sub>27</sub>O<sub>45</sub>S<sup>3+</sup> [M+3H<sup>+</sup>/3]:1152.1936, Found 1152.2644. *m/z* Calcd. for C<sub>175</sub>H<sub>225</sub>N<sub>27</sub>O<sub>45</sub>S<sup>4+</sup> [M+4H<sup>+</sup>/4]:864.3970, Found 864.3387.

### Protected-HMM Siderophore-*D*-WSPKYM-Daptomycin; **S25**



## E. Conjugate Synthesis (Global Deprotection to Final Conjugate):

**General Procedure for Global Deprotection:** TFA Source: (Aldrich ReagentPlus 99%) [note: Depending on the source of TFA, due to the presence of trace metals, distillation may be necessary to avoid excessive sulfide oxidation during global deprotections of these conjugates. TFA (Aldrich ReagentPlus 99%) did not lead to excessive sulfide oxidation under an argon atmosphere and did not require distillation.] TFA (4 mL) was added to a 20-mL scintillation vial under argon. The TFA was degassed by passing a stream of argon through a thin needle (22 G) into the liquid for 10 min. To a separate, 20-mL scintillation vial under argon was added EDT (50  $\mu$ L), triisopropylsilane (TIPS-H, 25  $\mu$ L), H<sub>2</sub>O (50  $\mu$ L), and degassed TFA (1.88 mL). To a separate, 20-mL scintillation vial containing the protected conjugate under argon was added the deprotection cocktail (EDT/TIPS-H/H<sub>2</sub>O/TFA) via syringe. (note: The syringe was flushed with argon 3x prior to the transfer.) The mixture was stirred at 23 °C for 1.5–2 h. The mixture was then concentrated under reduced pressure and azeotroped with benzene (3x2 mL), sonicating for 5 seconds after adding benzene. [note: Sonication in benzene led to the rapid formation of a dry, white solid upon evaporation. Azeotroping with benzene was necessary to remove residual TFA, which accelerates the oxidation of the conjugates in air. Exposure to oxygen was minimized by backfilling the rotary evaporator with argon when releasing the vacuum].

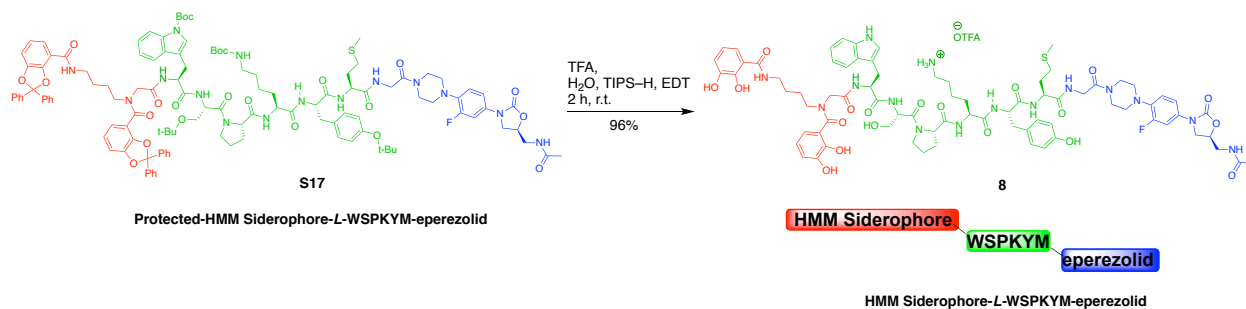
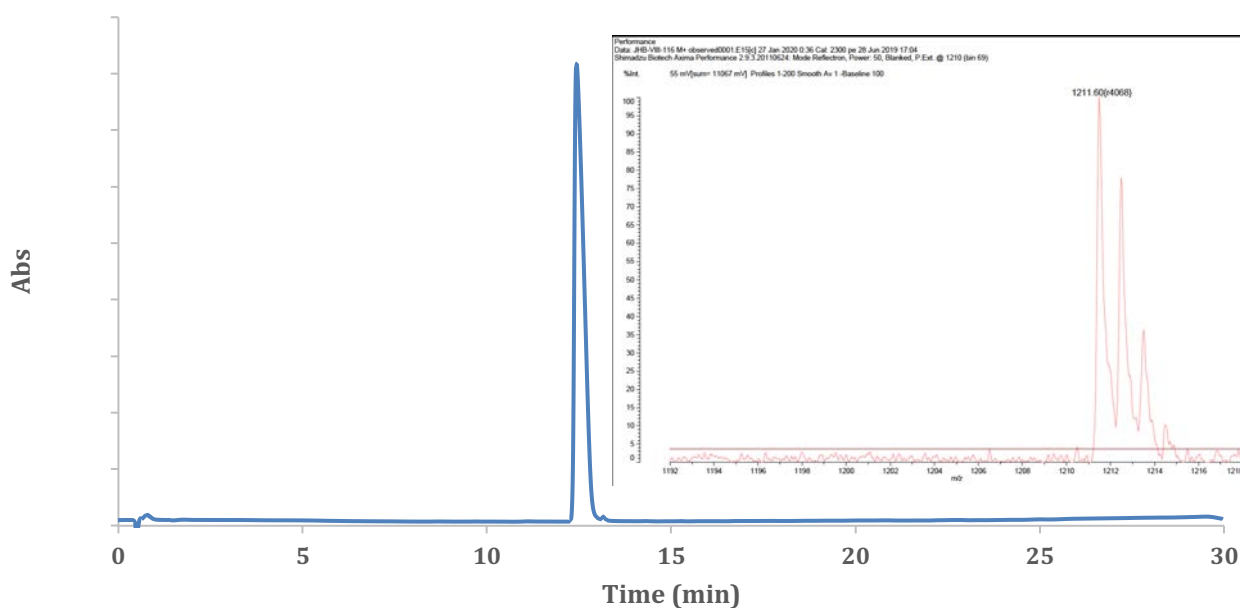


HMM Siderophore *L*-Linker Conjugate (no antibiotic)

**HMM Siderophore *L*-Linker Conjugate (no antibiotic), Conjugate 11:** Conjugate 11 (6.9 mg, 85% yield) was provided from compound 3 (11.4 mg, 6.15  $\mu$ mol) by the *General Procedure for Global Deprotection* described above. Purification of conjugate 11 was accomplished by a wash strategy. The crude, solid product was washed with ether (3x2 mL), sonicated, and filtered through

a cotton plug. The ether washes, which only contained impurities, were discarded. The crude product was then washed with H<sub>2</sub>O (3x2 mL), 10% CH<sub>3</sub>CN/H<sub>2</sub>O (2x1 mL), and 40% CH<sub>3</sub>CN/H<sub>2</sub>O (4x1 mL). Each solvent system was filtered through a cotton plug into a separate flask. The washes were concentrated under reduced pressure and analyzed by analytical HPLC and LC/MS. From the 40% CH<sub>3</sub>CN/H<sub>2</sub>O was isolated 6.9 mg (85%) of conjugate **11** as a white solid. An additional 2.9 mg (impure) of **11** was isolated from the 10% CH<sub>3</sub>CN/H<sub>2</sub>O wash. **11**: MALDI-TOF (Reflectron mode): *m/z* Calcd. for C<sub>59</sub>H<sub>74</sub>N<sub>10</sub>O<sub>16</sub>S [M+H<sup>+</sup>]:1211.50, Found 1211.60.

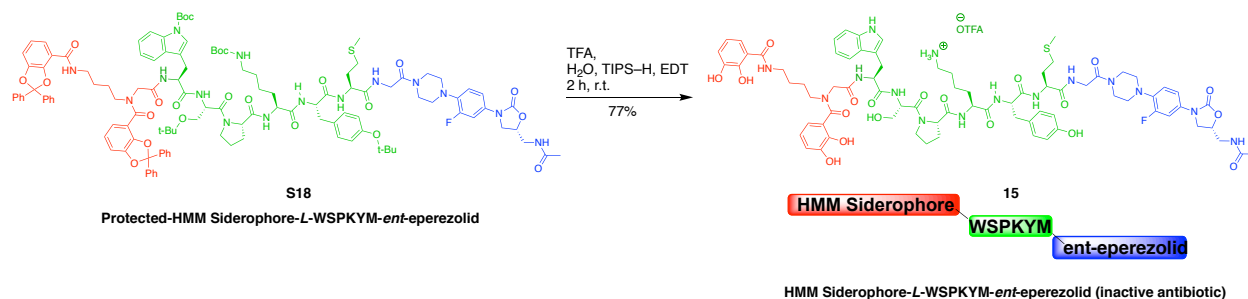
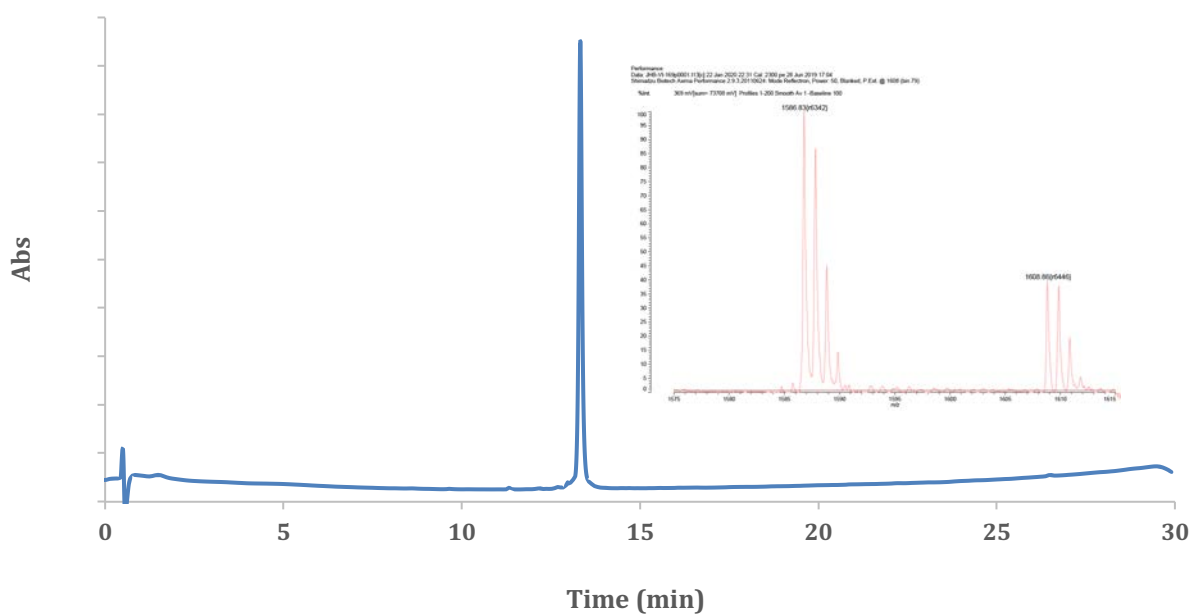
HMM Siderophore-*L*-WSPKYM-OH (no antibiotic);  
conjugate **11**



**HMM Siderophore-*L*-WSPKYM-eperezolid, Conjugate 8:** Conjugate **8** (9.7 mg, 96% yield) was provided from compound **S17** (13.3 mg, 5.97 μmol) by the *General Procedure for Global Deprotection* described above. Purification of conjugate **7** was accomplished by a wash strategy.

The crude, solid product was washed with ether (3x1 mL), sonicated, and filtered through a cotton plug. The ether washes, which only contained impurities, were discarded. The crude product was then washed with H<sub>2</sub>O (3x1 mL), 40% CH<sub>3</sub>CN/H<sub>2</sub>O (2x1 mL), and 80% CH<sub>3</sub>CN/H<sub>2</sub>O (2x1 mL). Each solvent system was filtered through a cotton plug into a separate flask. The 40% CH<sub>3</sub>CN/H<sub>2</sub>O and 80% CH<sub>3</sub>CN/H<sub>2</sub>O washes provided 8.6 mg (96%) of eperezolid conjugate **8** as a white solid. **8**: **MALDI-TOF (Reflectron mode)**: *m/z* Calcd. for C<sub>77</sub>H<sub>96</sub>FN<sub>15</sub>O<sub>19</sub>S [M+H<sup>+</sup>]:1586.67, Found 1586.83.; *m/z* Calcd. for C<sub>77</sub>H<sub>96</sub>FN<sub>15</sub>NaO<sub>19</sub>S [M+Na<sup>+</sup>]:1608.66, Found 1608.86.

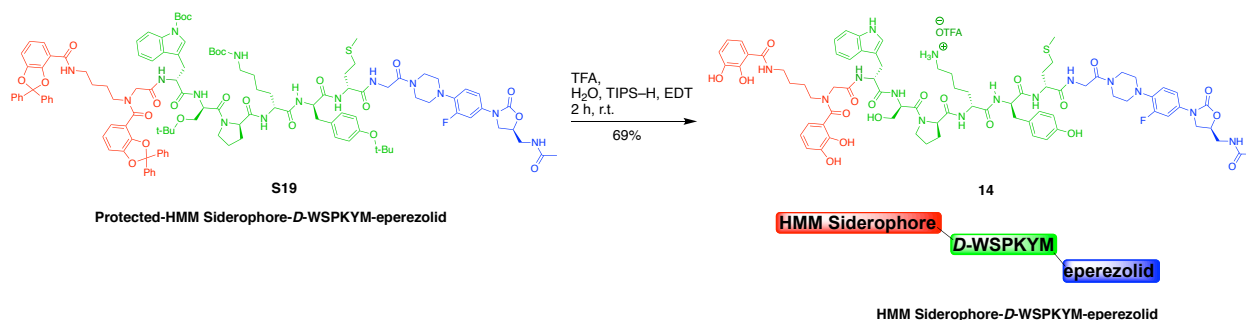
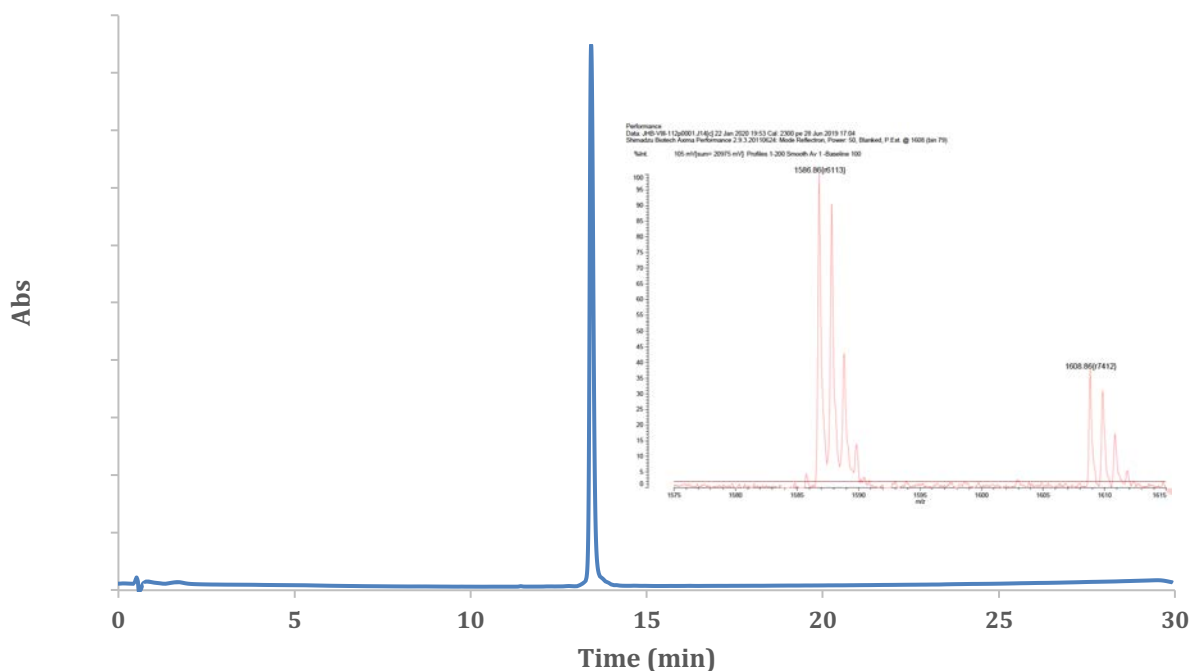
### HMM Siderophore-*L*-WSPKYM-Eperezolid, conjugate **8**



**HMM Siderophore-*L*-WSPKYM-*ent*-eperezolid** (*ent*-eperezolid = inactive antibiotic), **Conjugate 15**: Conjugate **15** (11.8 mg, 77% yield) was provided from compound **S18** (20.2 mg, 9.07 μmol) by the *General Procedure for Global Deprotection* described above. Purification of conjugate **15** was accomplished by a wash strategy. The crude, solid product was washed with ether (3x1 mL), sonicated, and filtered through a cotton plug. The ether washes, which only contained

impurities, were discarded. The crude product was then washed with H<sub>2</sub>O (3x1 mL), 40% CH<sub>3</sub>CN/H<sub>2</sub>O (2x1 mL), and 80% CH<sub>3</sub>CN/H<sub>2</sub>O (2x1 mL). Each solvent system was filtered through a cotton plug into a separate flask. The 40% CH<sub>3</sub>CN/H<sub>2</sub>O and 80% CH<sub>3</sub>CN/H<sub>2</sub>O washes provided 11.8 mg (77%) of *ent*-eperezolid conjugate **15** as a white solid. **15**: MALDI-TOF (Reflectron mode): *m/z* Calcd. for C<sub>77</sub>H<sub>96</sub>FN<sub>15</sub>O<sub>19</sub>S [M+H<sup>+</sup>]:1586.67, Found 1586.86.; *m/z* Calcd. for C<sub>77</sub>H<sub>96</sub>FN<sub>15</sub>NaO<sub>19</sub>S [M+Na<sup>+</sup>]:1608.66, Found 1608.86.

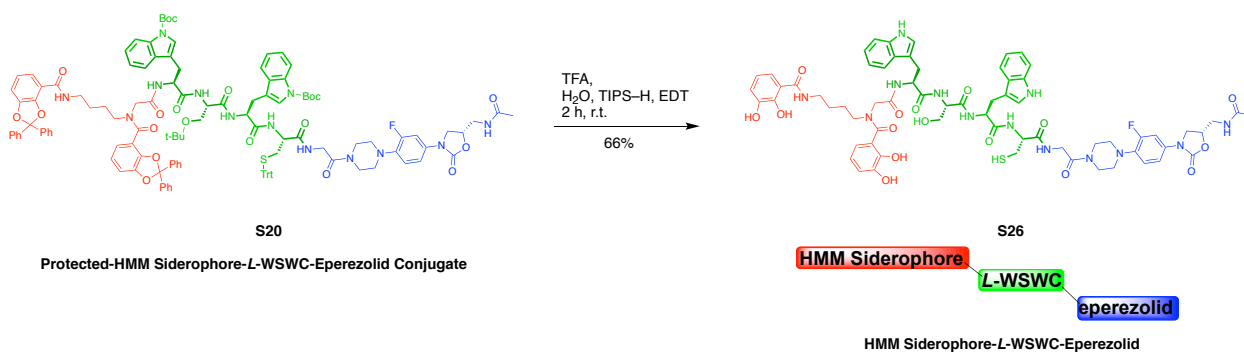
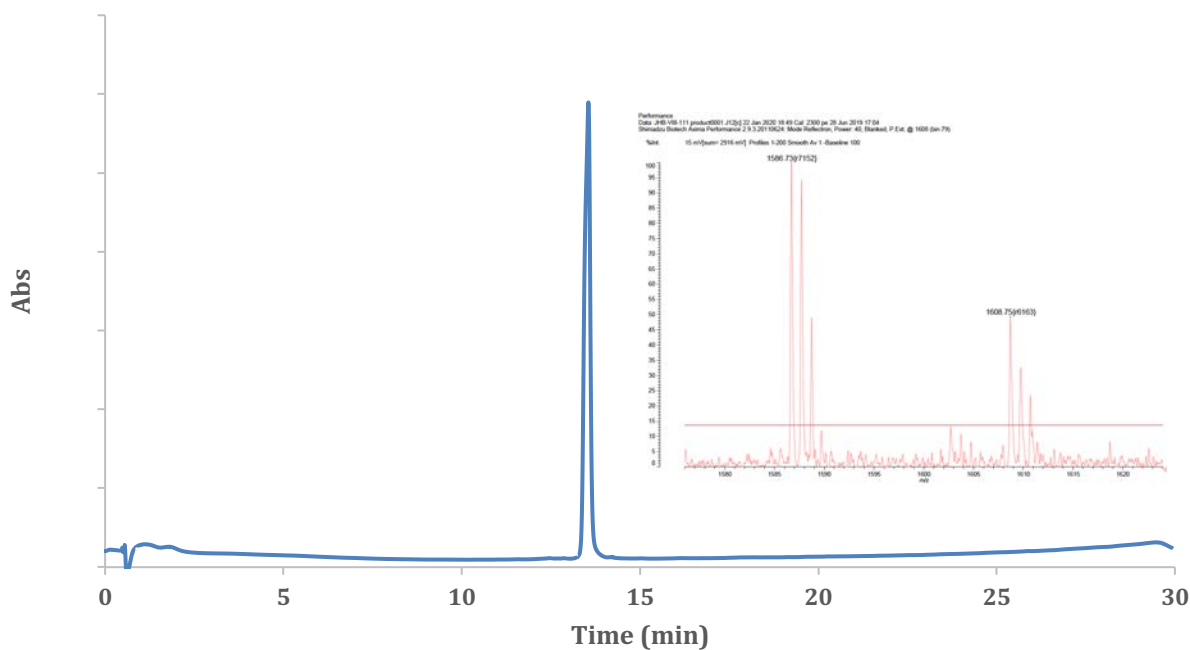
### HMM Siderophore-*L*-WSPKYM-*ent*-Eperezolid; Conjugate **15**



**HMM Siderophore-*D*-WSPKYM-eperezolid, Conjugate **14**:** Conjugate **14** (9.5 mg, 69% yield) was provided from compound **S19** (18.1 mg, 8.13 μmol) by the *General Procedure for Global Deprotection* described above. Purification of conjugate **14** was accomplished by a wash strategy. The crude, solid product was washed with ether (3x1 mL), sonicated, and filtered through a cotton

plug. The ether washes, which only contained impurities, were discarded. The crude product was then washed with H<sub>2</sub>O (3x1 mL), 50% CH<sub>3</sub>CN/H<sub>2</sub>O (2x1 mL), and 80% CH<sub>3</sub>CN/H<sub>2</sub>O (2x1 mL). Each solvent system was filtered through a cotton plug into a separate flask. The 50% CH<sub>3</sub>CN/H<sub>2</sub>O and 80% CH<sub>3</sub>CN/H<sub>2</sub>O washes provided 9.5 mg (69%) of *D*-linker conjugate **14** as a white solid. **14: MALDI-TOF (Reflectron mode):** *m/z* Calcd. for C<sub>77</sub>H<sub>96</sub>FN<sub>15</sub>O<sub>19</sub>S [M+H<sup>+</sup>]: 1586.67, Found 1586.73.; *m/z* Calcd. for C<sub>77</sub>H<sub>96</sub>FN<sub>15</sub>NaO<sub>19</sub>S [M+Na<sup>+</sup>]: 1608.66, Found 1608.75.

### HMM Siderophore-*D*-WSPKYM-Eperezolid; conjugate **14**

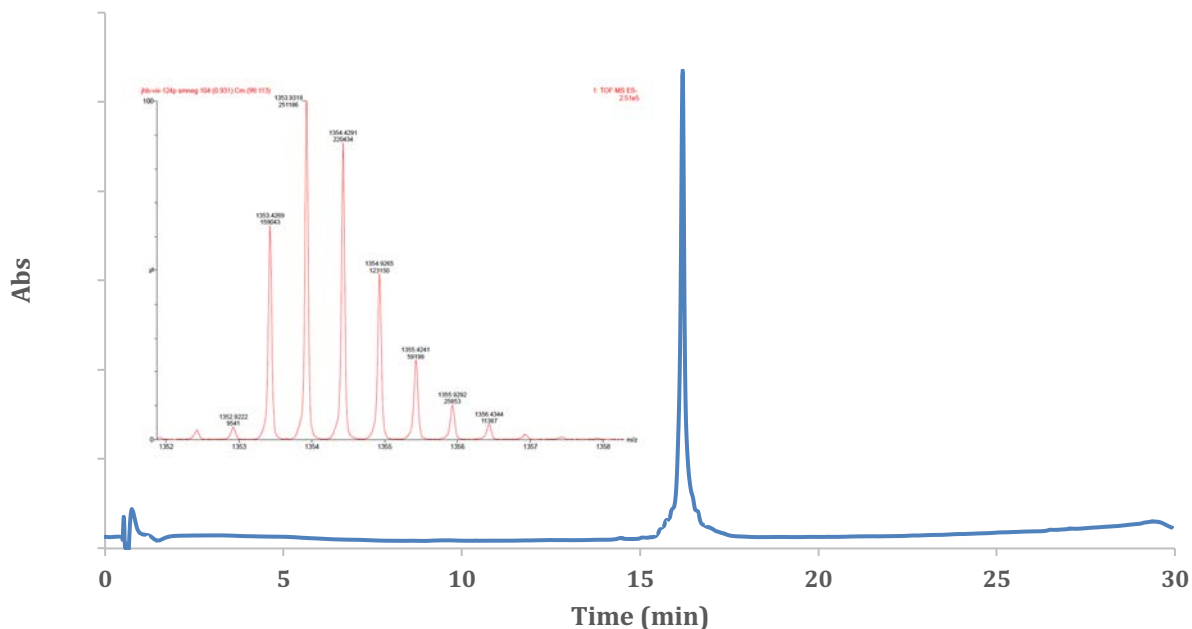


**HMM Siderophore-*L*-WSWC-eperezolid, Conjugate S26:** Conjugate **S26** (3.3 mg, 66% yield) was provided from compound **S20** (8.1 mg, 3.7 μmol) by the *General Procedure for Global Deprotection* described above. Purification of conjugate **S26** was accomplished by a wash strategy followed by HPLC. The crude, solid product was washed with ether (3x2 mL), sonicated, and

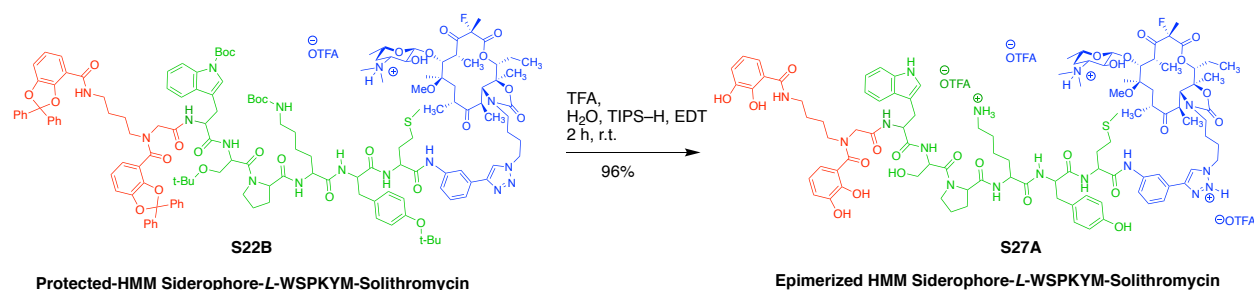


filtered through a cotton plug. The ether washes, which only contained impurities, were discarded. The crude product was then washed with 20% CH<sub>3</sub>CN/H<sub>2</sub>O (1x1 mL), 65% CH<sub>3</sub>CN/H<sub>2</sub>O (2x1 mL), 80% CH<sub>3</sub>CN/H<sub>2</sub>O (1x2 mL), and DMSO (1x 0.5 mL). Each solvent system was filtered through a cotton plug into a separate flask. The majority of the product was isolated in the DMSO wash with a minor impurity (~5:1 as determined by analytical HPLC analysis). Further purification by prep HPLC (5 to 95% CH<sub>3</sub>CN/H<sub>2</sub>O with 0.1 % TFA, C4 column) provided 3.3 mg (66%) of **S26** as a white solid. **S26**: HRMS: *m/z* Calcd. for C<sub>66</sub>H<sub>74</sub>FN<sub>13</sub>O<sub>16</sub>S [M-2H<sup>-</sup>]:1353.4936, Found 1353.4269.

### HMM Siderophore-*L*-WSWC-Eperezolid; Conjugate **S26**



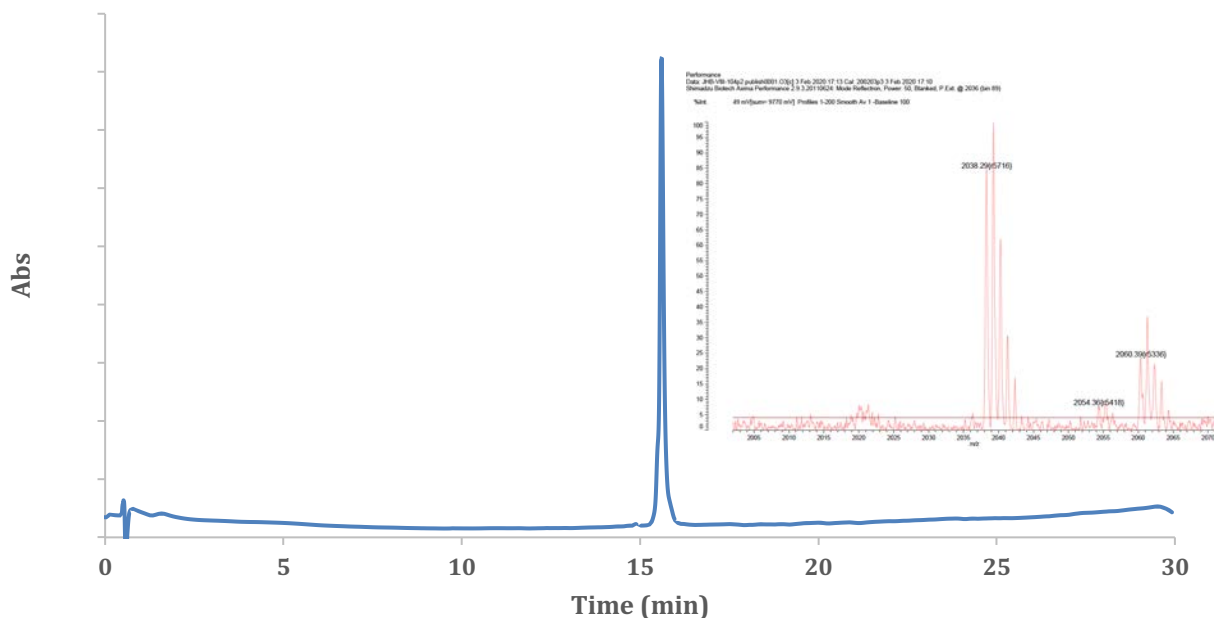


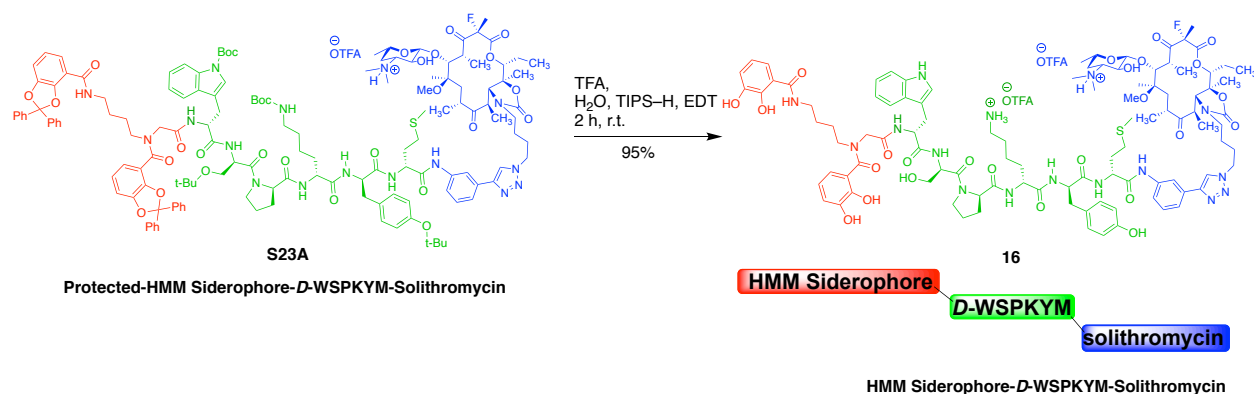


**Epimerized HMM Siderophore-*L*-WSPKYM-Solithromycin, Conjugate S27A:** Conjugate **S27A** (0.6 mg, 96% yield) was provided from compound **S22B** (0.7 mg, 0.26  $\mu\text{mol}$ ) by the *General Procedure for Global Deprotection* described above. Purification of conjugate **S27A** was accomplished by a wash strategy. The crude, solid product was washed with ether (3x2 mL), sonicated, and filtered through a cotton plug. The ether washes, which only contained impurities, were discarded. The crude product was then washed with H<sub>2</sub>O (1x0.5mL), 10% CH<sub>3</sub>CN/H<sub>2</sub>O (2x1 mL), 40% CH<sub>3</sub>CN/H<sub>2</sub>O (2x1 mL), 80% CH<sub>3</sub>CN/H<sub>2</sub>O (2x1 mL), and DMSO (1 x 0.5 mL). Each solvent system was filtered through a cotton plug into a separate flask. The DMSO wash provided 0.6 mg (96%) of conjugate **S27A** as a white solid following concentration under reduced pressure.

**S31A: MALDI-TOF (Reflectron mode):**  $m/z$  Calcd. for C<sub>102</sub>H<sub>137</sub>FN<sub>16</sub>O<sub>25</sub>S [M+H<sup>+</sup>]:2037.96, Found 2038.29.  $m/z$  Calcd. for C<sub>102</sub>H<sub>137</sub>FN<sub>16</sub>NaO<sub>25</sub>S [M+Na<sup>+</sup>]:2059.95, Found 2060.39.

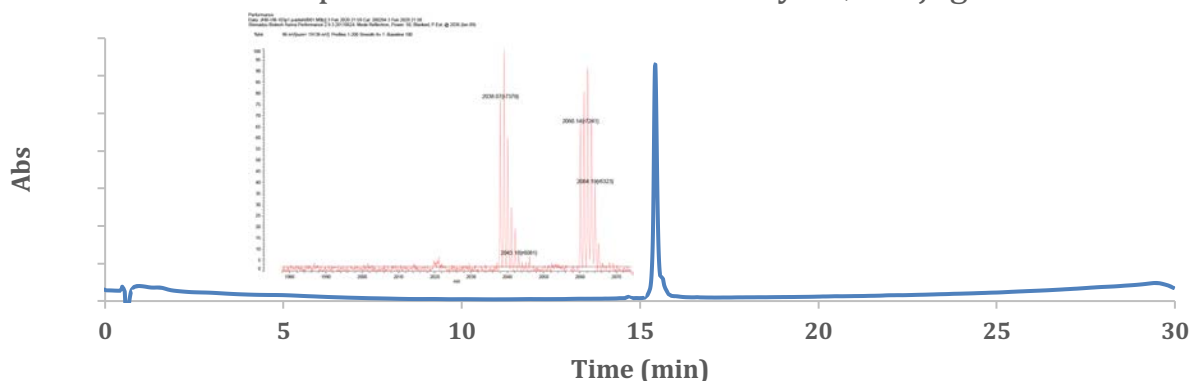
### Epimerized HMM Siderophore-*L*-WSPKYM-Solithromycin; **S27A**

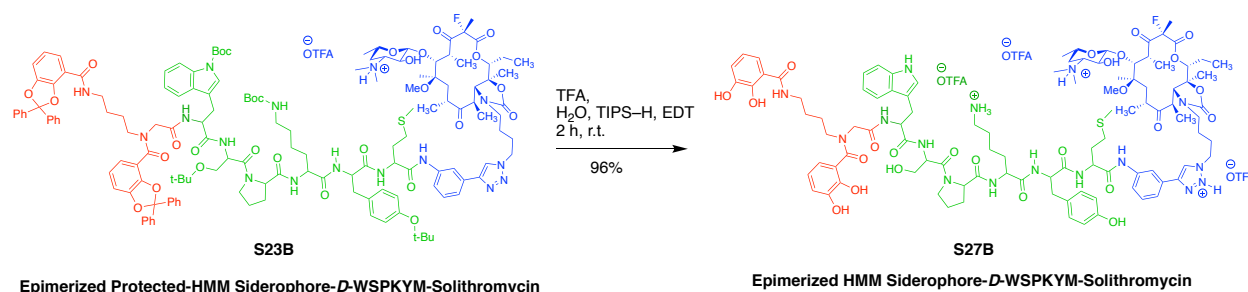




**HMM Siderophore-*D*-WSPKYM-Solithromycin, Conjugate 16:** *D*-Linker conjugate **16** (4.2 mg, 95% yield) was provided from compound **S23A** (5.2 mg, 1.9  $\mu$ mol) by the *General Procedure for Global Deprotection* described above. Purification of conjugate **16** was accomplished by a wash strategy. The crude, solid product was washed with ether (3x2 mL), sonicated, and filtered through a cotton plug. The ether washes, which only contained impurities, were discarded. The crude product was then washed with H<sub>2</sub>O (1x0.5 mL), 10% CH<sub>3</sub>CN/H<sub>2</sub>O (2x1 mL), 40% CH<sub>3</sub>CN/H<sub>2</sub>O (2x1 mL), 80% CH<sub>3</sub>CN/H<sub>2</sub>O (2x1 mL), and DMSO (1 x 0.5 mL). Each solvent system was filtered through a cotton plug into a separate flask. The CH<sub>3</sub>CN/H<sub>2</sub>O washes provided 4.2 mg (95%) of conjugate **16** as a white solid following concentration under reduced pressure. The product was purified by HPLC (5 to 95% CH<sub>3</sub>CN/H<sub>2</sub>O with 0.1 % TFA, C4 column) to remove trace oxidized product providing 4.3 mg (97%) of conjugate **16** for testing in bacteria. [It should be noted that the amount of product isolated from prep HPLC was roughly identical in quantity and purity to that isolated from the wash method.] **16: MALDI-TOF (Reflectron mode):** *m/z* Calcd. for C<sub>102</sub>H<sub>137</sub>FN<sub>16</sub>O<sub>25</sub>S [M+H<sup>+</sup>]:2037.96, Found 2038.07. *m/z* Calcd. for C<sub>102</sub>H<sub>137</sub>FN<sub>16</sub>NaO<sub>25</sub>S [M+Na<sup>+</sup>]:2059.95, Found 2060.14.

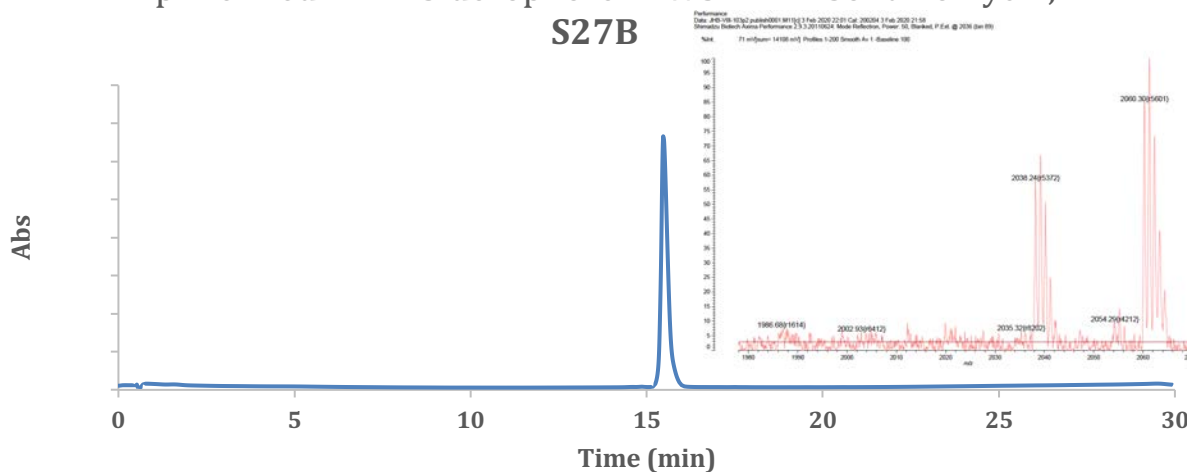
HMM Siderophore-*D*-WSPKYM-Solithromycin; conjugate **16**

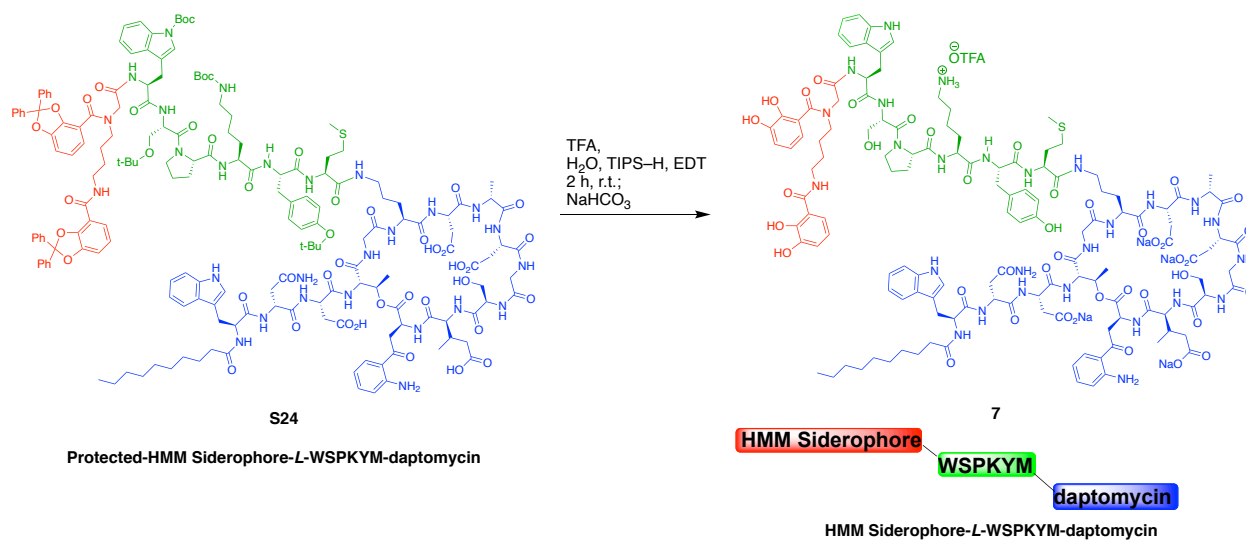




**Epimerized HMM Siderophore-*D*-WSPKYM-Solithromycin, Conjugate S27B:** Conjugate **S31B** (4.1 mg, 96% yield) was provided from compound **S23B** (4.8 mg, 1.8  $\mu$ mol) by the *General Procedure for Global Deprotection* described above. Purification of conjugate **S27B** was accomplished by a wash strategy. The crude, solid product was washed with ether (3x2 mL), sonicated, and filtered through a cotton plug. The ether washes, which only contained impurities, were discarded. The crude product was then washed with H<sub>2</sub>O (1x0.5 mL), 10% CH<sub>3</sub>CN/H<sub>2</sub>O (2x1 mL), 40% CH<sub>3</sub>CN/H<sub>2</sub>O (2x1 mL), 80% CH<sub>3</sub>CN/H<sub>2</sub>O (2x1 mL), and DMSO (1 x 0.5 mL). Each solvent system was filtered through a cotton plug into a separate flask. The CH<sub>3</sub>CN/H<sub>2</sub>O washes provided 4.1 mg (96%) of conjugate **S27B** as a white solid following concentration under reduced pressure. The product was purified by HPLC to remove trace oxidized product providing 4.1 mg (96%) of conjugate **S27B** for testing in bacteria. [note: The amount of product isolated from prep HPLC was roughly identical in quantity and purity to that isolated from the wash purification method]. **S27B: MALDI-TOF (Reflectron mode):** *m/z* Calcd. for C<sub>102</sub>H<sub>137</sub>FN<sub>16</sub>O<sub>25</sub>S [M+H<sup>+</sup>]:2037.96, Found 2038.24. *m/z* Calcd. for C<sub>102</sub>H<sub>137</sub>FN<sub>16</sub>NaO<sub>25</sub>S [M+Na<sup>+</sup>]:2059.95, Found 2060.30.

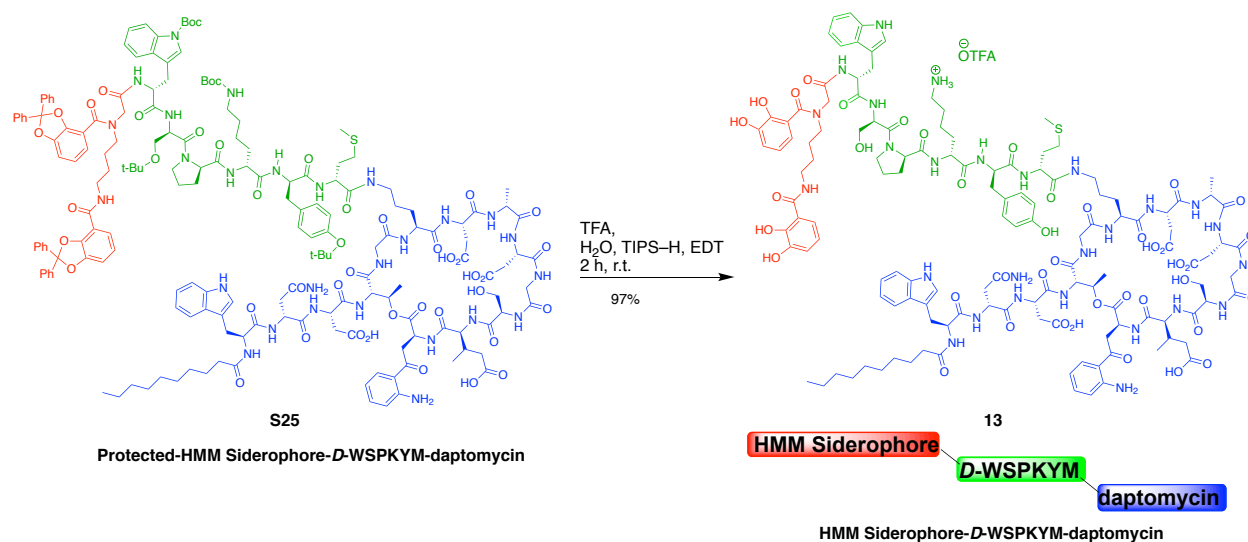
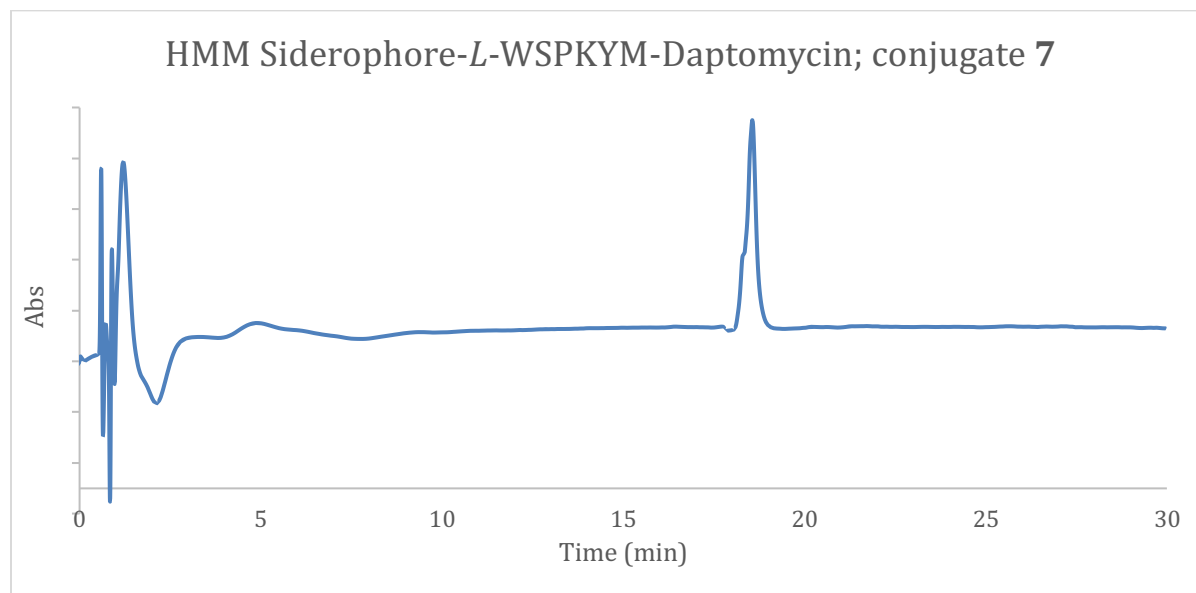
Epimerized HMM Siderophore-*D*-WSPKYM-Solithromycin,  
**S27B**





**HMM Siderophore-L-WSPKYM-daptomycin, conjugate 7:** Conjugate 7 (5.0 mg, 1.7  $\mu$ mol, crude) was provided from compound S24 (5.8 mg, 1.7  $\mu$ mol) by the *General Procedure for Global Deprotection* described above. Purification of conjugate 7 was accomplished by a wash strategy. [Note: Due to the solubility properties of our compound, HPLC purification was not practical using the standard solvent system (CH<sub>3</sub>CN/H<sub>2</sub>O with 0.1% TFA). Thus, a wash purification of this product was implemented, which provided the more soluble sodium salt. Such strategies have been discussed previously for compounds involving daptomycin].<sup>S9</sup> A 1-L stock solution of aqueous 5 mM NaHCO<sub>3</sub> was prepared from anhydrous NaHCO<sub>3</sub> (420.1 mg, 5 mmol) in MilliQ water (1 L). To a 1-dram (3.7-mL) vial containing the crude conjugate 7 (5.0 mg, 1.7  $\mu$ mol) was added a 0.005 M aqueous solution of NaHCO<sub>3</sub> (1.7 mL, 8.5  $\mu$ mol). The crude product was extracted with CH<sub>2</sub>Cl<sub>2</sub> (3x1 mL), and the aqueous layer was collected, along with a precipitate from the CH<sub>2</sub>Cl<sub>2</sub> extracts. The aqueous layer and the precipitate were combined in the original 1-dram vial and lyophilized. **Purification was accomplished by the following wash protocol:** **1)** The solid was washed with CH<sub>3</sub>CN (1 mL) and decanted. The acetonitrile contained mostly impurities. **2)** The solid was then washed with CH<sub>3</sub>CN/H<sub>2</sub>O (5:1, 1 mL) and decanted. The decanted wash was concentrated under reduced pressure and analyzed by LC/MS to reveal mostly product with minor impurities. An additional wash of this material with CH<sub>3</sub>CN/H<sub>2</sub>O (5:1, 1 mL) provided a pure sample of conjugate 7 (1.0 mg), which was used for testing in bacteria. **3)** The solid was then washed with CH<sub>3</sub>CN/H<sub>2</sub>O (1:1, 1 mL) and decanted to provide 1.3 mg of pure conjugate 7 after concentrating under reduced pressure, and this material was used for testing in bacteria. Total product isolated: 2.3 mg (64% yield). Conjugate 7: **LC/MS (positive mode):** *m/z* Calcd. for

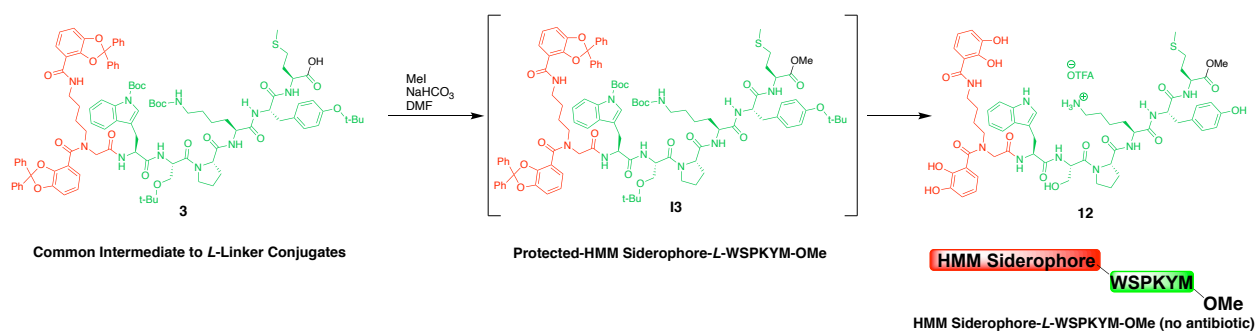
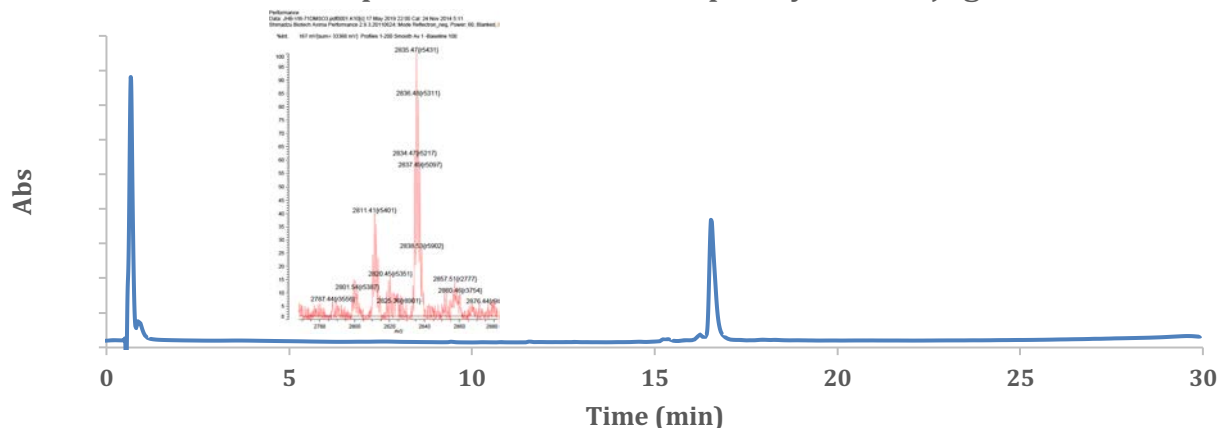
$C_{131}H_{175}N_{27}O_{41}S^{2+}$   $[M+2H^+/2]:1407.6$ , Found 1407.3. **HRMS:**  $m/z$  Calcd. for  $C_{131}H_{175}N_{27}O_{41}S^{2+}$   $[M+2H^+/2]:1407.6091$ , Found 1407.0343.



**HMM Siderophore-*D*-WSPKYM-daptomycin, conjugate 13:** Conjugate 13 (0.9 mg, 97% yield) was provided from compound **S25** (1.1 mg, 0.32  $\mu\text{mol}$ ) by the *General Procedure for Global Deprotection* described above. Purification of conjugate **13** was accomplished by a wash strategy. The crude, solid product was washed with ether (3x2 mL), sonicated, and filtered through a cotton plug. The ether washes, which only contained impurities, were discarded. The crude product was then washed with  $\text{H}_2\text{O}$  (1x0.5 mL), 20%  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (1x1 mL), 50%  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (1x1 mL), 80%  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (1x1 mL), and DMSO (1 x 0.5 mL). Each solvent system was filtered through a cotton plug into a separate flask. The DMSO wash provided 0.9 mg (97%) of conjugate **13** as a

solid following lyophilization. **13: MALDI-TOF (Reflectron mode, negative):**  $m/z$  Calcd. for  $C_{131}H_{173}N_{27}O_{41}S$   $[M-H]^+$ :2811.20, Found 2811.41  $m/z$  Calcd. for  $C_{131}H_{173}N_{27}NaO_{41}S$   $[M+Na-H]^+$ :2834.19, Found 2834.47. **HRMS:**  $m/z$  Calcd. for  $C_{131}H_{175}N_{27}O_{41}S^{2+}$   $[M+2H^+/2]$ :1407.6091, Found 1407.0880.  $m/z$  Calcd. for  $C_{131}H_{176}N_{27}O_{41}S^{3+}$   $[M+3H^+/3]$ :938.7418, Found 938.4005.

### HMM Siderophore-*D*-WSPKYM-Daptomycin; conjugate **13**

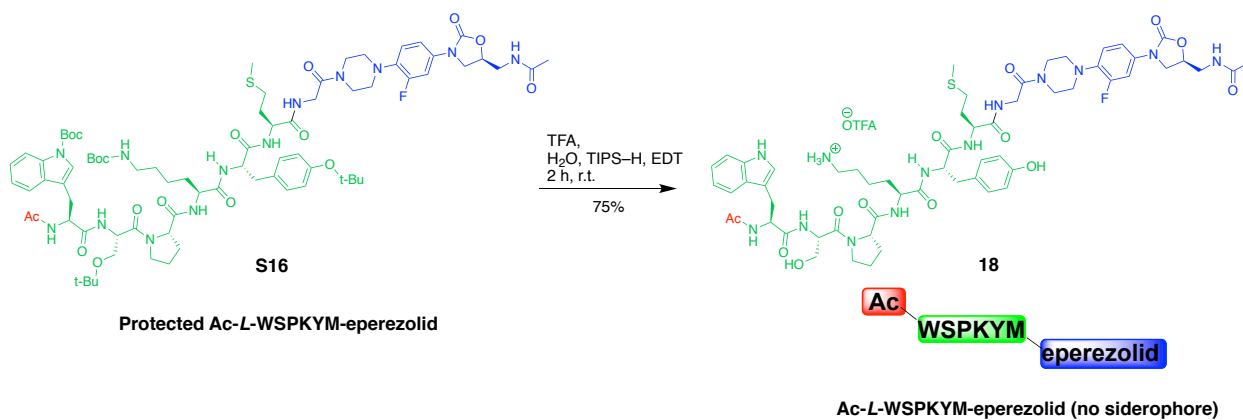
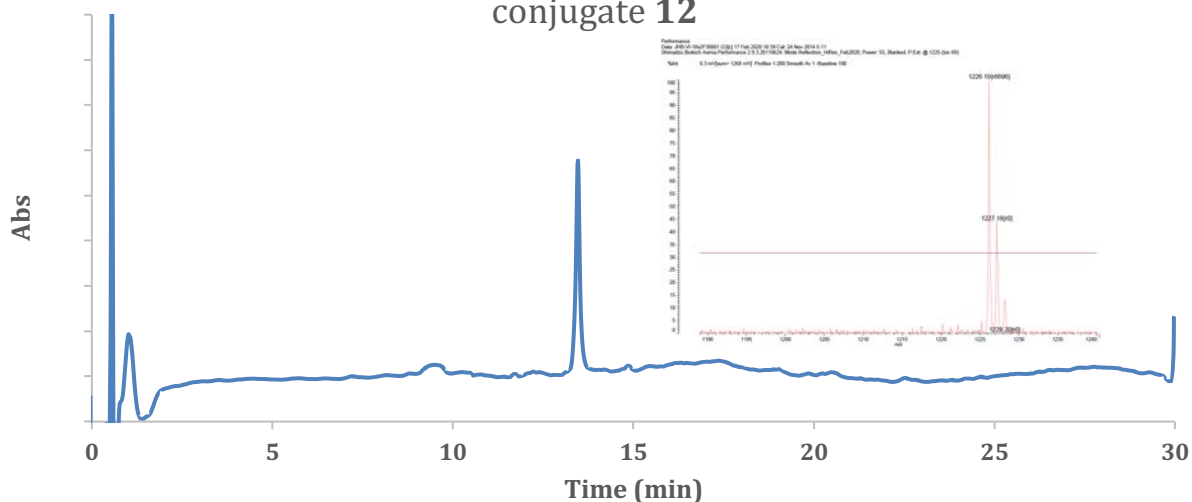


**HMM Siderophore-*L*-WSPKYM-OMe (no antibiotic), 12:** To a flame-dried, 5-mL round-bottom flask containing common intermediate **3** (11.0 mg, 5.94  $\mu$ mol) was added  $NaHCO_3$  (10 mg, 119  $\mu$ mol) and DMF (0.59 mL).  $CH_3I$  (23.7  $\mu$ L, 380  $\mu$ mol) was added, and the mixture was stirred for 2.5 h. Additional MeI (30  $\mu$ L, 480  $\mu$ mol) was added, and the mixture was stirred for an additional 1.5 h, at which point TLC analysis (6% MeOH/ $CH_2Cl_2$  with 4% AcOH) suggested completion. The mixture was concentrated under reduced pressure at 40  $^\circ C$  to provide 11.2 mg of a mixture containing protected intermediate **13** and an undesired bis-methylated product. [note: Methylations of Boc-protected lysines may occur under these conditions leading to bis-methylation of compound **3**].<sup>S10</sup> The flask containing **13** was purged with argon. The *General Procedure for Global Deprotection* described above followed by HPLC purification (5 to 95%



CH<sub>3</sub>CN/H<sub>2</sub>O with 0.1% TFA, 20 min) provided conjugate **12** (1.7 mg, 23% yield over 2 steps). A bis-methylated product (3.8 mg, 52%) was also isolated. **12: MALDI-TOF (Reflectron mode)** *m/z* Calcd. for C<sub>60</sub>H<sub>76</sub>N<sub>10</sub>O<sub>16</sub>S [M+H<sup>+</sup>]:1225.52, Found 1226.19.

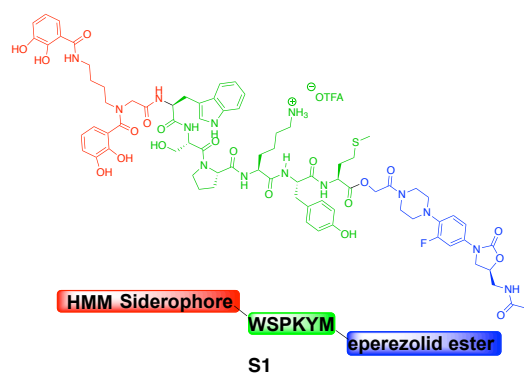
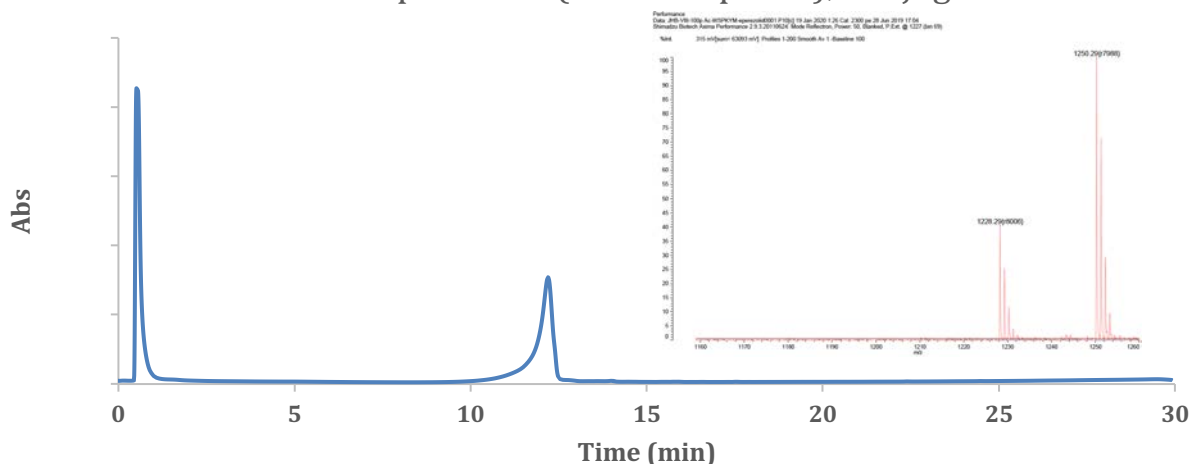
HMM Siderophore-*L*-WSPKYM-OMe (no antibiotic);  
conjugate **12**



**Ac-*L*-WSPKYM-eperezolid (no siderophore), conjugate 18:** Conjugate **18** (13.9 mg, 75% yield) was provided from compound **S16** (21.2 mg, 13.8 μmol) by the *General Procedure for Global Deprotection* described above. Purification of conjugate **18** was accomplished by a wash strategy. The crude, solid product was washed with ether (3x2 mL), sonicated, and filtered through a cotton plug. The ether washes, which only contained impurities, were discarded. The crude product was then washed with H<sub>2</sub>O (3x2 mL), 20% CH<sub>3</sub>CN/H<sub>2</sub>O (2x1 mL), and 80% CH<sub>3</sub>CN/H<sub>2</sub>O (4x1 mL), and 50% CH<sub>3</sub>CN/H<sub>2</sub>O (4x1 mL). Each solvent system was filtered through a cotton plug into a separate flask and concentrated under reduced pressure. The 50% CH<sub>3</sub>CN/H<sub>2</sub>O and

80% CH<sub>3</sub>CN/H<sub>2</sub>O washes provided 13.9 mg (75%) of acetylated epervezolid conjugate **18**. **18**: **MALDI-TOF (Reflectron mode)**: *m/z* Calcd. for C<sub>59</sub>H<sub>78</sub>FN<sub>13</sub>O<sub>13</sub>S [M+H<sup>+</sup>]:1228.55, Found 1228.29; *m/z* Calcd. for C<sub>59</sub>H<sub>78</sub>FN<sub>13</sub>NaO<sub>13</sub>S [M+Na<sup>+</sup>]:1250.54, Found 1250.29. Product retention time: 12.19 min. Initial peak is DMSO.

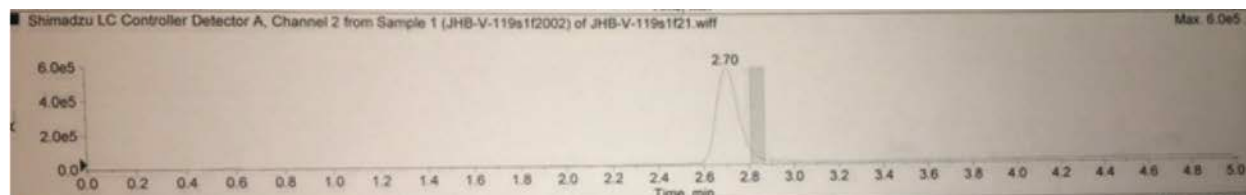
### Ac-*L*-WSPKYM-Epervezolid (no siderophore); conjugate **18**



### HMM Siderophore-*L*-WSPKYM-Epervezolid

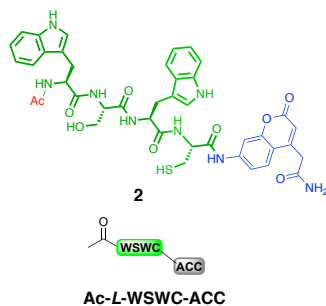
**Ester, compound S1:** Compound S1 was prepared in a similar manner described for the preparation of epervezolid amide conjugate **8**, but in this case using a catalytic amount of DMAP for esterification with epervezolid-OH (**S2**). **LC/MS (positive mode)**: *m/z* Calcd. for C<sub>79</sub>H<sub>96</sub>F<sub>4</sub>N<sub>14</sub>O<sub>22</sub>S [M+H<sup>+</sup>]: 1587.7, Found

1587.8. Cald. for C<sub>77</sub>H<sub>97</sub>FN<sub>14</sub>O<sub>20</sub>S<sup>2+</sup> [M+2H<sup>+</sup>/2]: 794.3, Found 794.4.



## F. Synthesis of ACC Conjugates:

The 7-Amino-4-carbamoylmethylcoumarin (ACC)<sup>S11,S12</sup> conjugates, Ac-L-WSWC-ACC **2** and Ac-L-WSPKYM-ACC **1**, were prepared from Fmoc-7-Aminocoumarin-4-Acetic Acid-Rink Amide AM resin (Fmoc-ACC resin) by the following general procedure: (described below for the case of conjugate **2**)



**Ac-L-WSWC-ACC (2):** To a 50-mL solid phase peptide synthesis vessel containing Fmoc-ACC resin (319.1 mg, 47.9  $\mu\text{mol}$ ) was added DMF (8 mL), and the resin was swelled by mixing with nitrogen for 15 min. Fmoc-deprotection on resin proceeded under the standard conditions described below. The stock solutions of Fmoc-Cys(Trt)-OH (2.6 mL, 300 mM), HATU (2.6 mL, 300 mM), and 2,4,6-collidine (1.3 mL, 600 mM) were added to the ACC resin. The resin was then mixed with nitrogen for 12 h. The process was repeated. After loading the first amino acid (P1), the resin was capped with a 1.2 M solution of  $\text{Ac}_2\text{O}$  in DMF (6.4 mL), which was mixed with the resin for 15 min. The resin was then washed with DMF (4x6 mL). Following the first amino acid loading onto ACC resin, the remaining amino acids were coupled by the general procedure described below.

**Stock Solutions:** Fmoc-Cys(Trt)-OH (300 mM, 504.7 mg) in DMF (2.87 mL), HATU (300 mM, 680.8 mg) in DMF (6 mL), 2,4,6-collidine (600 mM, 0.48 mL) in DMF (6 mL).

**General Procedure for Fmoc Deprotection on ACC resin:** Fmoc deprotection was achieved by mixing 40% 4-methylpiperidine (10 mL) with the ACC resin for 3 min, followed by mixing 20% 4-methylpiperidine for 10 min. The resin was then washed with DMF (4x6 mL), with mixing for 3 min each.

**Stock solutions preparation:** Fmoc-W(Boc)-OH (1.8654 g, 11.8 mL DMF, 300 mM), Fmoc-S(tBu)-OH (679.1 mg in 5.9 mL DMF, 300 mM), HATU (3.4 g in 30 mL DMF, 300 mM), NMM (2.1 mL in 16 mL DMF, 1.2 M), 2,4,6-collidine (1.27 mL in 16 mL DMF, 600 mM),  $\text{Ac}_2\text{O}$  (1.58 mL in 14 mL DMF, 1.2 M).

**General Procedure for Amino Acid Coupling on ACC resin:**

**Coupling 1:** To the Fmoc-deprotected resin was added a 300 mM solution of amino acid in DMF (2.6 mL), a 300 mM solution of HATU in DMF (2.6 mL), and a 1.2 M solution of *N*-methylmorpholine in DMF (1.3 mL). The contents were mixed for 20 min with nitrogen, and the solvent was removed.

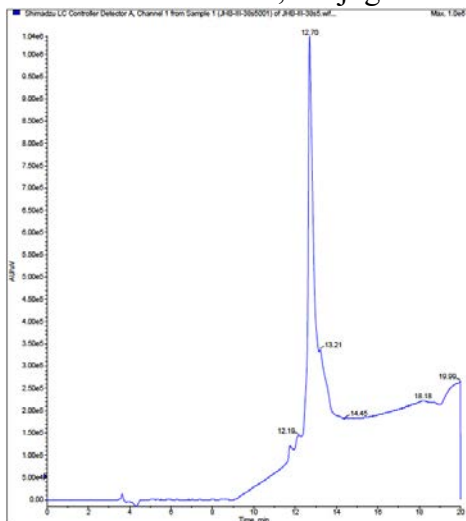
**Coupling 2:** To the resin was added a 300 mM solution of amino acid in DMF (2.6 mL), a 300 mM solution of HATU in DMF (2.6 mL), and a 600 mM solution of 2,4,6-collidine in DMF (1.3 mL). The contents were mixed for 30 min with nitrogen, and the solvent was removed.

**As an alternative to couplings 1 and 2,** a Biotage peptide synthesizer could be used to couple amino acids P2-P6 (15 equiv, 500 mM) with HCTU (15 equiv, 500 mM) and DIEA (30 equiv, 500 mM) using microwave-assisted synthesis (75 °C, 5 min per step). Deprotections with 20% 4-methylpiperidine were conducted at 70 °C (3 min per step). Swelling of the starting P1-conjugated ACC resin was achieved in DMF (70 °C, 20 min). Scales of 45 μmol-0.15 mmol were conducted.

**Acetylation of the N-Terminus:** To the peptide resin was added a 1.2 M solution of Ac<sub>2</sub>O in DMF (6.4 mL). The contents were mixed with nitrogen for 15 min, and the solvent was removed. The resin was then washed with DMF (4x6 mL) and CH<sub>2</sub>Cl<sub>2</sub> (3x6 mL).

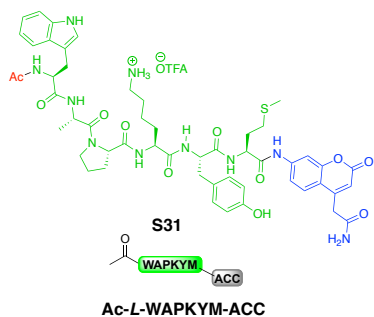
**Resin Cleavage and Deprotection:** The resin was washed with MeOH (3x8 mL) and CH<sub>2</sub>Cl<sub>2</sub> (3x8 mL). After drying the resin under reduced pressure for 30 min, the 50-mL solid phase peptide vessel was then purged with argon. EDT (200 μL), H<sub>2</sub>O (160 μL), TIPS-H (160 μL), and TFA (6.1 mL) were then added in sequential order to the dry resin. Without removing the argon balloon and septa, the vessel was then shaken with a Mistral Multi-Shaker® for 1.2h. Upon completion, the TFA solution was filtered into a round-bottom flask, and the resin was washed with Et<sub>2</sub>O/hexanes (1:1, 180 mL). The Et<sub>2</sub>O/hexanes filtrate was combined with the TFA filtrate. The solution was cooled to 0 °C, and the precipitated solid was filtered, washed with chilled Et<sub>2</sub>O/Hexanes (1:1, 2x5 mL), and purified by HPLC (5 to 95% CH<sub>3</sub>CN/H<sub>2</sub>O with 0.1 % TFA, 50 minutes) to provide 1.9 mg of ACC conjugate **2**. Conjugate **2**: LC/MS (positive mode): *m/z* Calcd. for C<sub>41</sub>H<sub>42</sub>N<sub>8</sub>O<sub>9</sub>S [M+H<sup>+</sup>]:823.3, Found 823.6.

Ac-L-WSWC-ACC, conjugate **2**



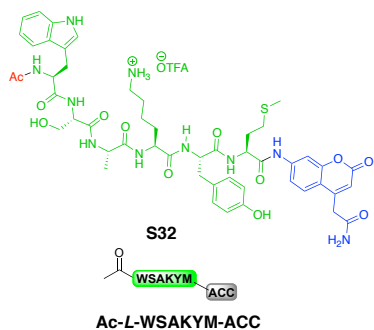
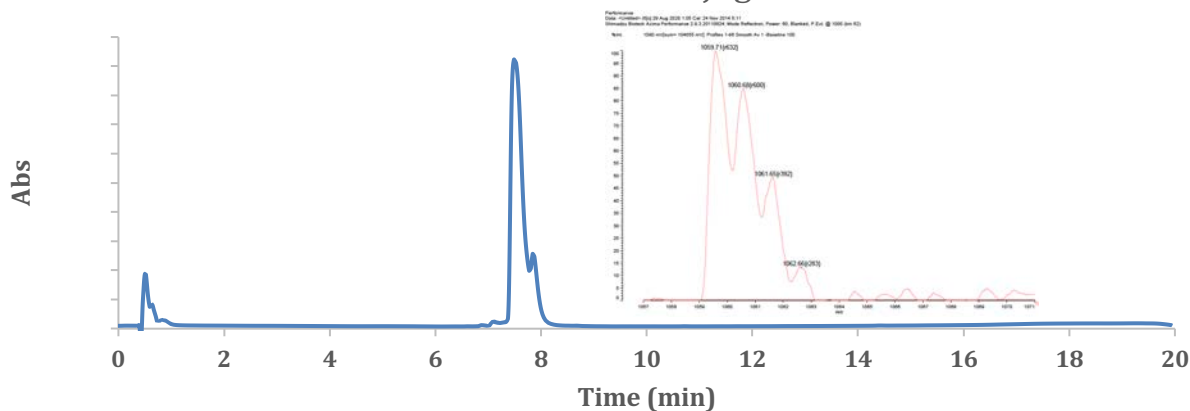






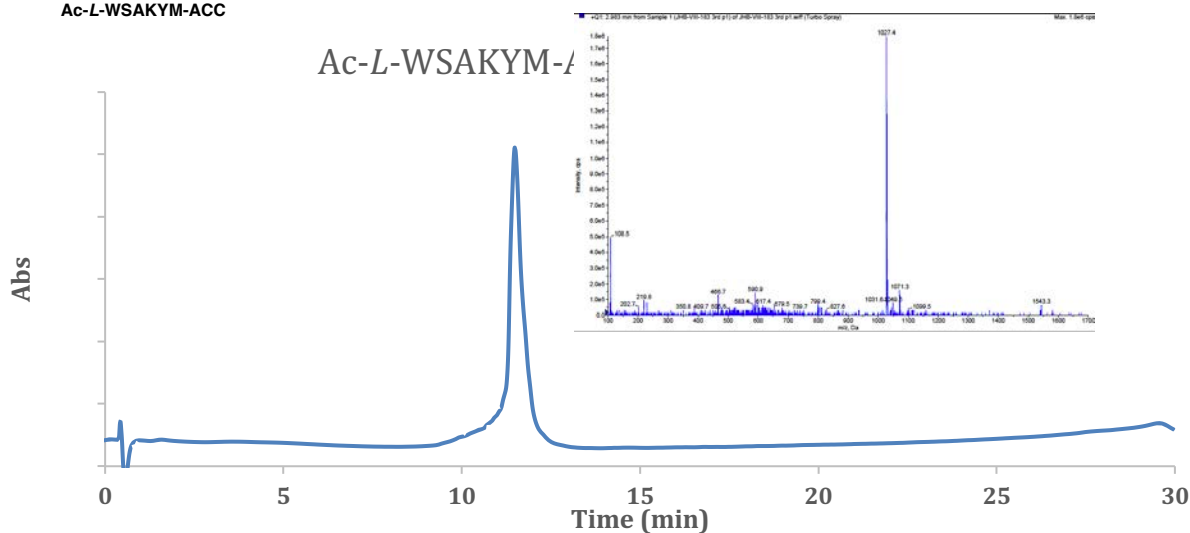
**Ac-L-WAPKYM-ACC (S31):** Conjugate **S31** was prepared by the general procedure described above. **LC/MS (positive mode):**  $m/z$  Calcd. for  $C_{52}H_{64}N_{10}O_{11}S$   $[M+H^+]$ : 1037.5, Found 1037.4. **MALDI-TOF (Reflectron mode):**  $m/z$  Calcd. for  $C_{52}H_{64}N_{10}NaO_{11}S^+$   $[M+Na^+]$ : 1059.44, Found 1059.71.

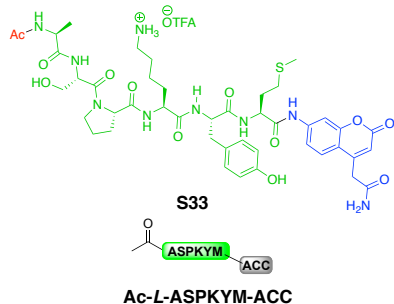
### Ac-L-WAPKYM-ACC; conjugate **S31**



**Ac-L-WSAKYM-ACC (S32):** Conjugate **S32** was prepared by the general procedure described above. **LC/MS (positive mode):**  $m/z$  Calcd. for  $C_{50}H_{62}N_{10}O_{12}S$   $[M+H^+]$ : 1027.4, Found 1027.4.

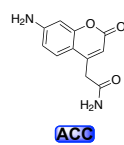
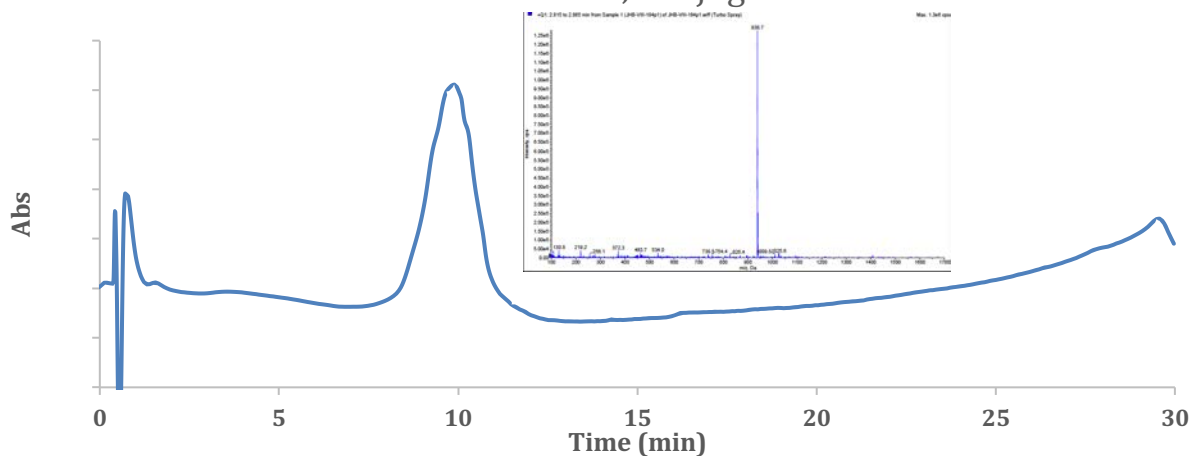
### Ac-L-WSAKYM-ACC





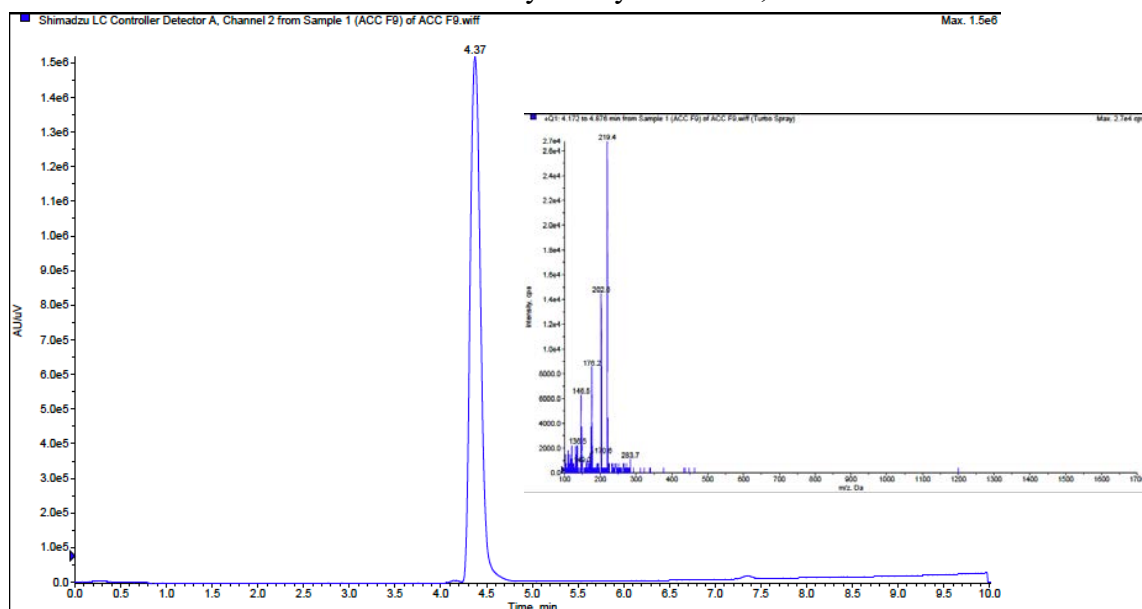
**Ac-L-ASPKYM-ACC (S33):** Conjugate **S33** was prepared by the general procedure described above. **LC/MS (positive mode):**  $m/z$  Calcd. for  $C_{44}H_{59}N_9O_{12}S$   $[M+H^+]$ : 938.4, Found 938.7.

### Ac-L-ASPKYM-ACC; conjugate **S33**



**7-Amino-4-carbamoylmethylcoumarin (ACC)**<sup>S11,S12</sup>: **ACC** was prepared from Fmoc-ACC resin in two steps: 1) Fmoc deprotection (see above), and 2) resin cleavage and deprotection (see above). Purification by HPLC (5 to 40%  $CH_3CN/H_2O$  with 0.1 % TFA, 30 minutes). **LC/MS (positive mode):**  $m/z$  Calcd. for  $C_{11}H_{10}N_2O_3$   $[M+H^+]$ : 219.1, Found 219.4.

### 7-Amino-4-carbamoylmethylcoumarin, ACC





## IV. MIC, PERIPLASMIC CLEAVAGE ASSAYS, AND CELL-FREE TRANSLATION

### A. Antibiotic Susceptibility Testing by the Broth Microdilution Method.

**Strains:** *E. coli* DCO was purchased from the Coli Genetic Stock Center (CGSC), and *A. baumannii* ATCC 1797 was purchased from ATCC. The remaining strains were obtained as gifts.

**Broth and Agar Preparation:** Mueller-Hinton II (MH-II, cation-adjusted) broth was prepared from solid MH-II (22 g) and MilliQ H<sub>2</sub>O (1 L), which was autoclaved in a presterilized 1-L glass bottle. MH-II agar plates were prepared from autoclaved MH-II (22 g), Bacto agar (17 g), and MilliQ water (1 L). The bacterial strains were obtained from glycerol stocks by streaking onto the MH-II agar plates (no antibiotics were introduced into the MH-II agar).

**Preparation of sterilized CaCl<sub>2</sub>\*2H<sub>2</sub>O Stock Solutions** (only for daptomycin-conjugates): In the case of daptomycin-containing conjugates, a stock solution of CaCl<sub>2</sub> (50mg/mL) was prepared from CaCl<sub>2</sub>\*2H<sub>2</sub>O (20 mL, 66.2 mg/mL). The solution was filter-sterilized utilizing a Steriflip Vacuum Filtration System with a Millipore Express PLUS Membrane (0.22 μm). In the case of daptomycin conjugates, the CaCl<sub>2</sub>\*2H<sub>2</sub>O solution (100 μL) was added to 48.3-mL aliquots of autoclaved MH-II broth to reach a final concentration of 100 μg/mL CaCl<sub>2</sub> in MH-II. Daptomycin is a calcium-dependent lipopeptide, which requires calcium to permeabilize the cell membrane.

**Preparation of sterilized 2,2'-dipyridyl (DP) and PBS stocks:** A sterile stock solution of 2,2'-bipyridyl solution (40 mL, 1 mg/mL) was prepared in a 50-mL Falcon tube and then was filter sterilized utilizing a Steriflip Vacuum Filtration System with a Millipore Express PLUS Membrane (0.22 μm). A sterile 1X PBS stock solution [10X PBS (10mL), and MilliQ WATER (90 mL)] was prepared and filter sterilized with an Olympus Plastics 250-mL vacuum-driven filter system with a PES membrane (0.22 μm). A 200 μM DP solution in autoclaved MH-II media was prepared from 1 mg/mL DP (1.56 mL) in MH-II (48.4 mL). A 600 μM DP solution in autoclaved MH-II media was prepared from 1 mg/mL DP (4.68 mL) in MH-II (45.3 mL) were prepared 24 h before use. [Note: Avoided the use of bovine serum albumin (BSA) in the PBS stock solution for siderophore conjugates, which may inhibit catechol-type siderophore mediated iron-transport].<sup>S13</sup>

**Bacterial Culture Preparation:** All autoclaved and sterilized solutions were transferred using sterilized Sarstedt Serological Pipettes (10-mL, 25-mL, and 50-mL), which are attached to an Eppendorf Easypet3. The autoclaved MH-II media contained DP (600  $\mu$ M, 200  $\mu$ M, 160  $\mu$ M, 129  $\mu$ M, or without DP). The culture tubes were then placed bottom-side-up in an incubator set at 37 °C without shaking for 18-20 h. A sterilized pipet tip was gently touched on the surface of a single colony and added to autoclaved MH-II broth (5 mL) in a sterilized 12-mL culture tube. The cultures was incubated for 18 h at 37 °C with shaking at 250 rpm. The cultures were diluted 200-fold into autoclaved MH-II broth (4-mL) with 2,2'-dipyridyl (DP) and incubated at 37 °C with shaking at 250 rpm for 3-5 h, or until an optical density at 600 nm ( $OD_{600}$ ) between 0.2 and 0.6 was achieved. An aliquot of the culture (1 mL) was transferred to a sterilized culture tube and diluted with PBS until an  $OD_{600}$  of 0.13. The cultures (11.5  $\mu$ L) were then diluted into MH-II media (15-mL) to a concentration of  $1 \times 10^5$  cells/mL. The diluted cultures (90  $\mu$ L/well) were then added to substrate (10  $\mu$ L at the following concentrations: 640  $\mu$ g/mL, 320  $\mu$ g/mL, 160  $\mu$ g/mL, 80  $\mu$ g/mL, 40  $\mu$ g/mL, 20  $\mu$ g/mL, and 10  $\mu$ g/mL) in sterilized 96-well plates to achieve a final substrate concentration of 64  $\mu$ g/mL, 32  $\mu$ g/mL, 16  $\mu$ g/mL, 8  $\mu$ g/mL, 4  $\mu$ g/mL, 2  $\mu$ g/mL, and 1  $\mu$ g/mL. [note: Polymyxin B was tested at the following final concentrations ( $\mu$ g/mL): 8, 4, 2, 1, 0.5, 0.25, 0.125.] The 96-well plates were then sealed with sterilized gas-permeable covers. The plates were incubated at 37 °C with shaking at 225 rpm for 16-20 h. Each well condition was prepared in triplicate. The turbidity of each well was examined and the MIC was counted as the lowest concentration that lacked turbidity. The  $OD_{600}$  was also measured.

### **B. Procedure for Isolating Periplasmic Extract:**

#### **Stock Preparations [20% sucrose/30mM Tris, 50 mM Tris, 50 mM Tris with 0.01% Tween]:**

A filter-sterilized, 1-L aqueous solution of 20% sucrose/30mM Tris base was prepared with sucrose (200 g) and Tris base (3.63 g). [Note: EDTA was not used in this procedure to avoid the elimination of metalloproteases from the resulting extract]. A 50-mL aqueous solution of 50 mM Tris base was prepared from solid Tris base (340 mg), along with a separate 50 mM Tris solution containing 0.01% Tween 20 (note: Tween prevents coagulation of proteins).

**Osmotic Shock Procedure (without EDTA):** An overnight culture on a MH-II plate (prepared as described above) at 37 °C for 18 h. Added a single colony to 100 mL of MH-II media and a

single colony to 100 mL of LB media in a sterile 2.5-L Erlenmeyer flask. The flask was incubated with shaking until an OD<sub>600</sub> of 0.5 was reached (~5-6 hours). Centrifuged the cultures at 4150 rpm for 40 min at 4 °C or at 15,000 rpm for 10 min at 4 °C. Poured-off the supernatant into bleach, so as not to disturb the pelleted cells. The filter-sterilized 20% sucrose/30mM Tris base (50 mL) was added to the cell pellets after removal of supernatant using a sterile 50-mL GeneMate serological pipet. The pellets were suspended with the pipet tip and subsequently vortexed to achieve a relatively homogenous suspension and mixed for 10 min at 4 °C. The suspension was then centrifuged at 4150 rpm for 30 min at 4 °C or at 15,000 rpm for 10 min at 4 °C. The supernatant was decanted-off so as not to disturb the pellets. Filter-sterilized cold water (50 mL) was added and vortexed to provide a homogeneous suspension, which was allowed to shake for 10 minutes at 4 °C. The suspension was then centrifuged at 4150 rpm for 30 min at 4 °C or at 15,000 rpm for 10 min at 4 °C. The supernatant (periplasmic extract) was filtered through a 500-mL vacuum filter (PES: 0.22 µm), ensuring that the filtrate remained at 4 °C by keeping the plastic filtration container on ice. The filtrate was then concentrated in an Amicon Ultra-15 Centrifugal Filter Ultracel -10K (15-mL) by centrifuging at 3600 rpm for 15 min at 4 °C. The process was repeated until the periplasmic extract reached a volume of 0.5-2 mL and a total protein concentration <2.5 mg/mL. A nanodrop was used to determine the protein concentrations. Depending on the concentration, the periplasmic extract may be diluted with aqueous Tris solution (pH=8). The periplasmic extract were then aliquoted (200 µL/Eppendorf tube) into 1.5-mL Eppendorf tubes and flash-frozen with liquid nitrogen. [Note: To avoid reduction in protease activity, glycerol was not added to the periplasmic extract prior to freezing. All experiments were conducted with a fresh 200 µL aliquot of periplasmic extract to avoid inconsistencies after multiple freeze-thaw cycles. Before use, the periplasmic extract was thawed slowly at 4 °C.]

**Total Protein/Periplasmic Extract** (Evaluated concentration at OD 280 nm using Nanodrop™):

*E. coli* K12 MG1655 (from LB media): 1.557 mg/mL; subsequent preparation: 745 µg/mL

*E. coli* K12 MG1655 (from MH-II media): 1.979 mg/mL

*A. nosocomialis* M2 (from LB media): 548 µg/mL

*A. nosocomialis* M2 (from MH-II media): 545 µg/mL

*A. baumannii* ATCC BAA-1797 (from LB media): 1.006 mg/mL

*A. baumannii* ATCC BAA-1797 (from MH-II media): 565 µg/mL

*E. coli* DCO (from LB media): 1.581 mg/mL  
*E. coli* DCO (from MH-II media): 1.906 mg/mL  
*E. coli* BW25113  $\Delta$ *bamB* $\Delta$ *tolC* (from LB media): 924  $\mu$ g/mL (batch 1); 1.962  $\mu$ g/mL (batch 2)  
*E. coli* BW25113  $\Delta$ *bamB* $\Delta$ *tolC* (from MH-II media): 919  $\mu$ g/mL  
*P. aeruginosa* PA01 (from LB media): 2.401 mg/mL  
*P. aeruginosa* PA01 (from MH-II media): 1.769 mg/mL  
*P. aeruginosa* ATCC10145 (from LB media): 2.002 mg/mL  
*P. aeruginosa* ATCC10145 (from MH-II media): 1.447 mg/mL  
*S. enterica* 14028s (from LB media): 939  $\mu$ g/mL  
*S. enterica* 14028s (from MH-II media): 1.257  $\mu$ g/mL  
*E. cloacae* ATCC 13047 (from LB media): 917  $\mu$ g/mL  
*E. cloacae* ATCC 13047 (from MH-II media): 1.549 mg/mL  
*E. aerogenes* ATCC 13048 (from LB media): 2.471  $\mu$ g/mL  
*E. aerogenes* ATCC 13048 (from MH-II media): 1.456  $\mu$ g/mL  
*K. pneumoniae* MGH78578 (from LB media): low protein yield <50  $\mu$ g/mL  
*K. pneumoniae* MGH78578 (from MH-II media): low protein yield <50  $\mu$ g/mL

### **C. ACC Cleavage in Periplasmic Extract: Spectroscopic Evaluation of Scarless Linkers.**

[Note: The assay and all stock solutions were prepared at 4 °C.] Solutions for substrates **1** and **2** (50  $\mu$ M) were prepared in Tris (without Tween 20) from 2 mg/mL stock solutions in DMSO. Each 50  $\mu$ M solution of **1** and **2** (25  $\mu$ L) was then transferred to a 96-well plate (opaque, 200  $\mu$ L/well volume) in triplicate over two rows. *As a control, each substrate was mixed in triplicate with 50 mM Tris (25  $\mu$ L, note: 0.01% Tween 20 was contained in Tris) without periplasmic extract.* After slow thawing of *E. coli* K12 MG1655 (1.557 mg/mL, 100  $\mu$ L) and *E. coli*  $\Delta$ *bamB* $\Delta$ *tolC* BW25113 (1.962 mg/mL, 100  $\mu$ L) periplasmic extracts over 30 min on ice, they were diluted in Tris (94.6  $\mu$ L and 145.3  $\mu$ L, respectively, note: 0.01% Tween 20 was contained in Tris) to provide 800  $\mu$ g/mL stock solutions of each extract. The 800  $\mu$ g/mL solutions of periplasmic extract (25  $\mu$ L) were then transferred to the 96-well plate (opaque, 200  $\mu$ L/well volume) containing **1** and **2** resulting in a maximum volume of 50  $\mu$ L/well. The final concentrations of periplasmic extract and substrate were 400  $\mu$ g/mL and 25  $\mu$ M, respectively. The plate was sealed with a transparent polyolefin silicone film (Thermofisher™ Nuc™ Sealing Tape, 12-565-513) and placed in a plate reader set

to 37 °C with shaking at 225 rpm for 8 h. ACC excitation wavelengths: 300-410 nm at 5 nm intervals. ACC excitation maximum: 355 nm. ACC emission wavelengths: 410-500 nm at 5 nm intervals. ACC Emission maximum is 460 nm. The data is plotted in Figure 2 of the manuscript, and refer to Figures S4-S8 for similar experiments. Protease inhibitors were pre-incubated with periplasmic extract for 5-10 min at r.t. As shown in Figure S9 (page S17), HPLC traces of peptide **1** at 326 nm (at time t=0 and t=8h) suggests ~100% linker cleavage over the time course of the reaction.

#### **D. Cleavage of Eperezolid-NH<sub>2</sub> Conjugate (5) and Daptomycin Conjugate (4) in Periplasmic Extract.**

The periplasmic extract [25 µL, 400 µg/mL total protein for *E. coli* K12 MG1655 and *A. nosocomialis*; see total protein list above in section **B** for other strains] was mixed with the daptomycin conjugate **7** (25 µL, 117.6 µM) or the eperezolid conjugate **8** (25 µL, 117.6 µM) in a 1.5-mL Eppendorf tube. The reaction mixture was mixed at 1050 rpm in a table-top incubator set at 37 °C for 11 h. After 11 h, 25 µL was removed and placed in a 400-µL glass insert fitted in a mass-spec vial, which was followed by the addition of MilliQ H<sub>2</sub>O (25 µL) and 1 M HCl (2 µL). The reaction mixture was analyzed by HPLC (54 minutes, 0 to 95% CH<sub>3</sub>CN/H<sub>2</sub>O with 0.1 % TFA, 254 nm). The % release of eperezolid-NH<sub>2</sub> (**5**) was evaluated by the following equation:

$$\% \text{ release of } \mathbf{5} \text{ from conj. } \mathbf{8} = \frac{\text{area under the curve for } \mathbf{5} \text{ (produced in the reaction mixture)}}{\text{area for } \mathbf{5} \text{ at } 58.8 \mu\text{M}},$$

where 58.8 µM is the maximum amount of **5** that could be produced in the reaction mixture. These data are provided in Section VIII, pages S133-136. Evidence for unmodified daptomycin release is denoted by peaks that overlap with daptomycin (Section VIII, pages S115-S120, *e.g.* *A. nosocomialis* and *A. baumannii*). Due to limitations in the mass spec detector, we were unable to assign a mass to peaks overlapping with daptomycin. For cases where daptomycin release was not apparent or insignificant, see Section VIII, pages S121-S134 (*e.g.* *E. coli*).

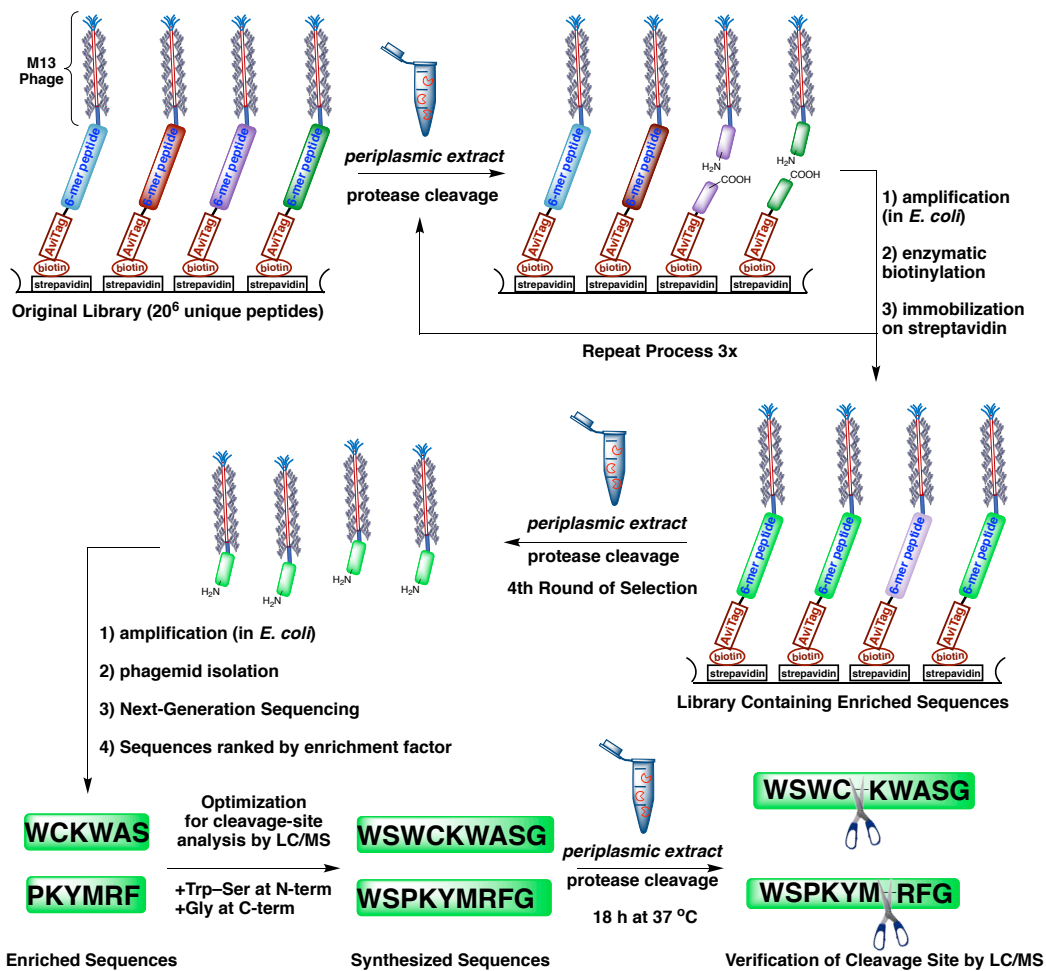
#### **E. In Vitro Translation.**

The ability of conjugates to inhibit the 70S *E. coli* ribosome was tested in vitro using the PURExpress, In-Vitro Protein Synthesis Kit (NEB), murine RNase inhibitor (NEB), and 7.5 ng/µl of template DNA encoding the fluorescent protein mEGFP. The volumes of each component in the reaction mixture were scaled down from the NEB protocol for a final reaction volume of 4 µL.



We then added water (40  $\mu$ L), 2 $\times$  biotinylation buffer (80  $\mu$ L, composition of biotinylation buffer: 0.1 M Tris, 10 mM MgCl<sub>2</sub>, 2 mM Biotin, pH 8.0) to wash the 30 kDa MWCO tube and transfer the remaining solution to a 1.5-mL Eppendorf tube. Additional water (20  $\mu$ L) and 2 $\times$  biotinylation buffer (20  $\mu$ L) was used to complete the transfer to the Eppendorf tube. We then added 0.1 M ATP (~6  $\mu$ L) and BirA enzyme (2  $\mu$ L, 3U/ $\mu$ L) to the phage solution (~200  $\mu$ L). The mixture was vortexed and cooled to 4 °C overnight for biotinylation. An anti-biotin western blot was then performed to confirm that phages were biotinylated.

## B. Substrate phage selection experiments.



Four rounds of substrate selection were conducted. In round one, the streptavidin-coated ELISA plate was blocked with 2% BSA for 30 min and washed three times with phosphate-buffered saline containing 0.1 % Tween 20 (PBST) buffer. The plate was then coated with  $>10^{10}$  phages (one well) by gently shaking the phages for 2 hours. The wells were then washed with PBST (6x), and then the wells were washed with PBST (0.3 mL, 3  $\times$  20 min) while incubating at

37 °C.

For the first round of cleavage selection, periplasmic extract (50 µL) was added to one well and shaken at 37 °C for 3 hours. Cleaved phages were amplified overnight in 2xYT (30 mL) at 37 °C with shaking. The second, third, and fourth rounds of selection were performed by repeating the procedure above with 25 uL extract + 25 uL PBS added to each well, with reduced cleavage times: 2 hours, 1 hour, and 30 minutes, respectively. The output phage titer is  $\sim 3 \times 10^8$  cfu for each round of selection. The final output phages were sequenced by Next Generation Sequencing.

Note: Output reads are the number of total reads of the same peptide sequence in the output library. Initial reads are the number of the total reads of the same peptide sequence in the initial naive library. Ranking is based on the ratio of output reads over initial reads. Read number 0.5 means the specific sequence was not found in the sequencing-result file. Sequences colored in red were selected for further validation.

K12 periplasmic extract selection results (Enrichment factor > 5000)			
Sequence	Output Reads	Initial reads	Enrichment factor
KNQSLG	10652	0.5	21304
GSDSSV	9239	0.5	18478
NHADVH	8138	0.5	16276
YSDSET	7764	0.5	15528
KSEMLS	7742	0.5	15484
WCKWAS	15307	1	15307
NKWNPS	7651	0.5	15302
SCYQCQ	7591	0.5	15182
TFATCK	7405	0.5	14810
ALWIYR	6805	0.5	13610
PKYMRF	13192	1	13192
RFQPPV	6467	0.5	12934
RVGLGA	6412	0.5	12824
MYPPTW	6396	0.5	12792
PGSQSL	11285	1	11285
QSFSID	5616	0.5	11232
VSPNRH	5371	0.5	10742

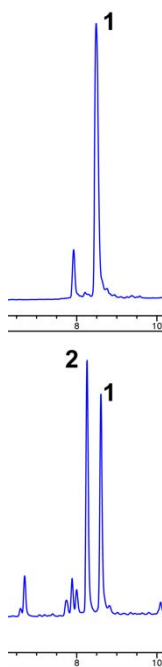


RTVYYP	5056	0.5	10112
YFQYHP	4856	0.5	9712
KNYVFQ	4385	0.5	8770
KHPSRR	4073	0.5	8146
NTHPAN	4030	0.5	8060
YTTEAF	3980	0.5	7960
SREASN	3912	0.5	7824
HLMFNC	3676	0.5	7352
WRPHPT	3622	0.5	7244
SLGFSK	3529	0.5	7058
LEIPRF	3448	0.5	6896
QNKPSV	3407	0.5	6814
DVNWSG	3187	0.5	6374
NLKREL	3185	0.5	6370
STLHQS	3028	0.5	6056
YKEYQT	2938	0.5	5876
SHNLNY	2851	0.5	5702
PCNTNR	2826	0.5	5652
NNAQNQ	2761	0.5	5522
VSYSR	2755	0.5	5510
FTADRC	2705	0.5	5410
LESEPG	2681	0.5	5362
LEPRSP	2581	0.5	5162
NRHART	2561	0.5	5122

**Sequences selected for validation.**

The peptides were synthesized as WSXXXXXXG, where XXXXXX represents the specific hexapeptide. Trp was added at the N-terminus to help the peptide bind with the reverse-phase HPLC column. The N-terminus is a free amine, while the C-terminus is a carboxylamide.

From K12 selection
KNQSLG
GSDSSV
NHADVH
KSEMLS
WCKWAS
PKYMRF



WSPKYMRFG cleavage with K12 extract. Cleavage Conditions: 50 mM Tris (pH 8.0), peptide (0.2 mg/mL), extract 0.1 mg/mL (total protein concentration based on OD 280), 37 °C, 18 hours. Cleaved peptide **2** is **WSPKYM**.

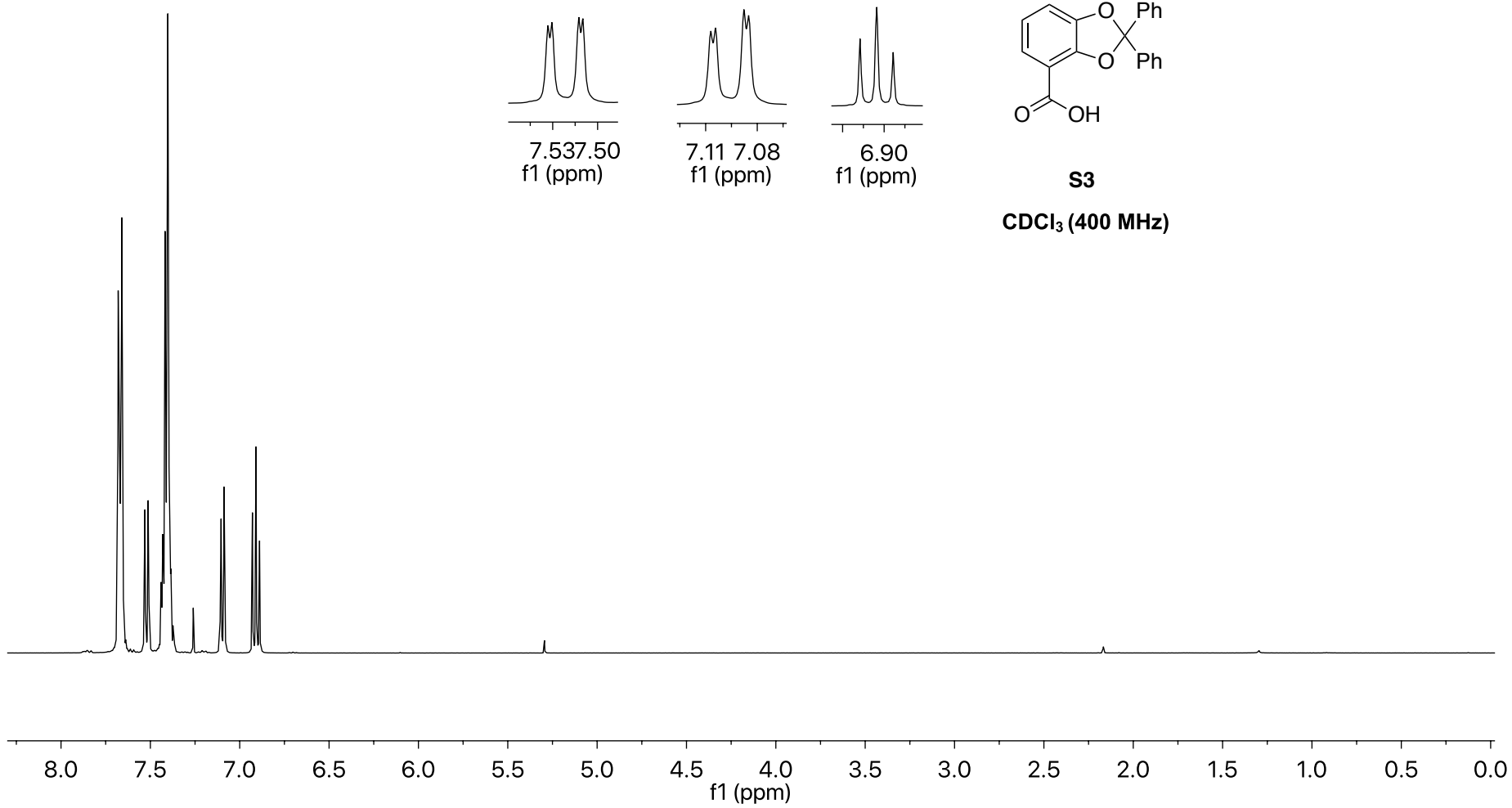
See **Table S5** for cleavage-site analysis of all six sequences.

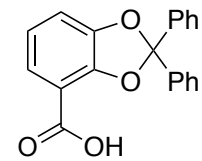
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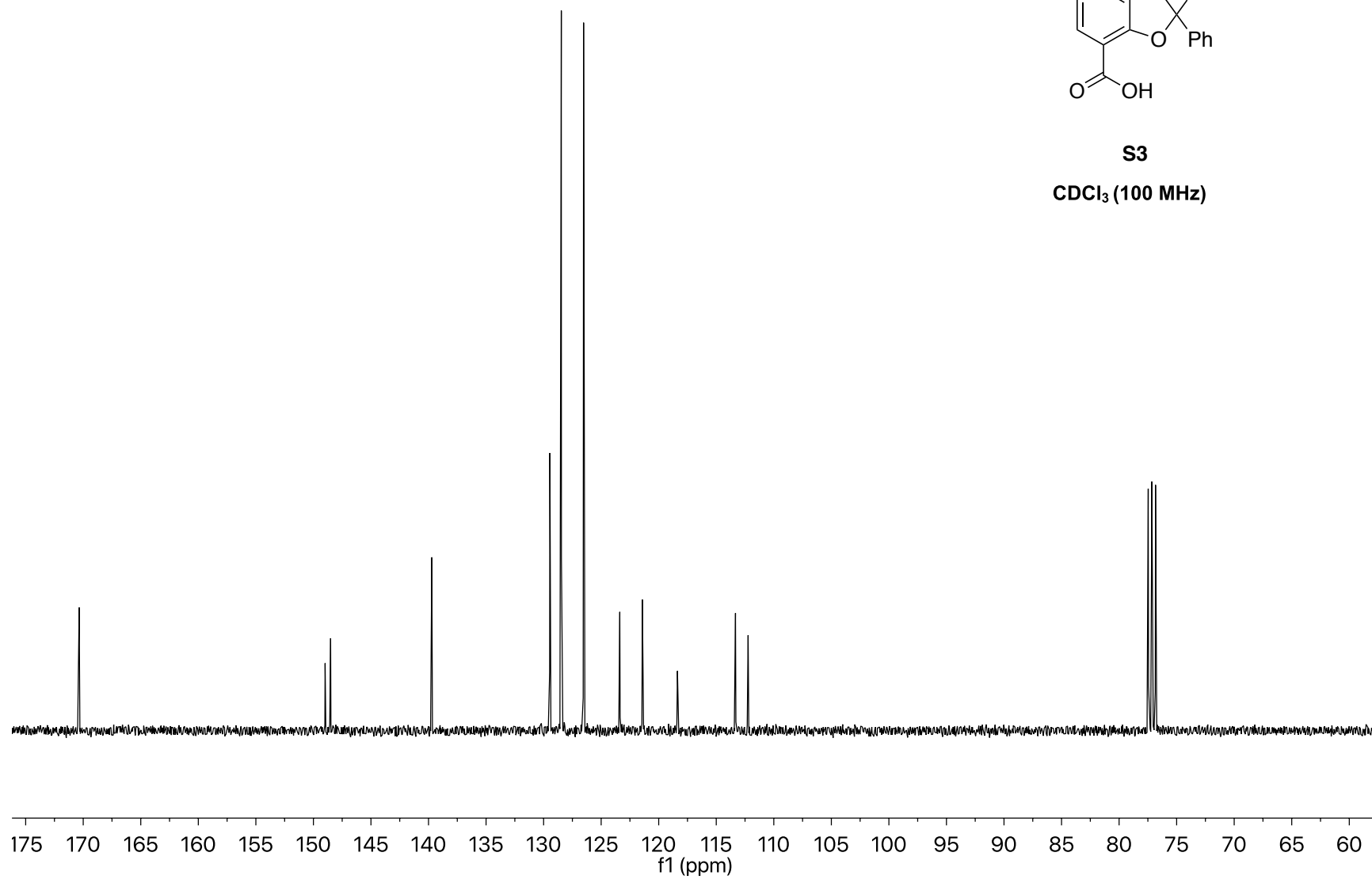
## VII: Select NMR Spectra



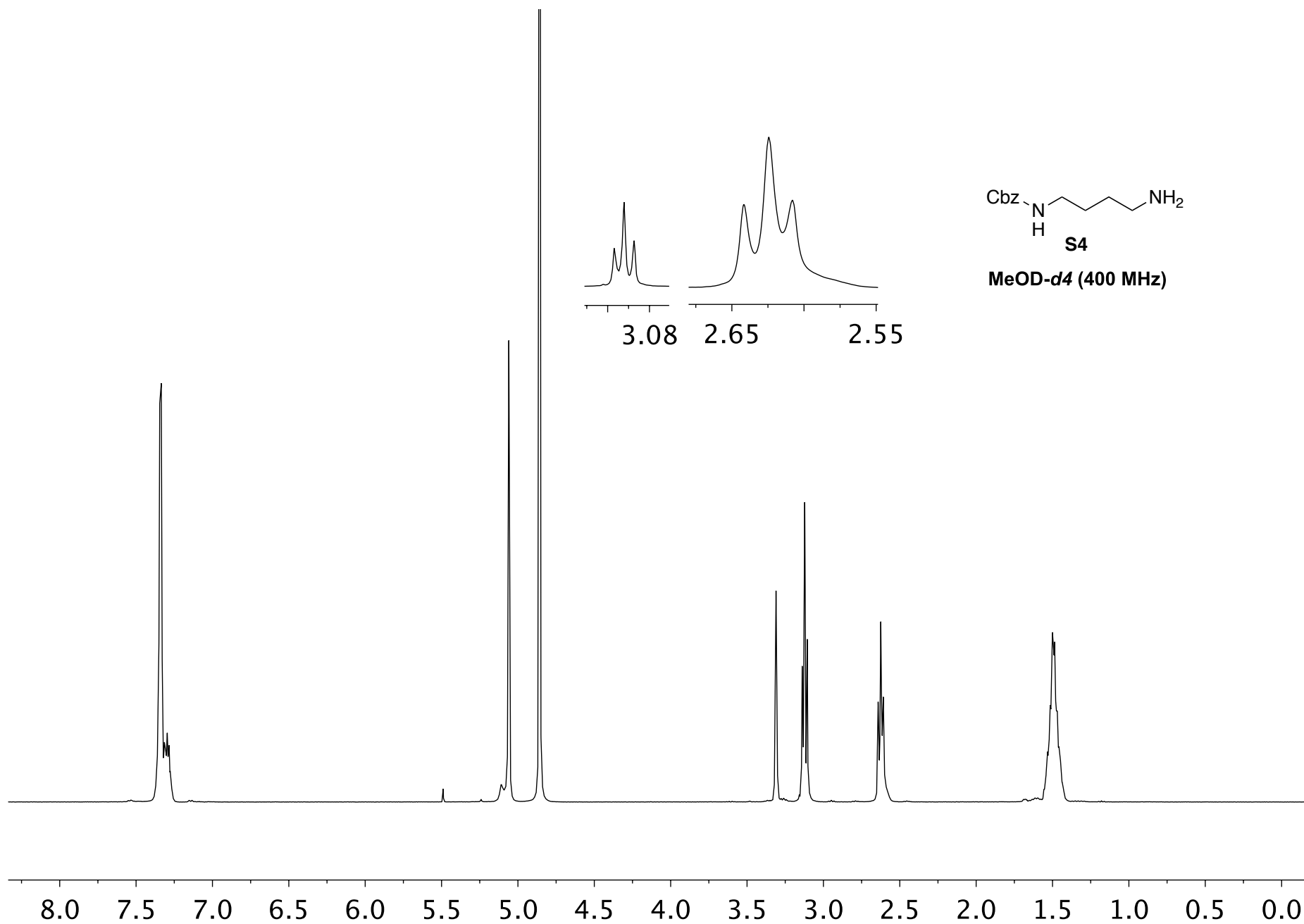


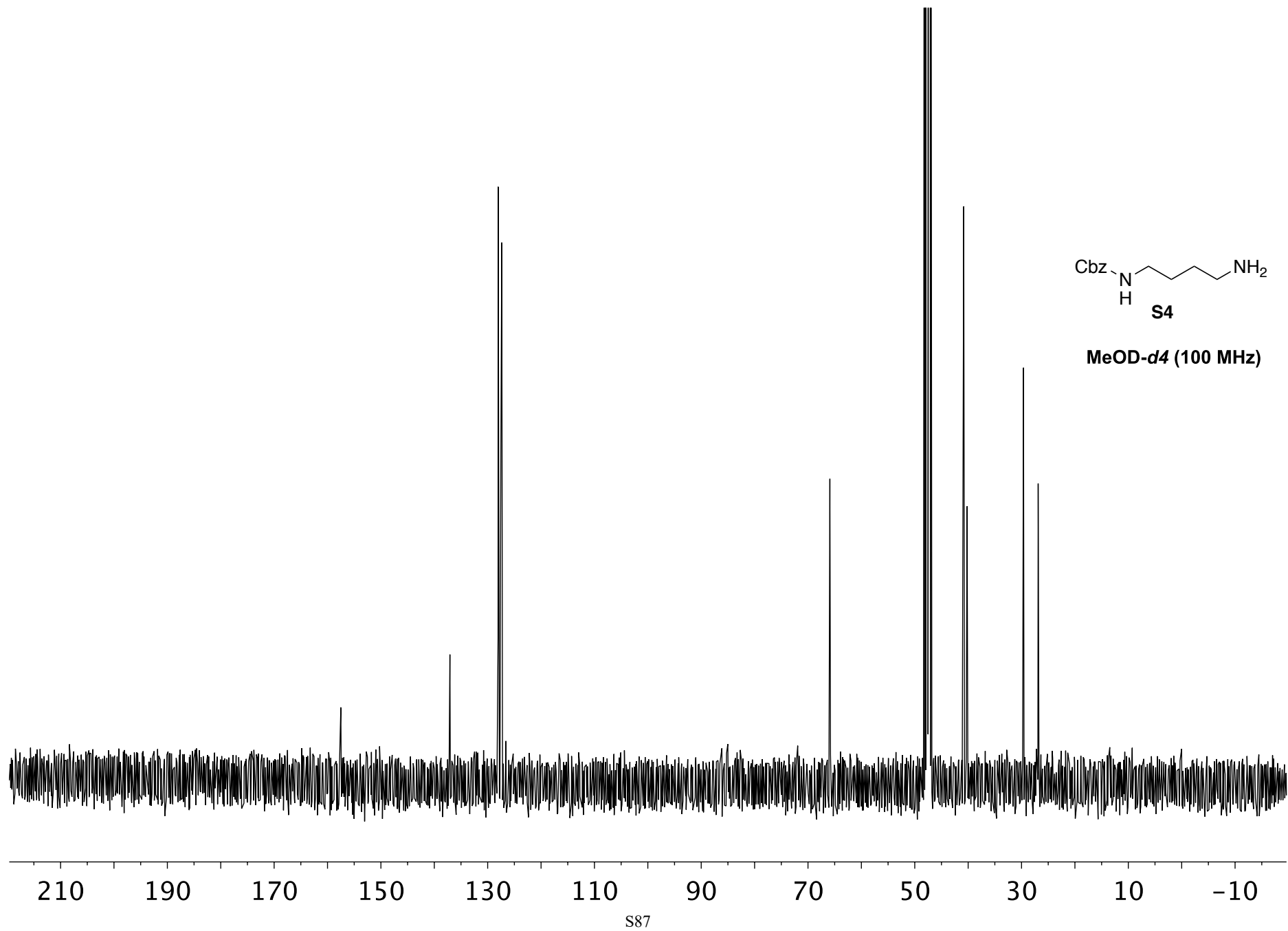
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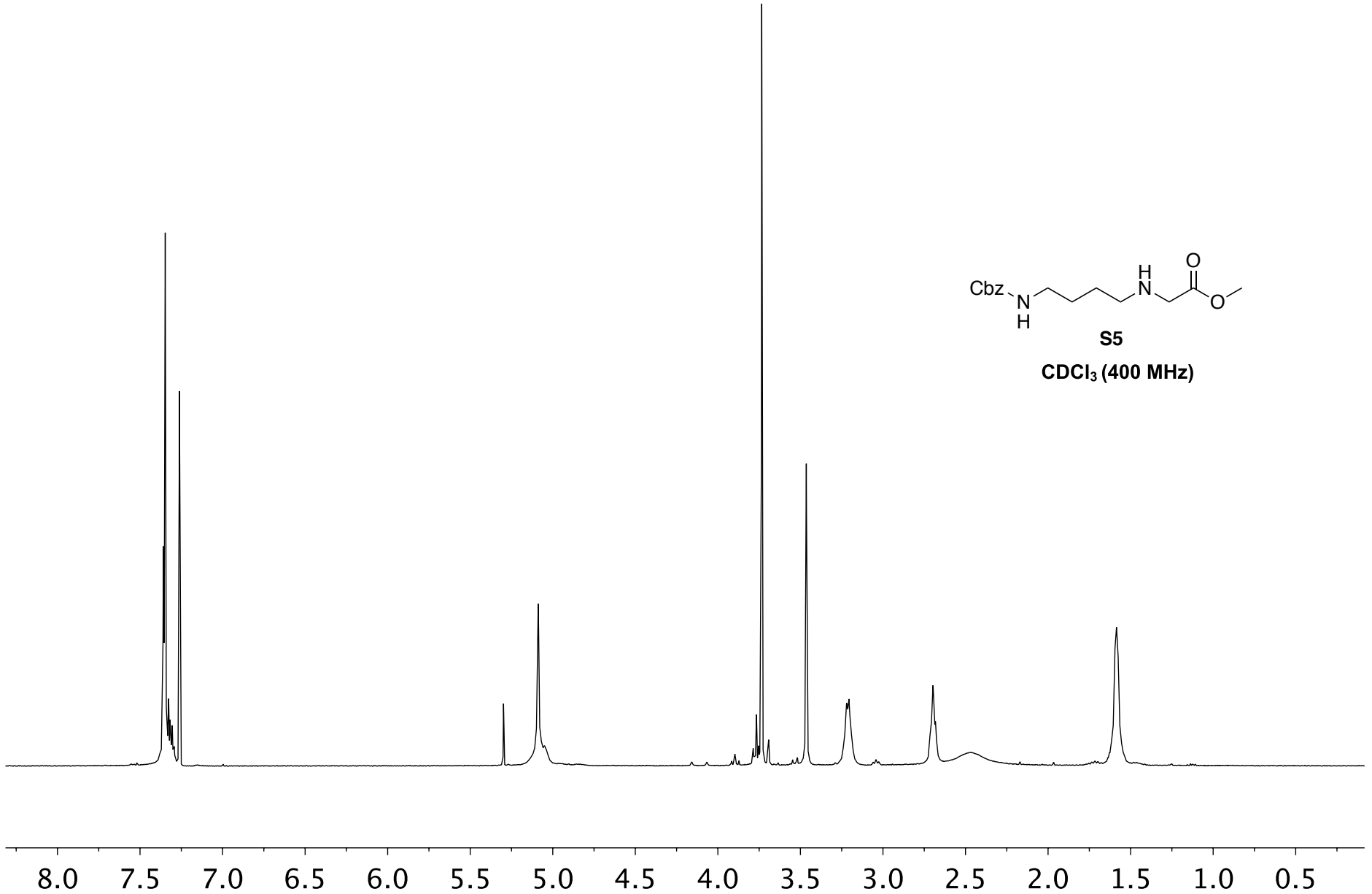
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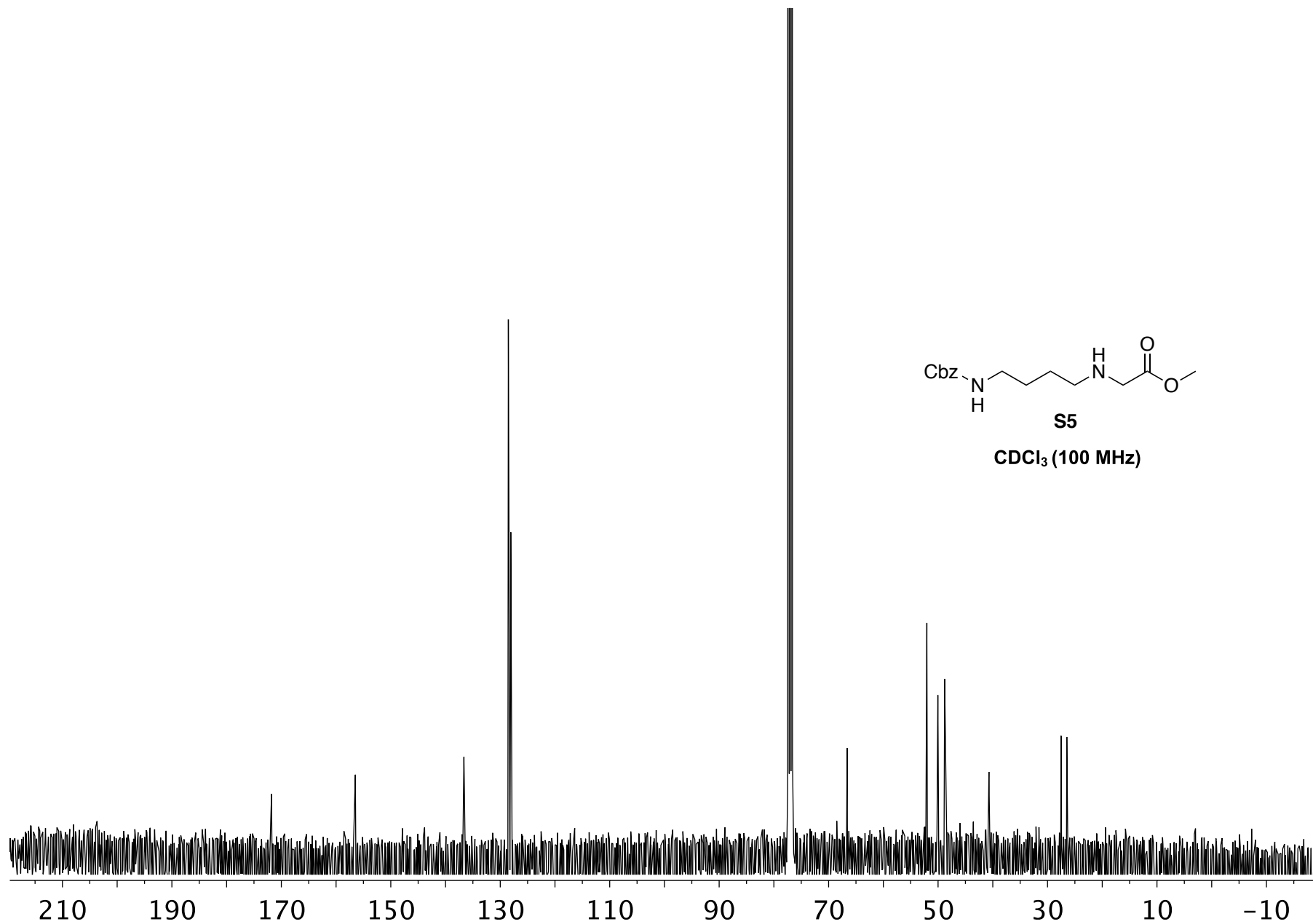
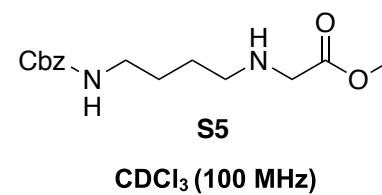
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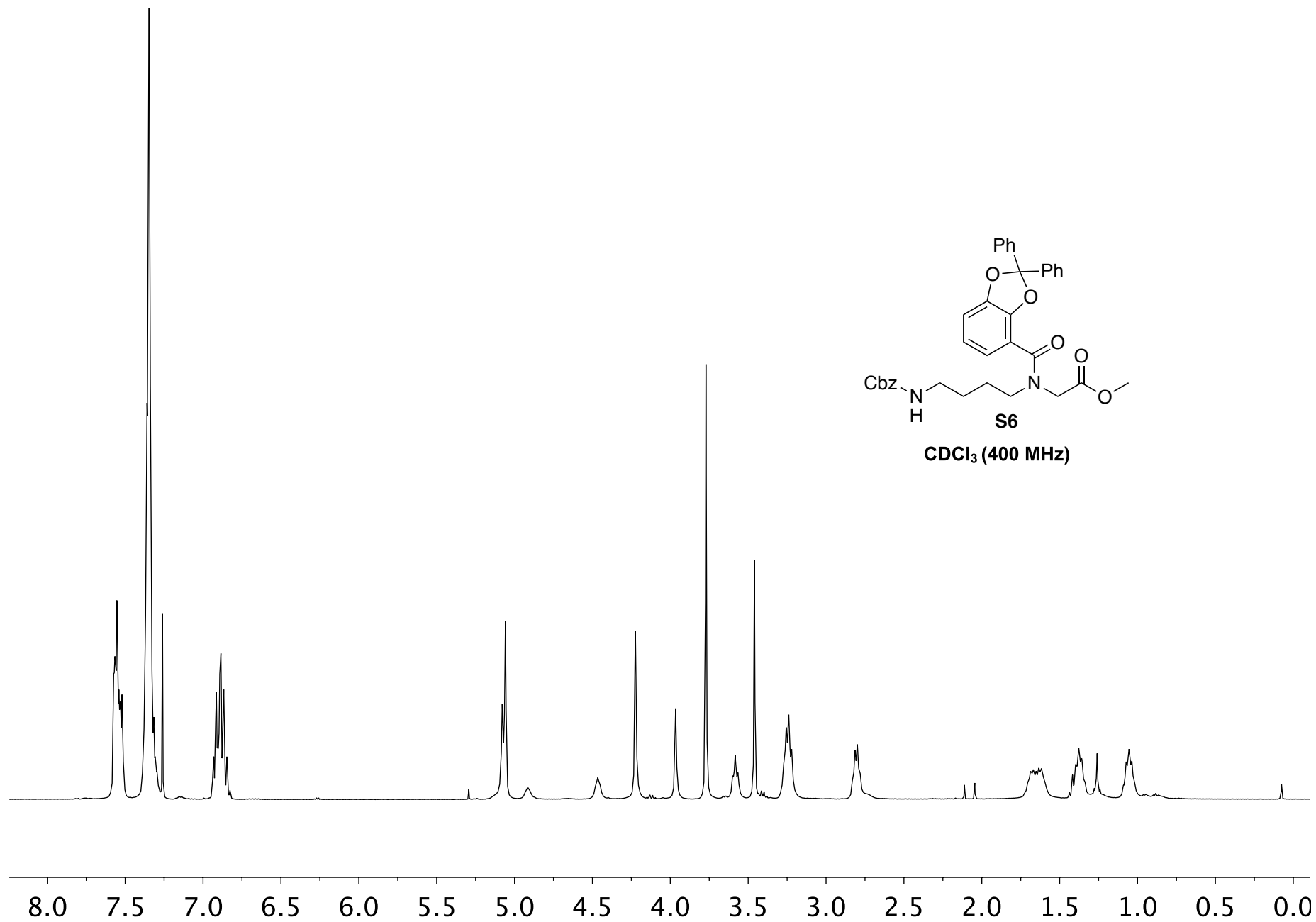
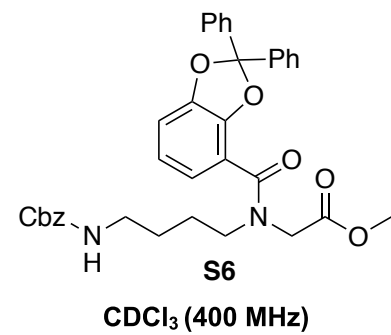


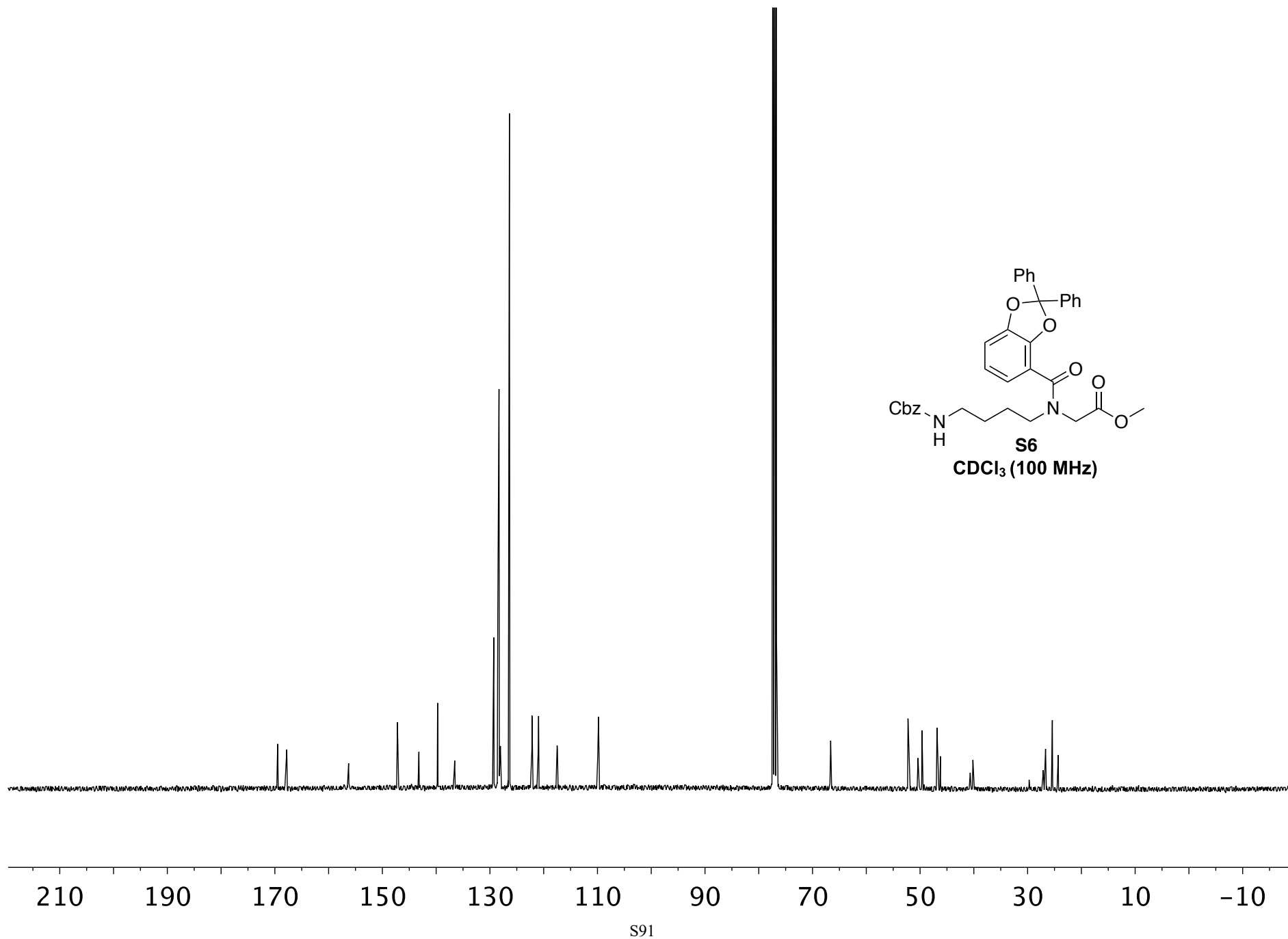


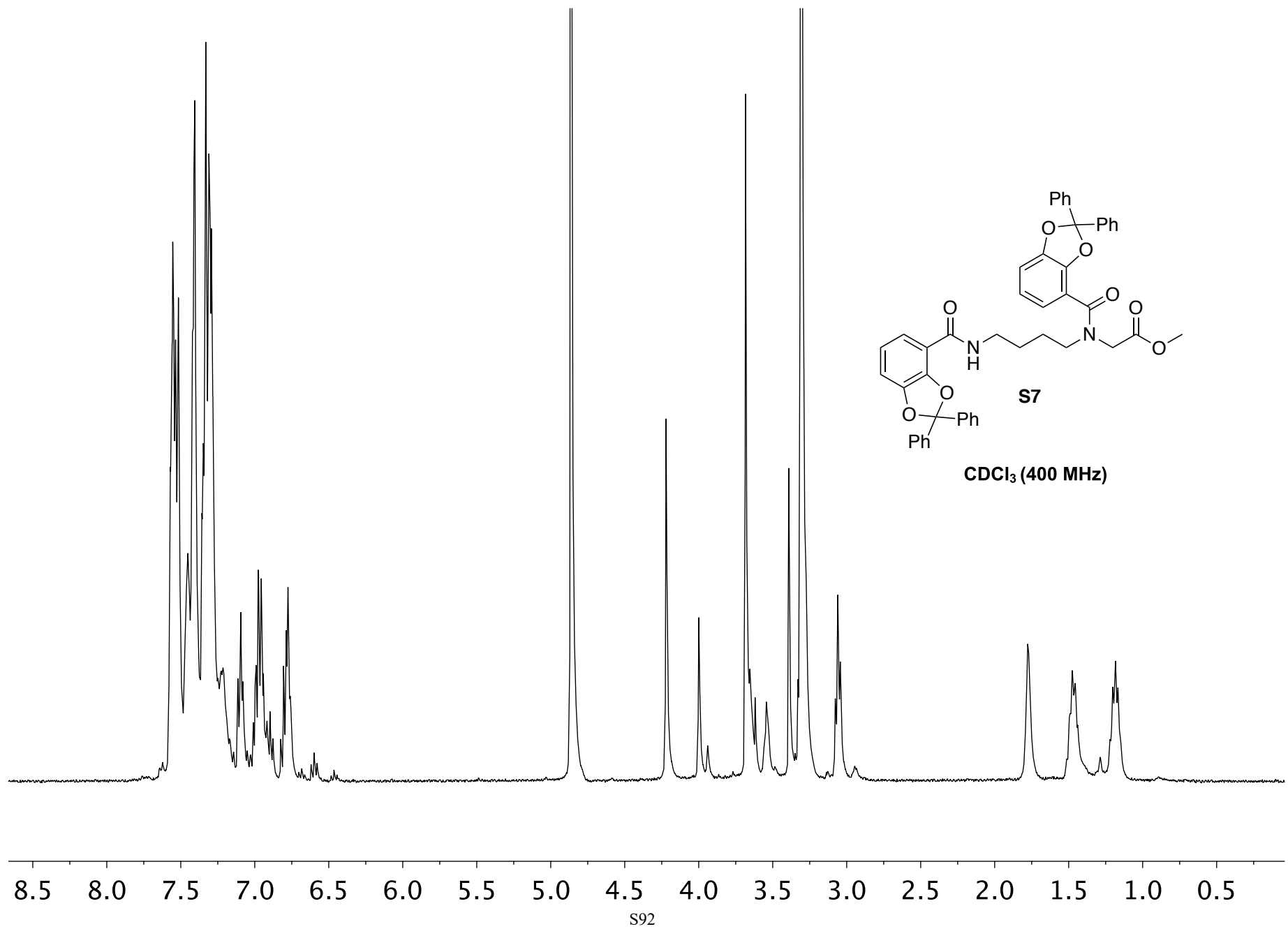


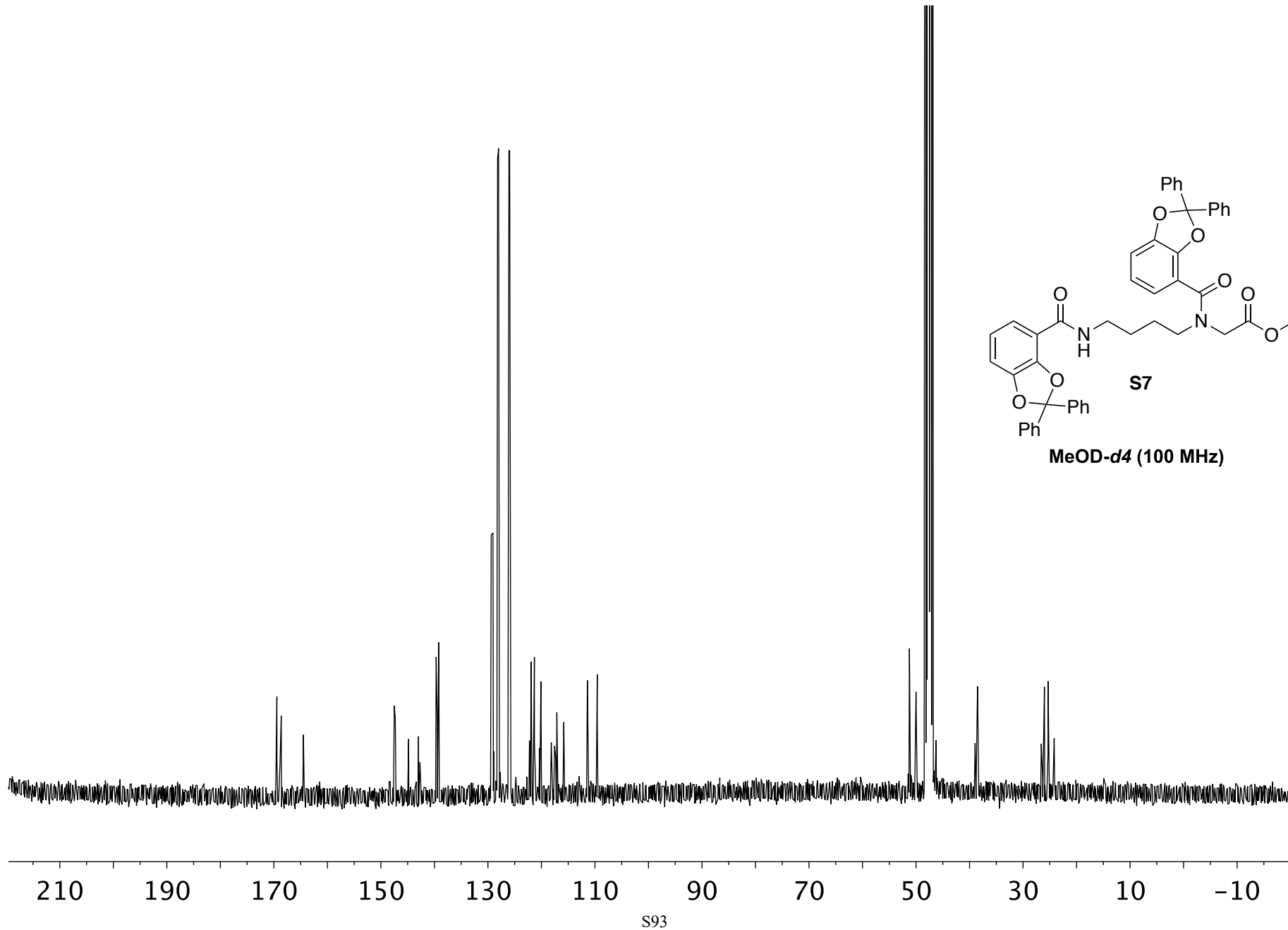


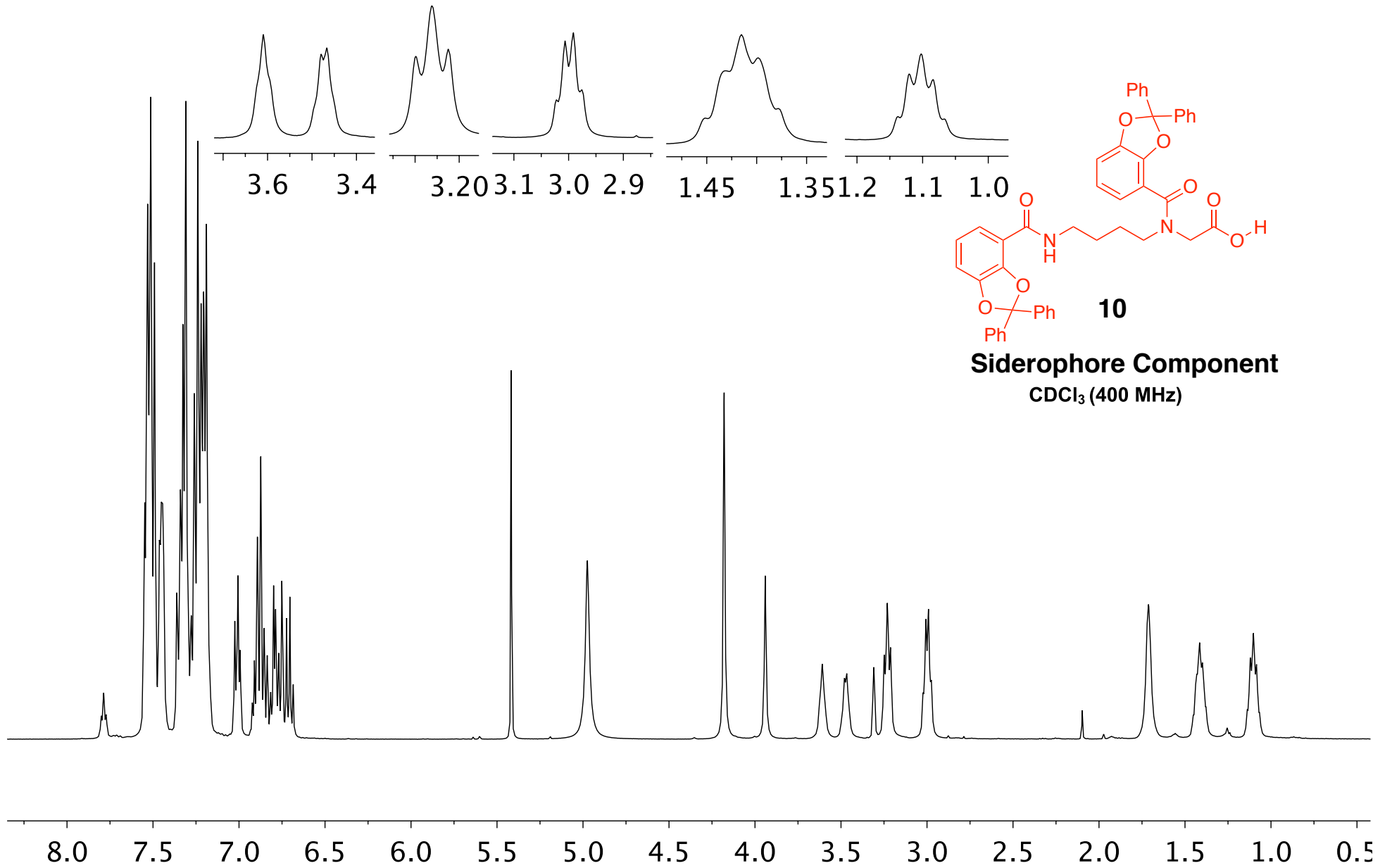


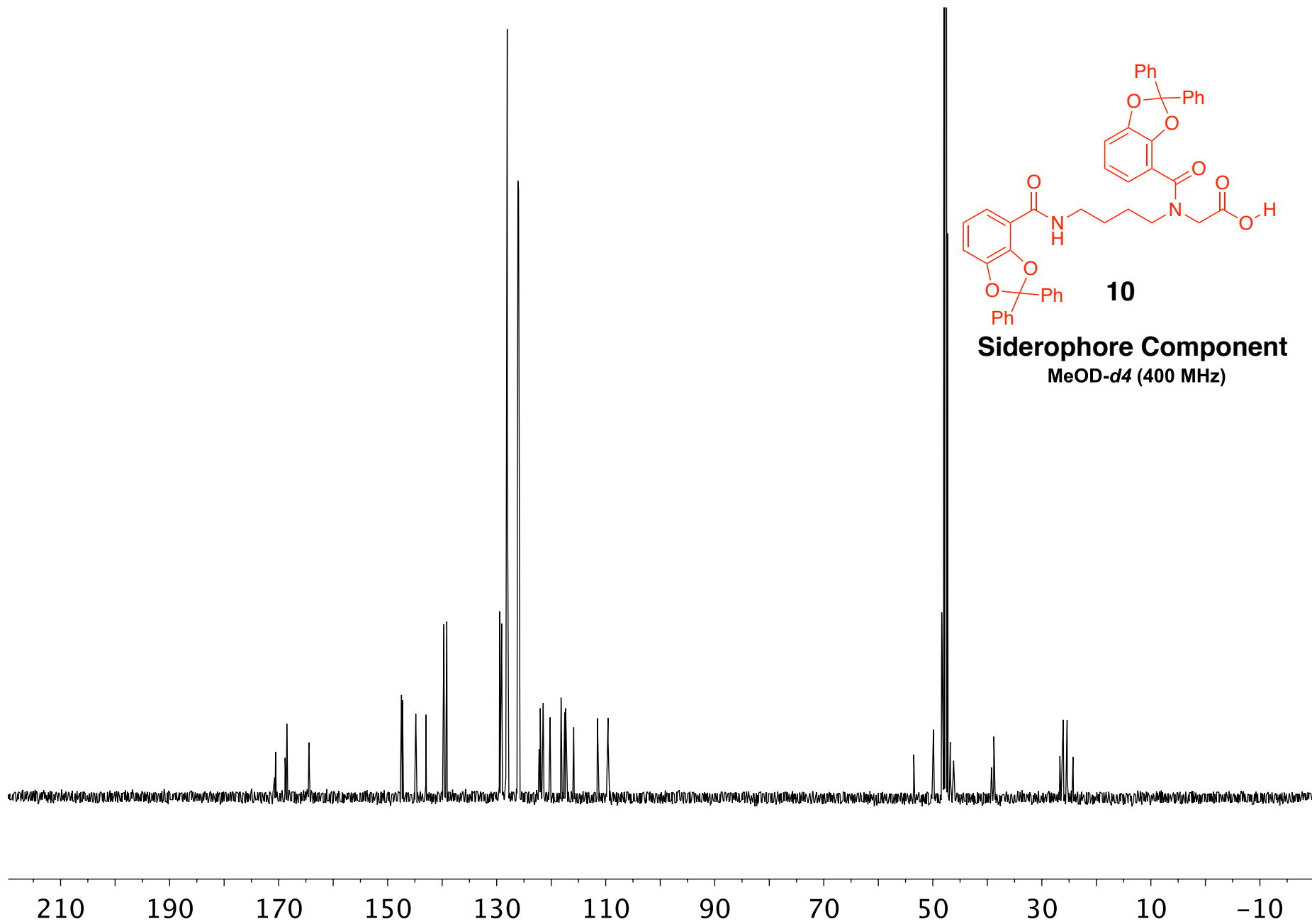




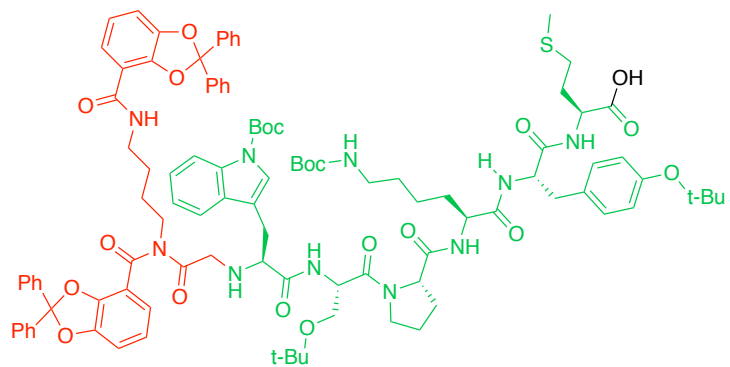






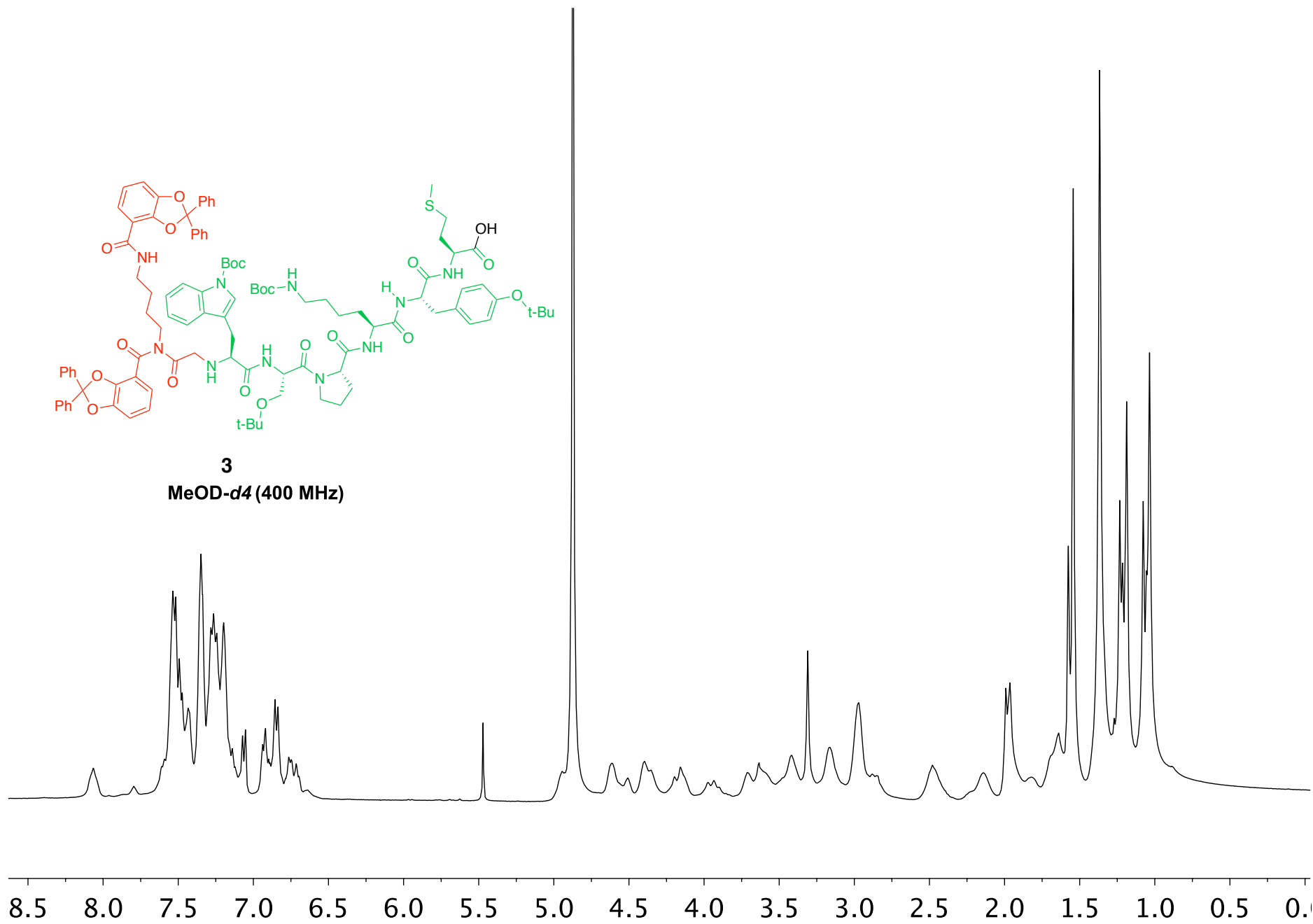


S95



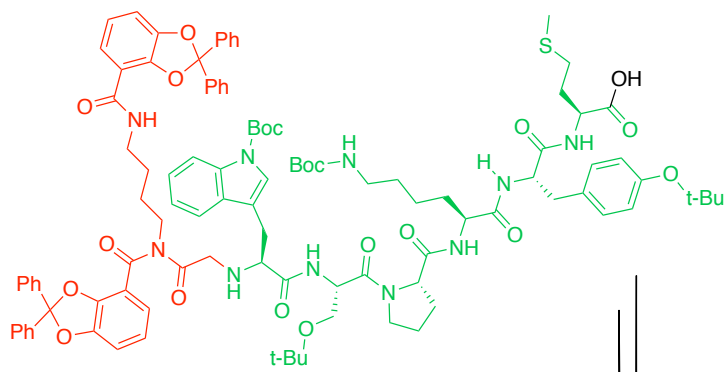
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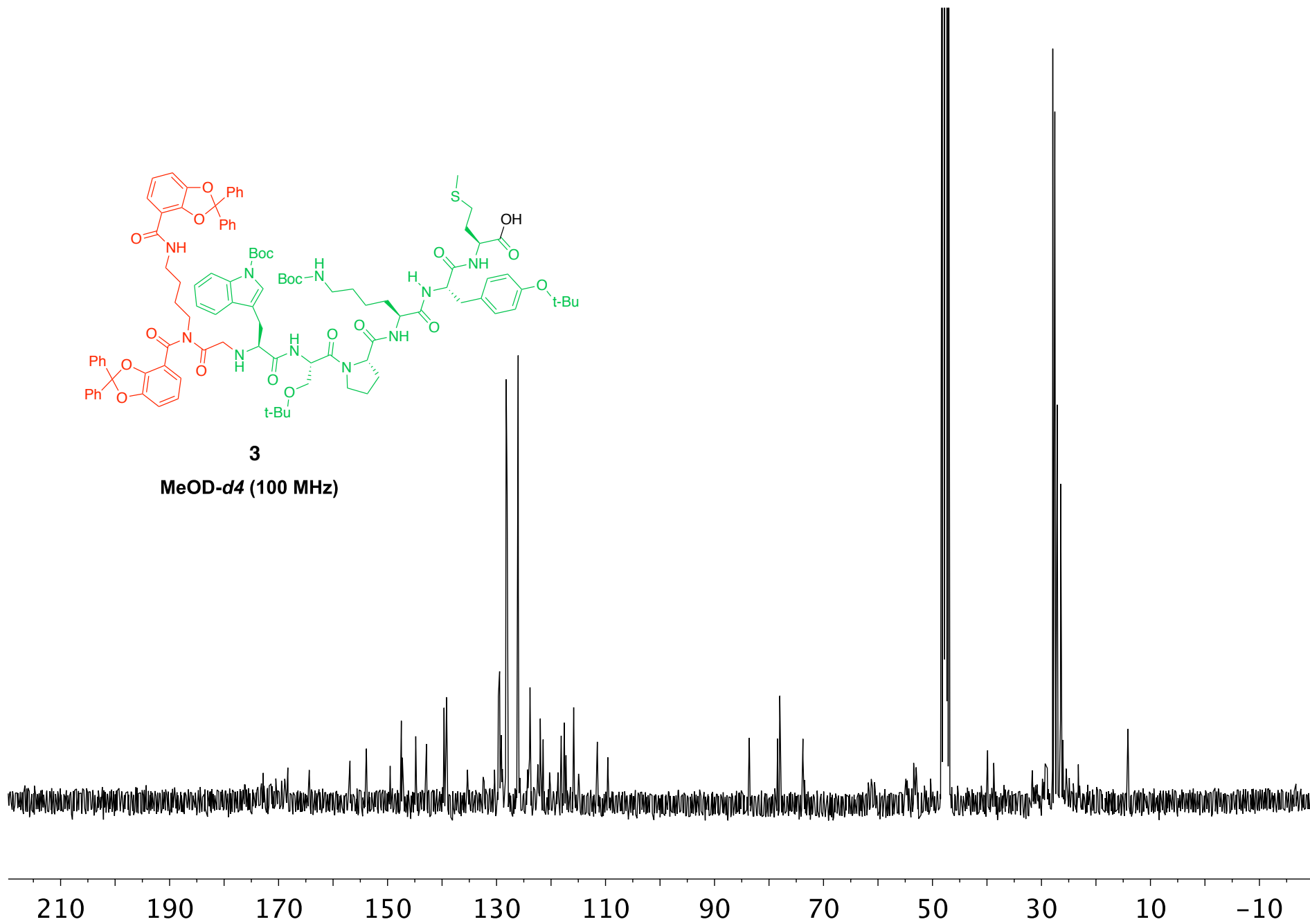
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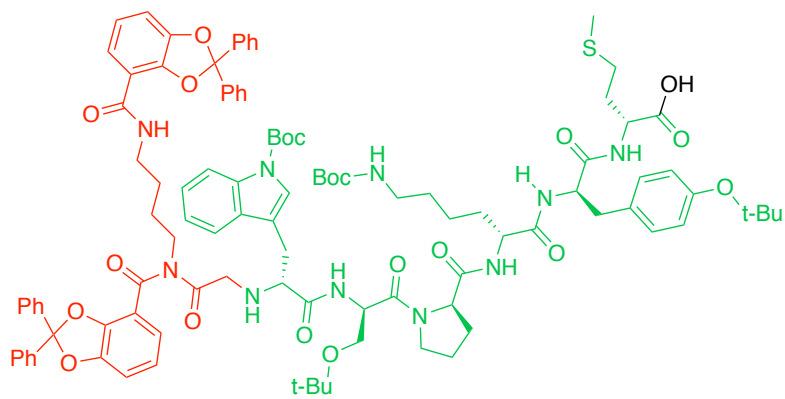




**3**

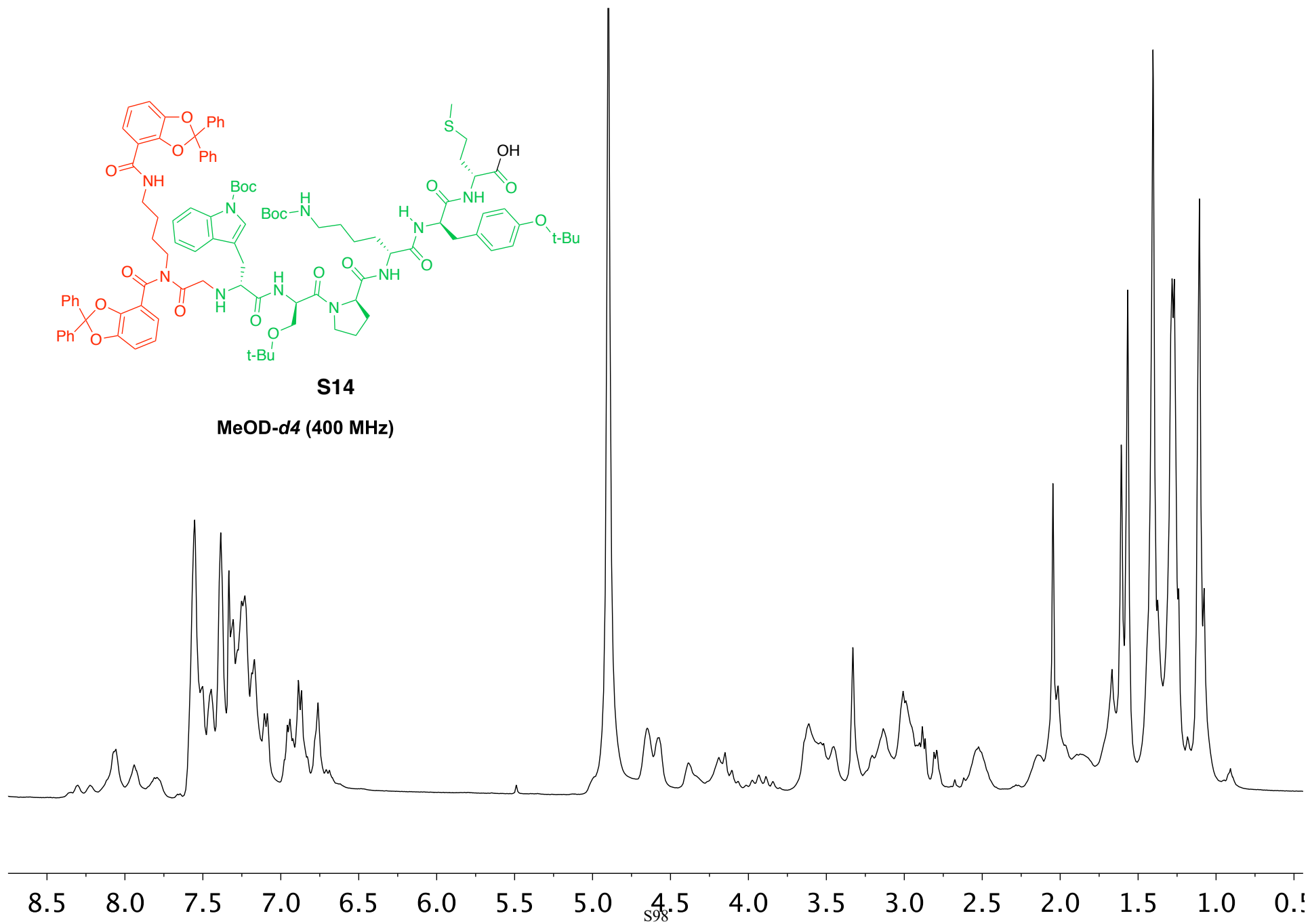
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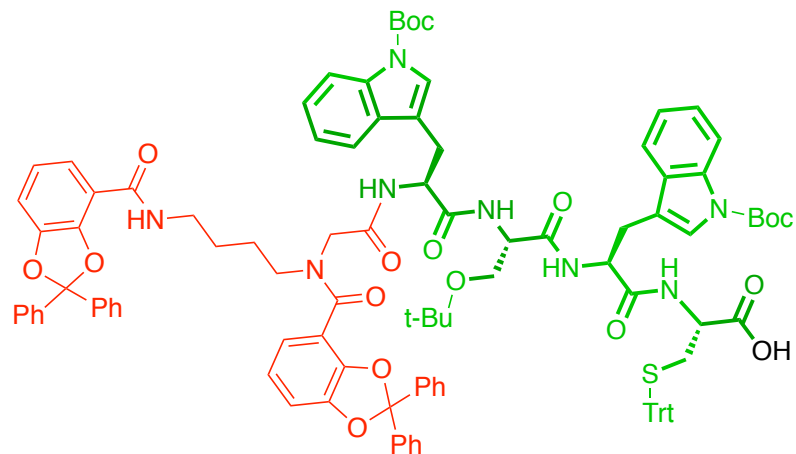


**S14**

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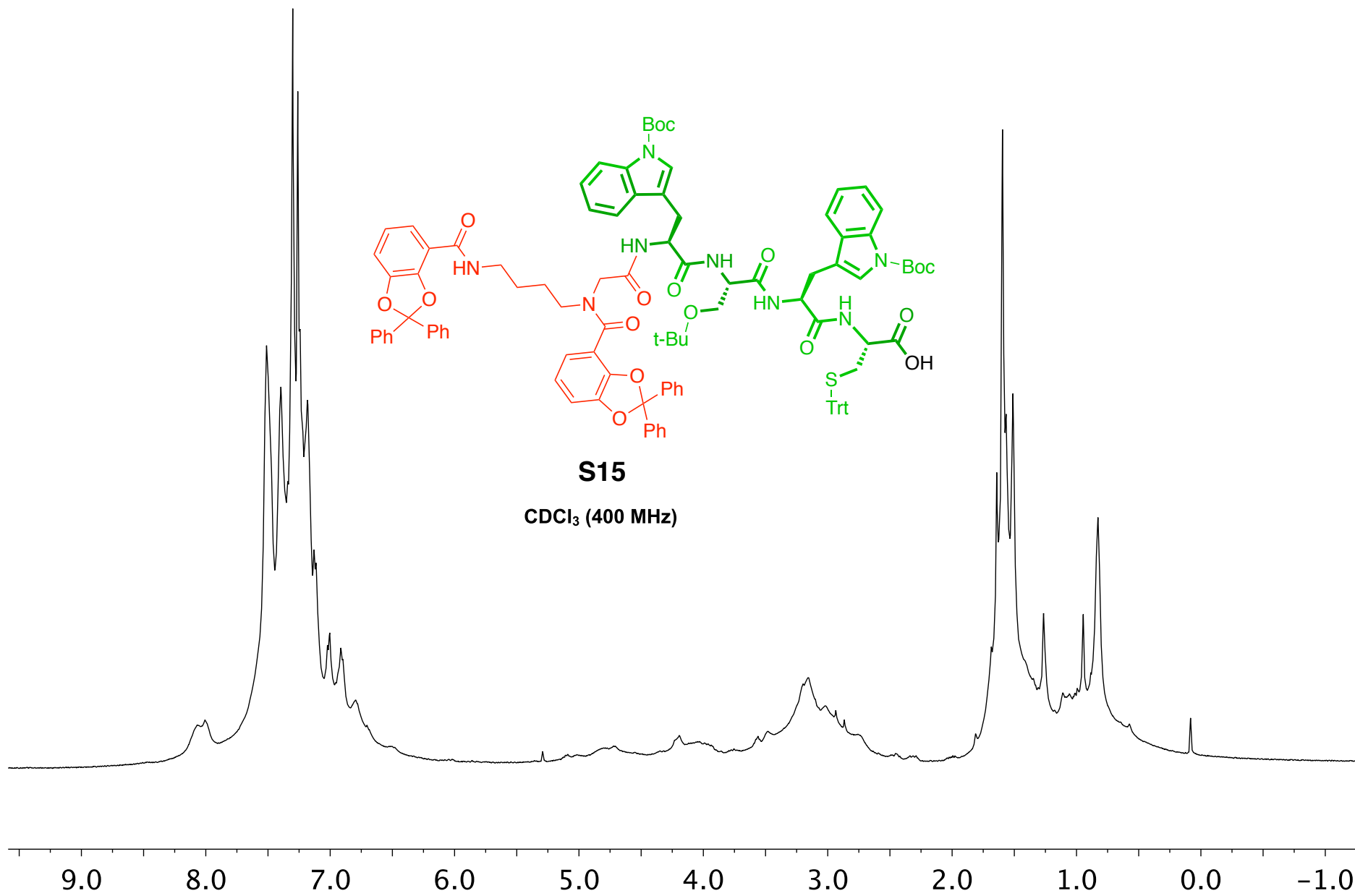




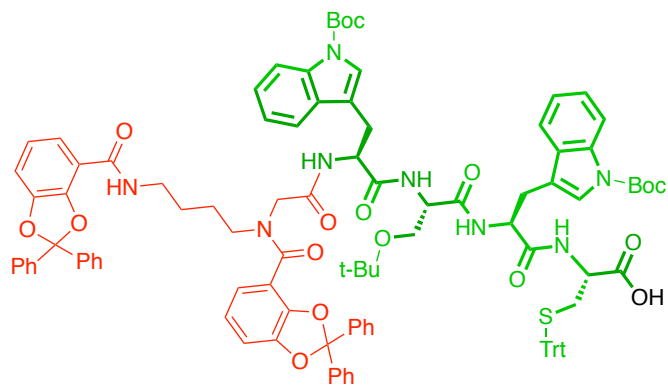


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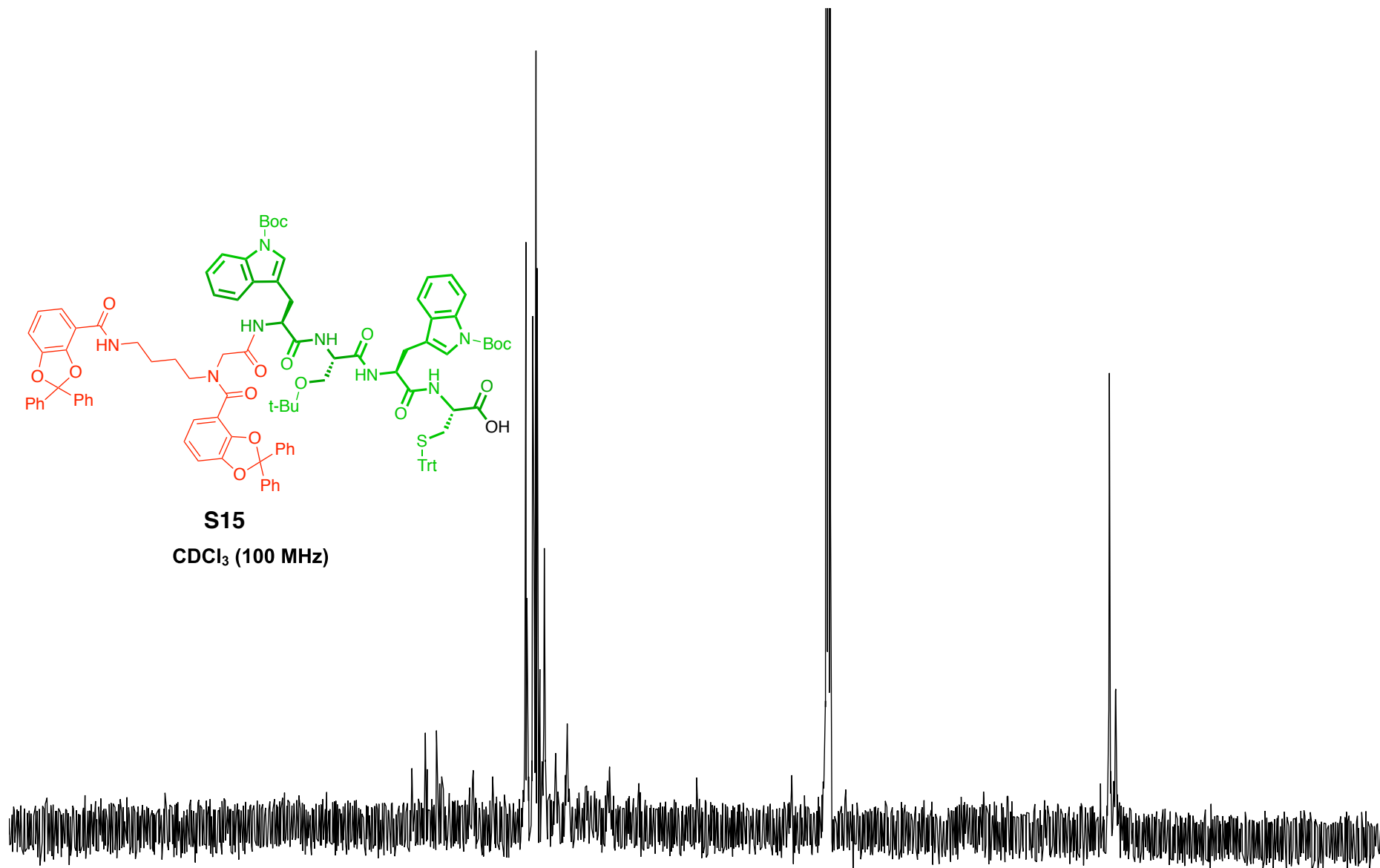


S100



**S15**

**CDCl<sub>3</sub> (100 MHz)**



210

190

170

150

130

110

90

70

50

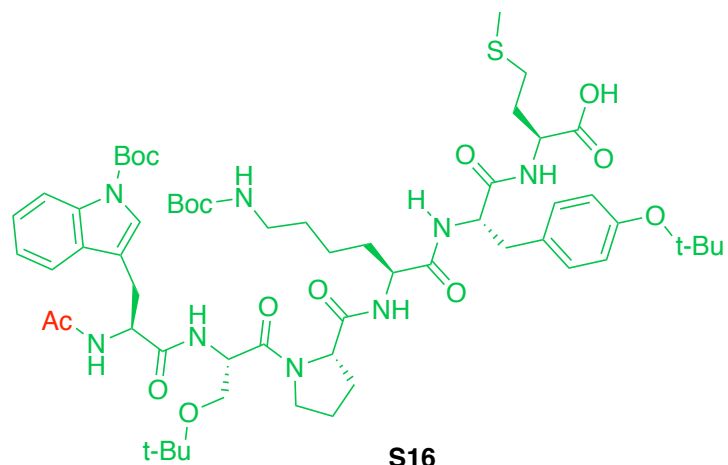
30

10

-10

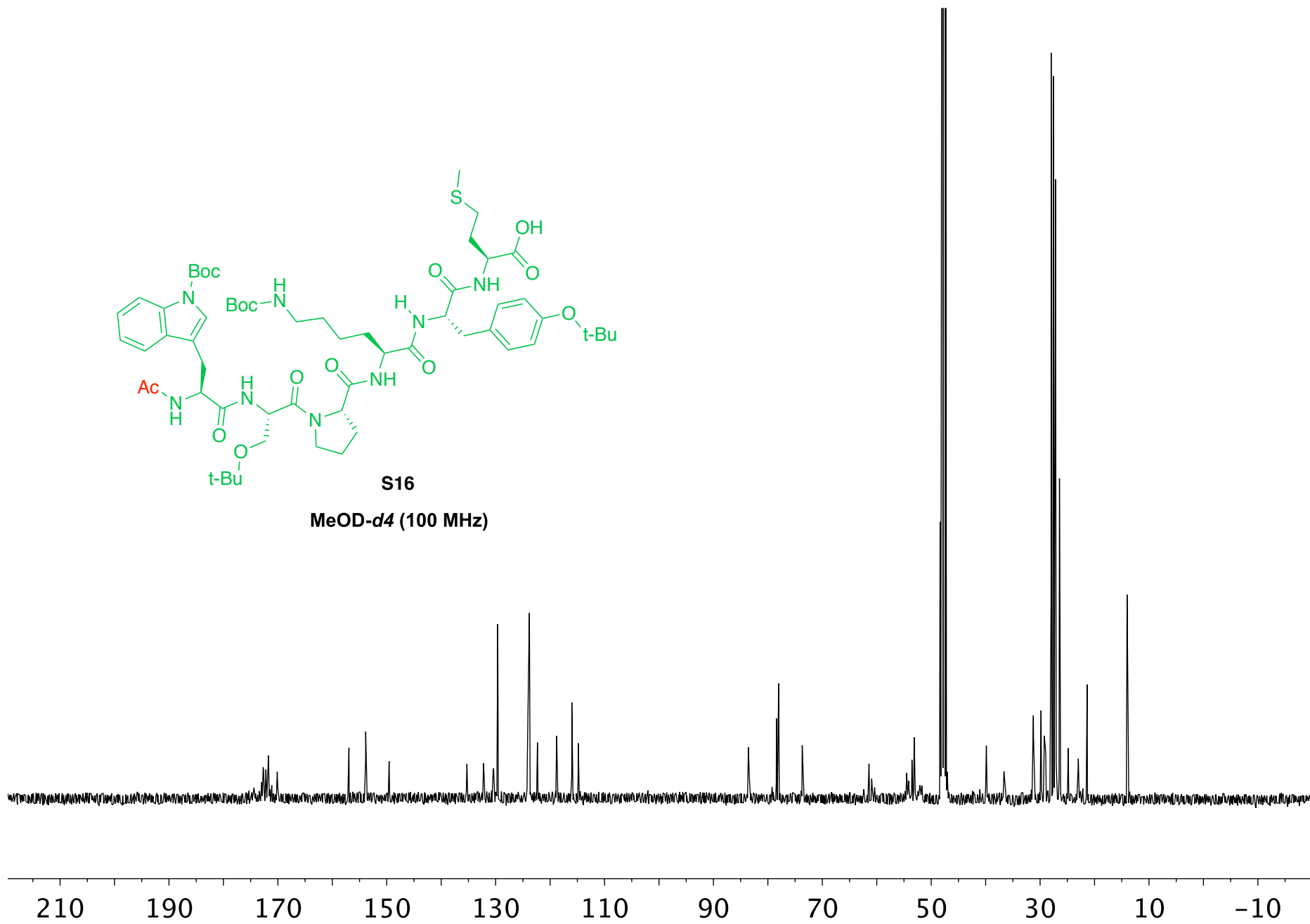
S101



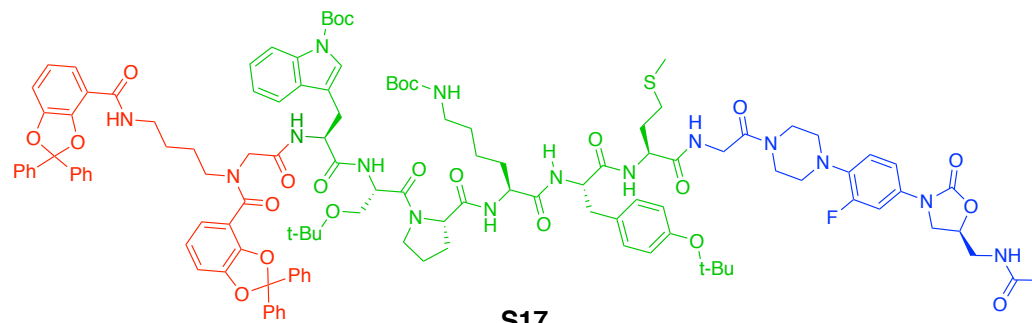


S16

MeOD-*d*4 (100 MHz)

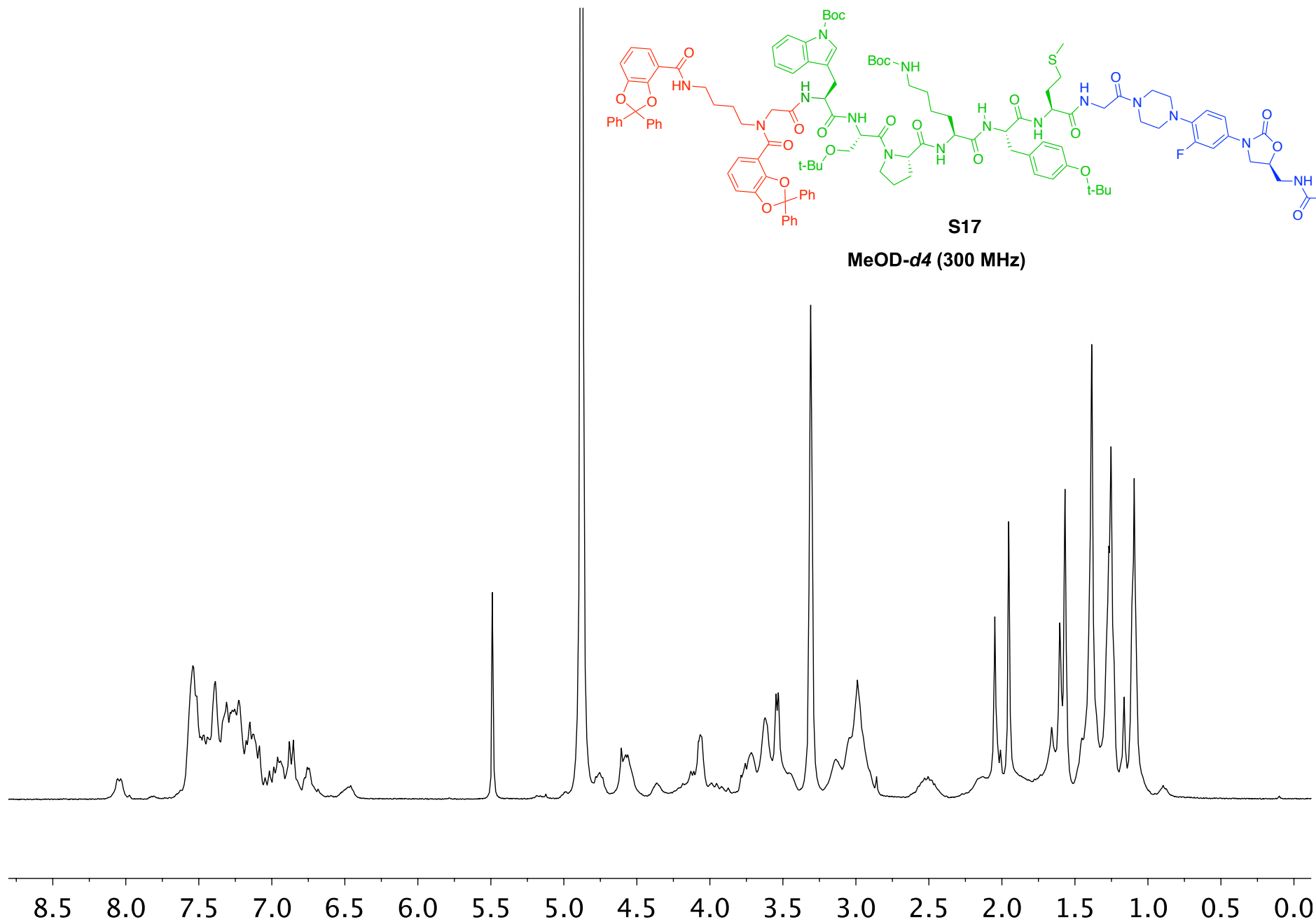


S103



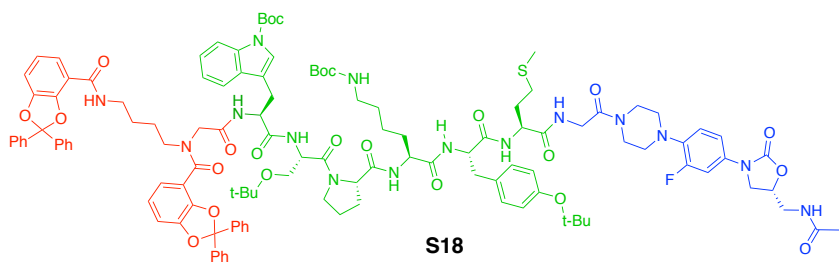
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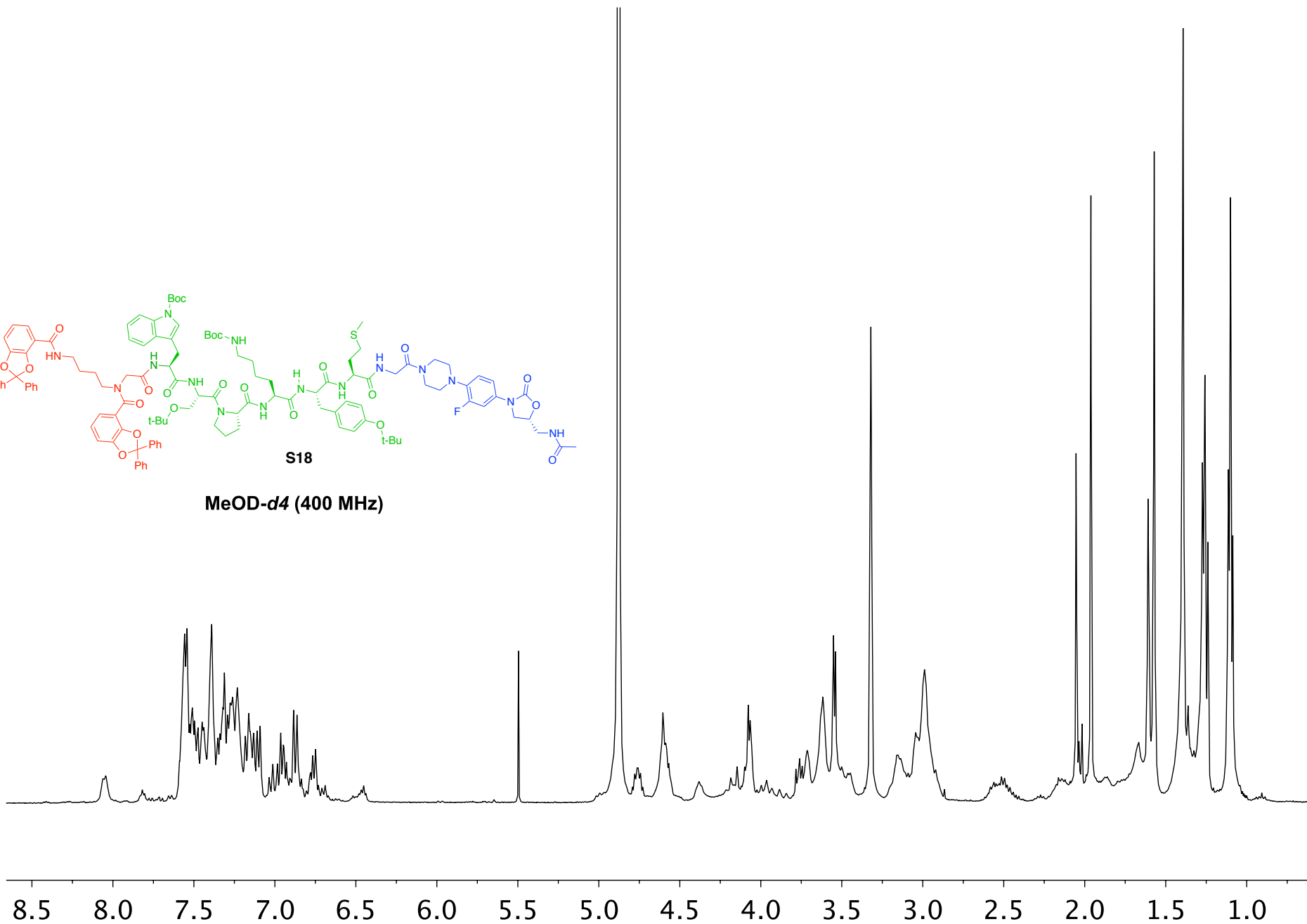
S104





S18

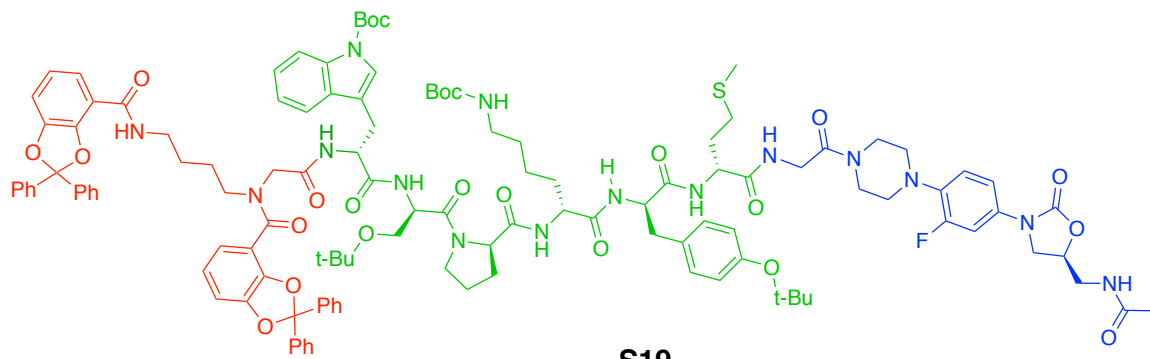
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S105

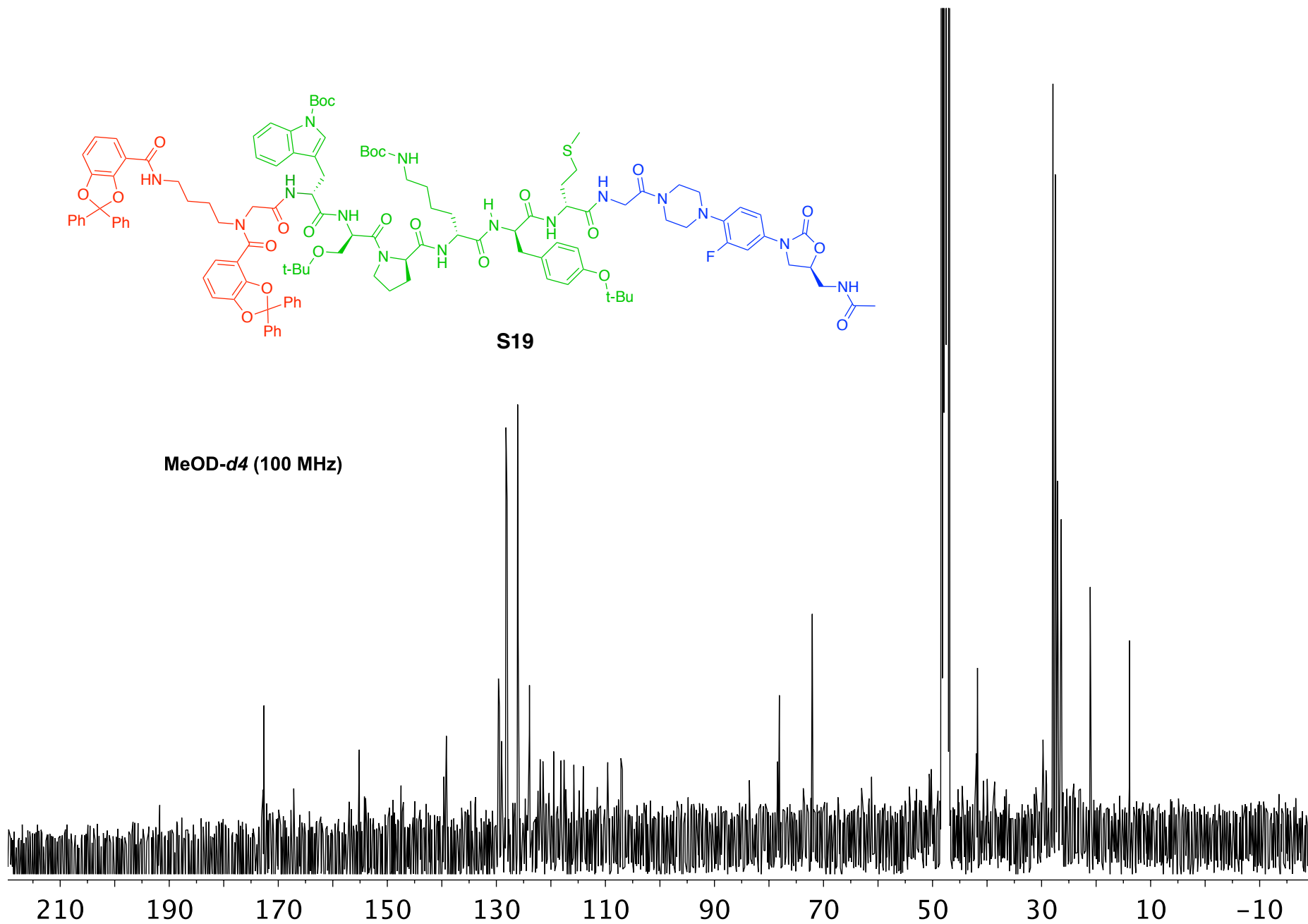


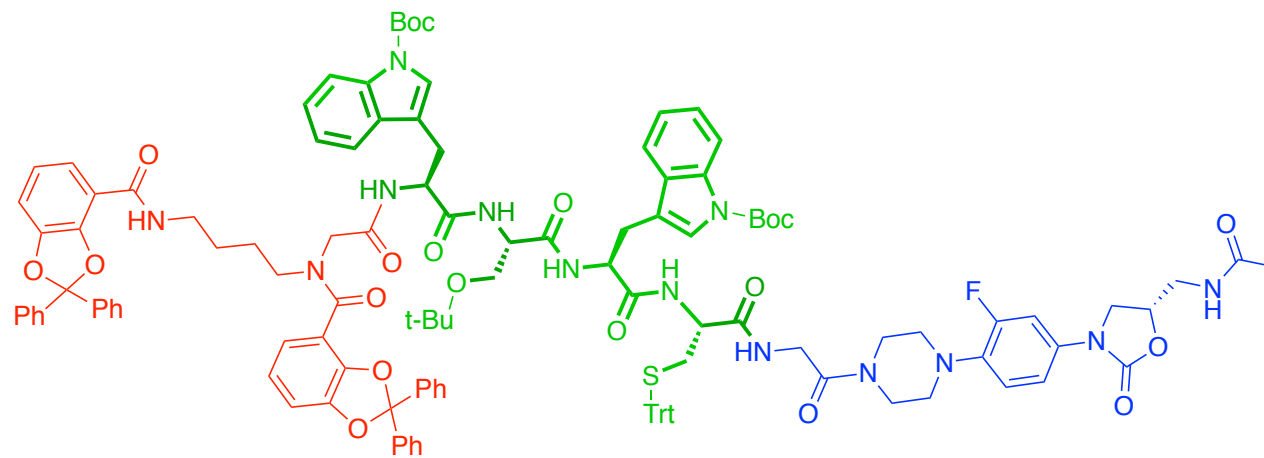




S19

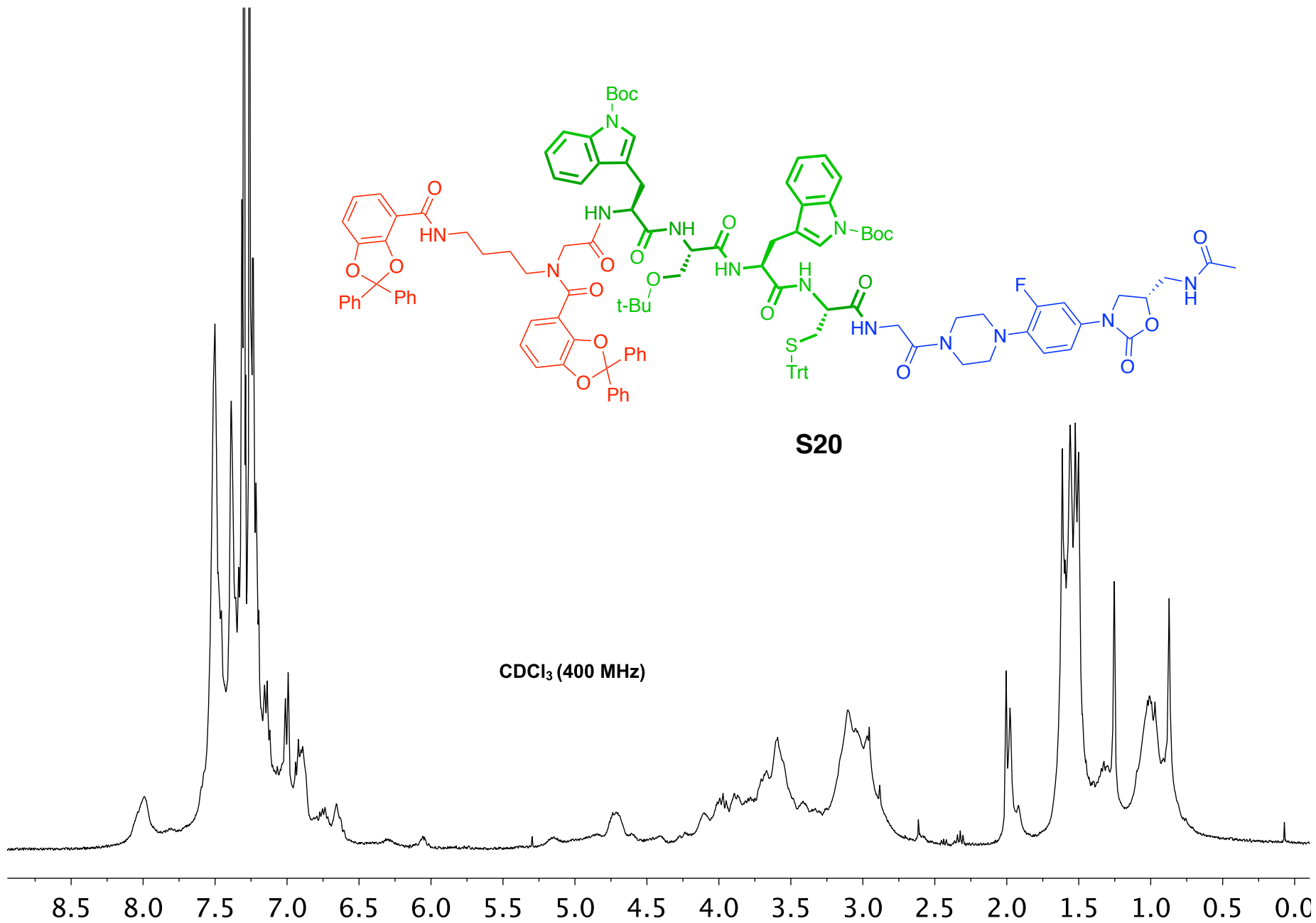
MeOD-*d*4 (100 MHz)

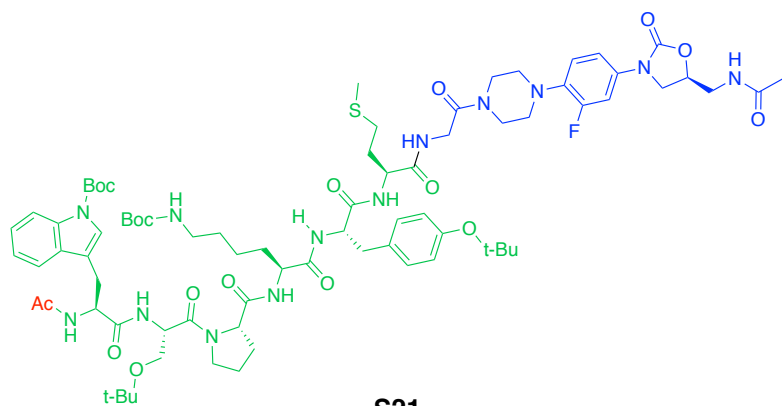




**S20**

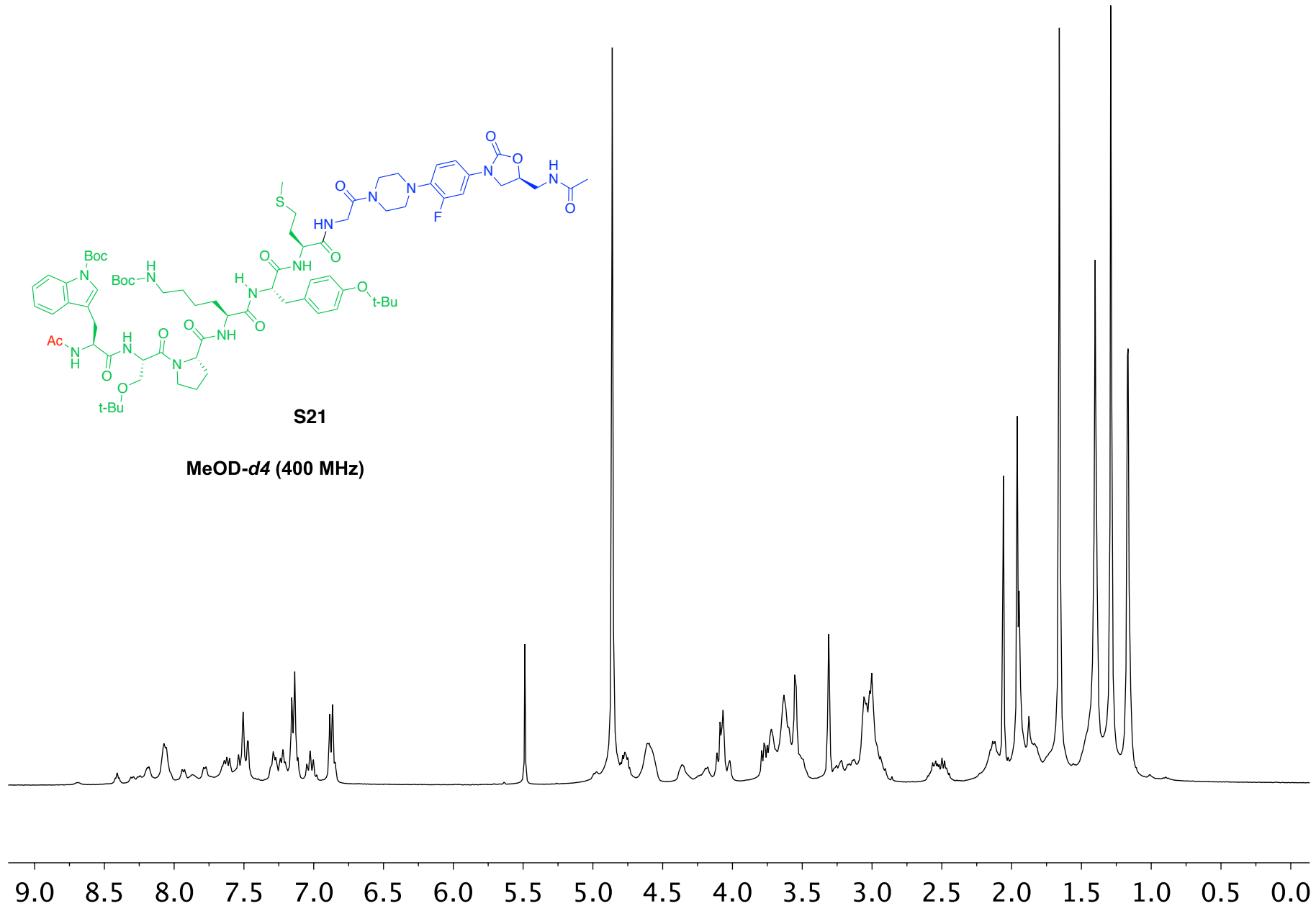
CDCl<sub>3</sub> (400 MHz)



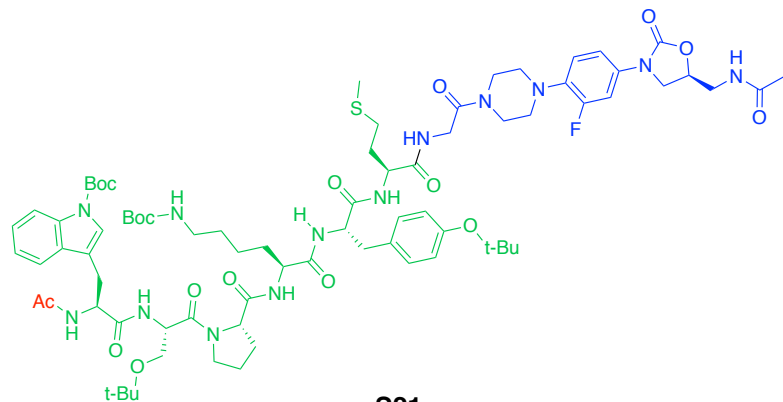


S21

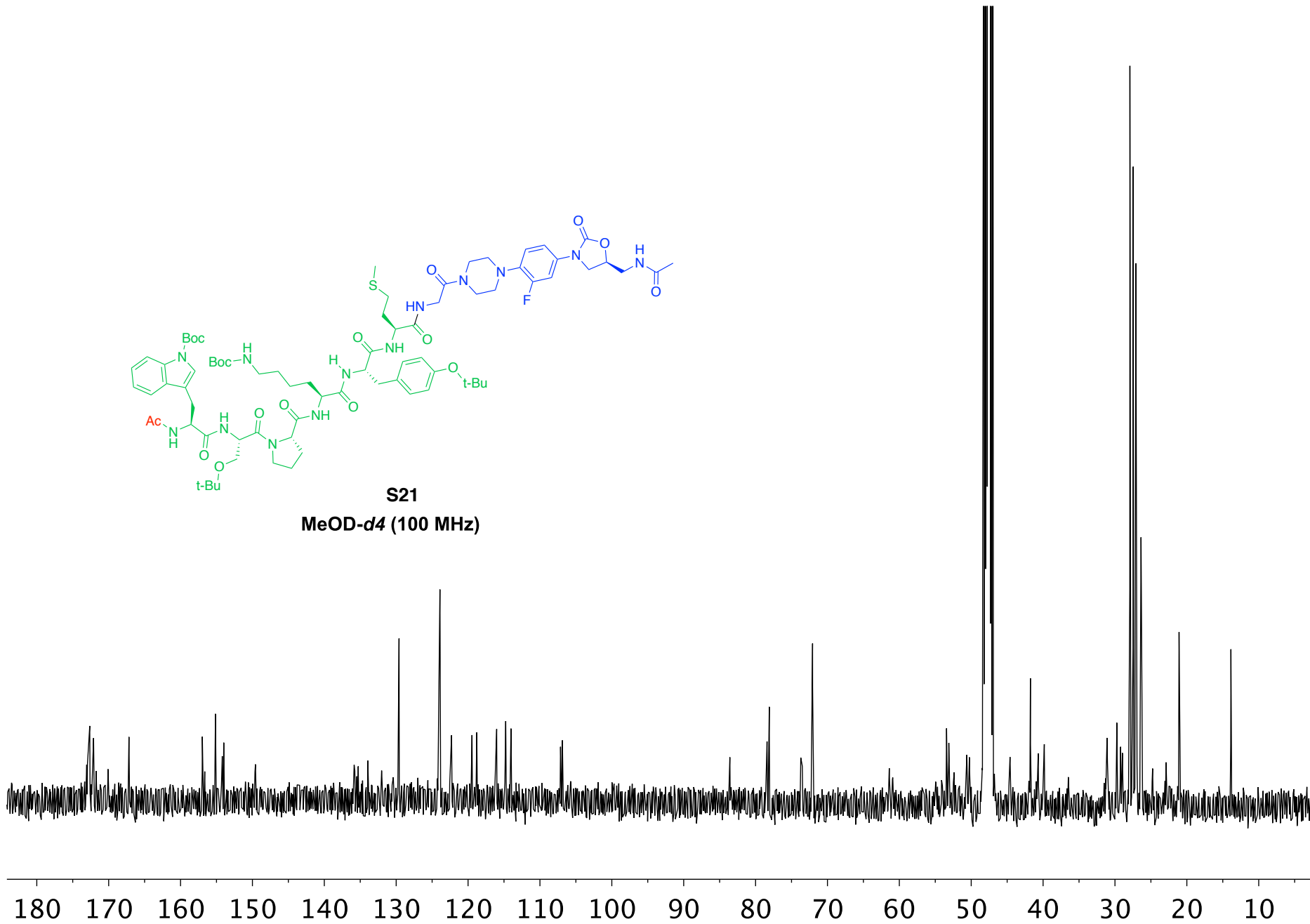
MeOD-*d*4 (400 MHz)

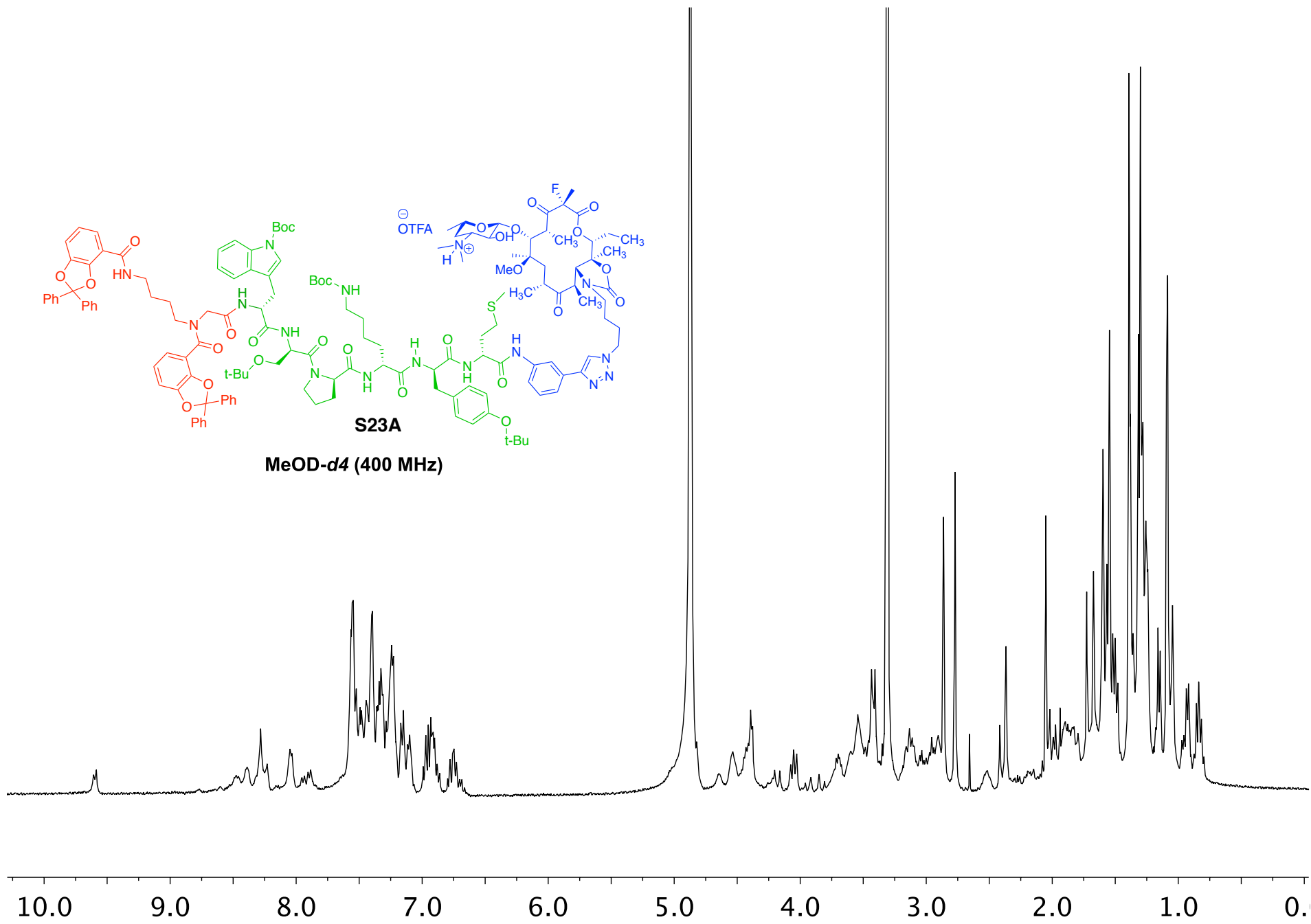


S110

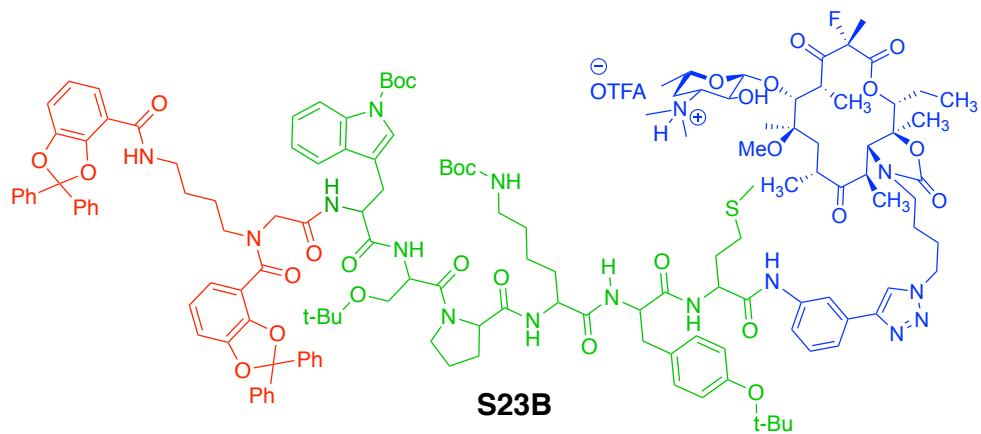


**S21**  
**MeOD-*d*4 (100 MHz)**



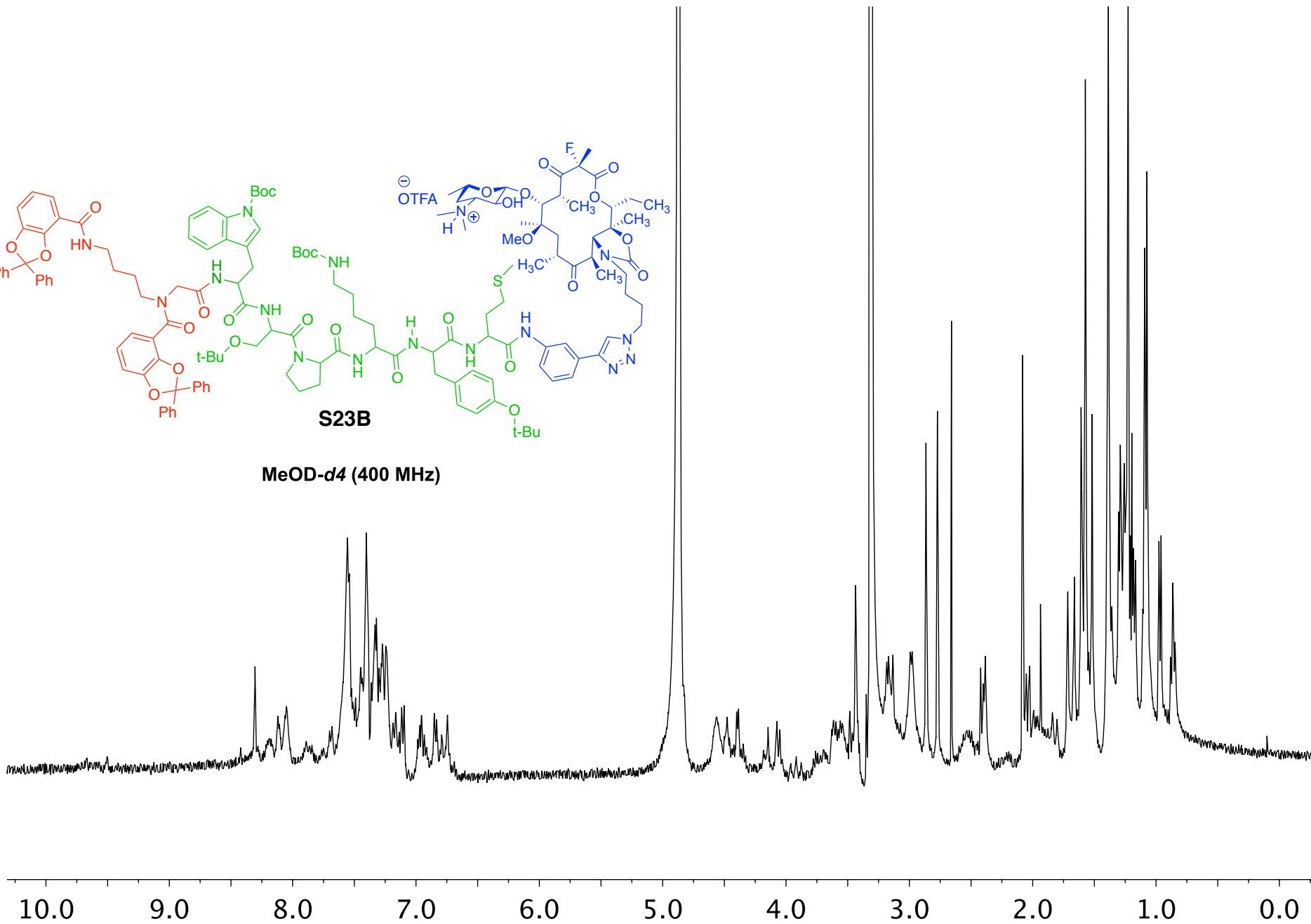




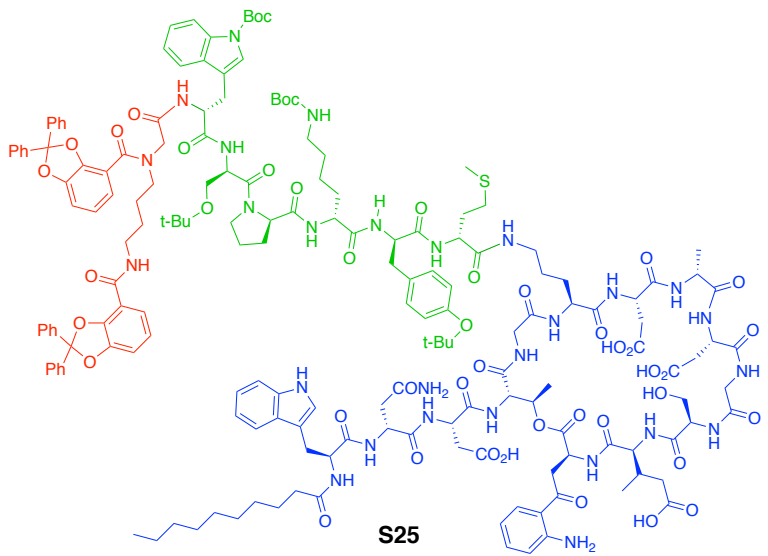


**S23B**

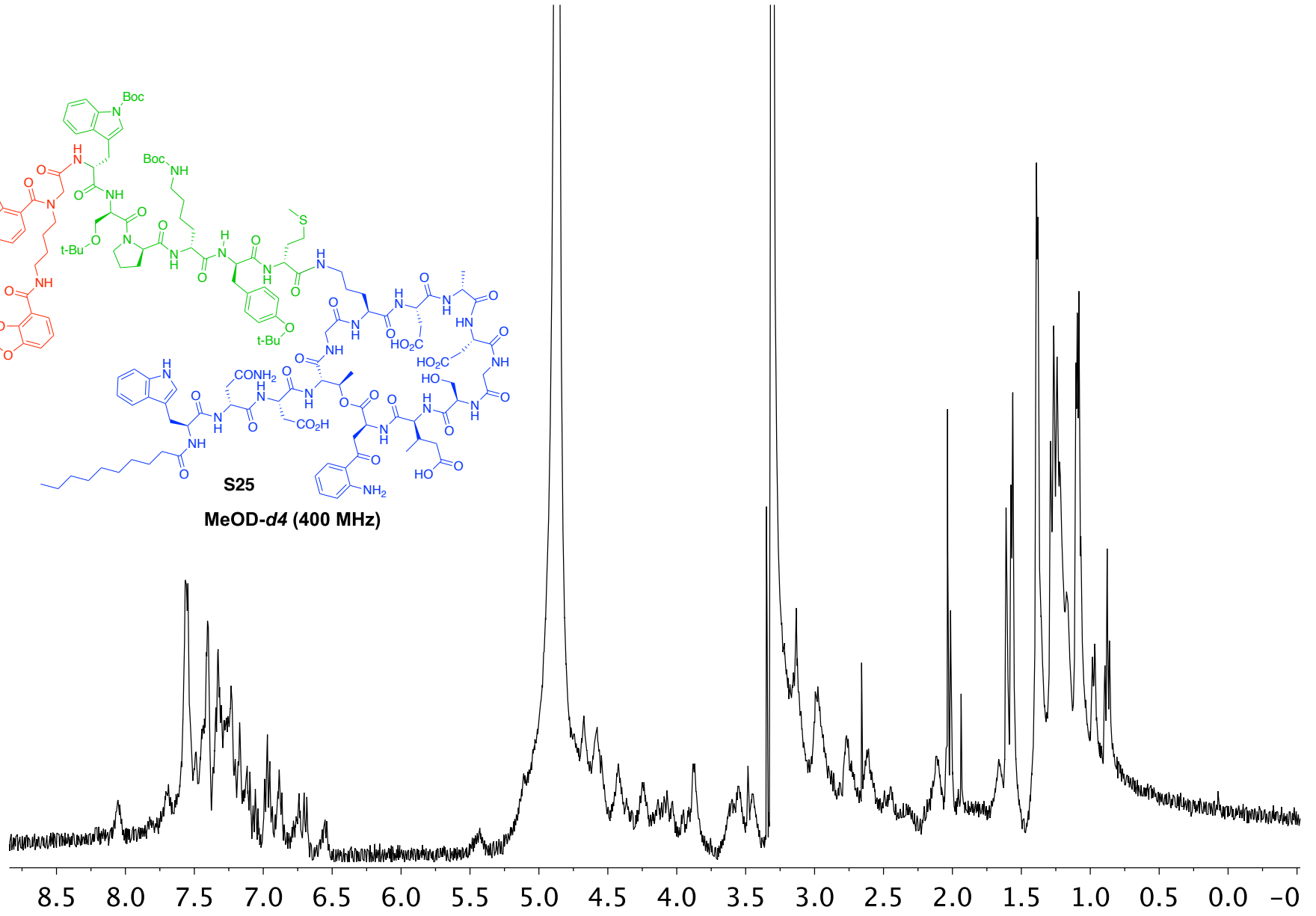
**MeOD-*d*4 (400 MHz)**



S113

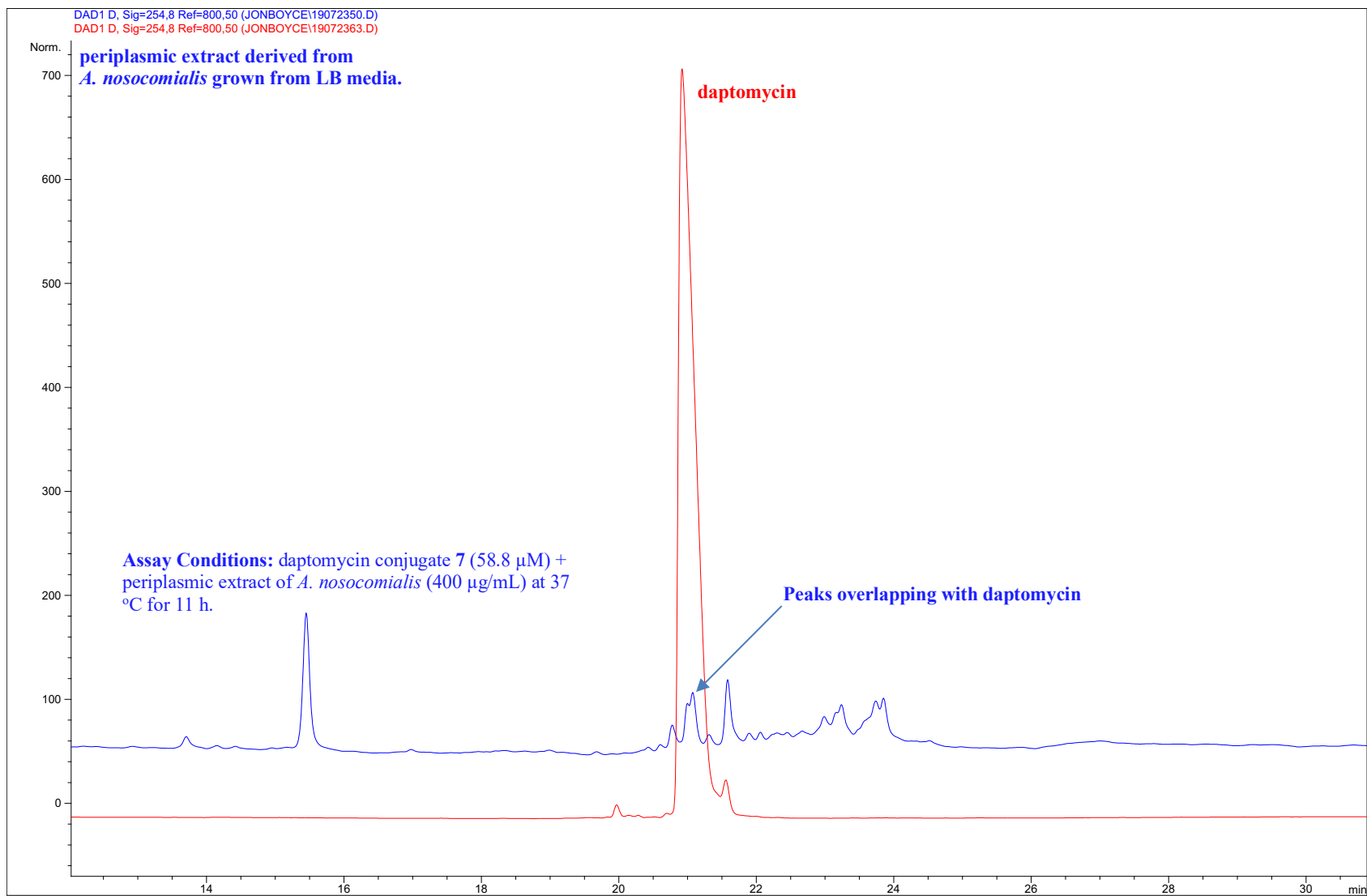


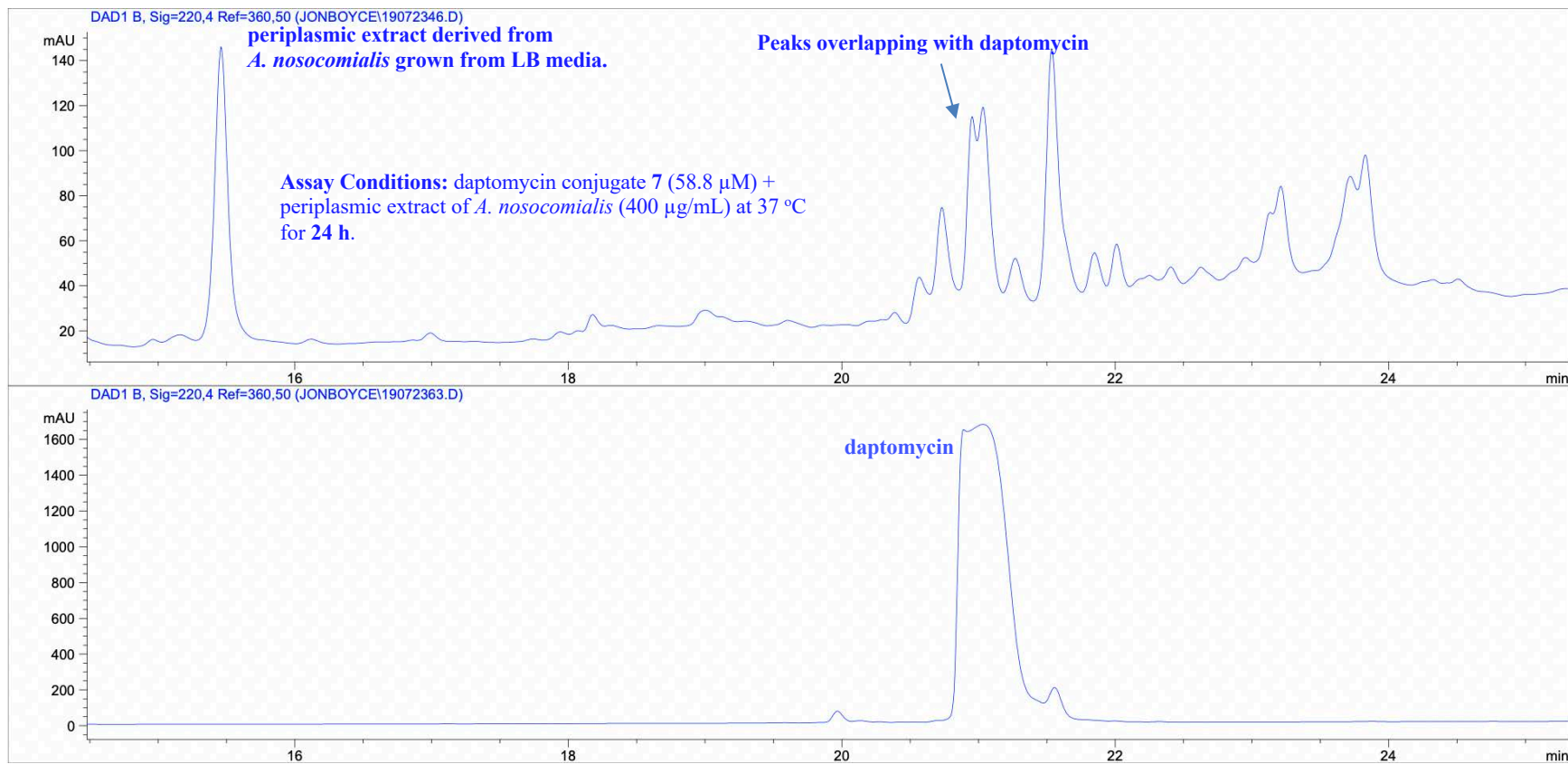
**S25**  
**MeOD-*d*4 (400 MHz)**

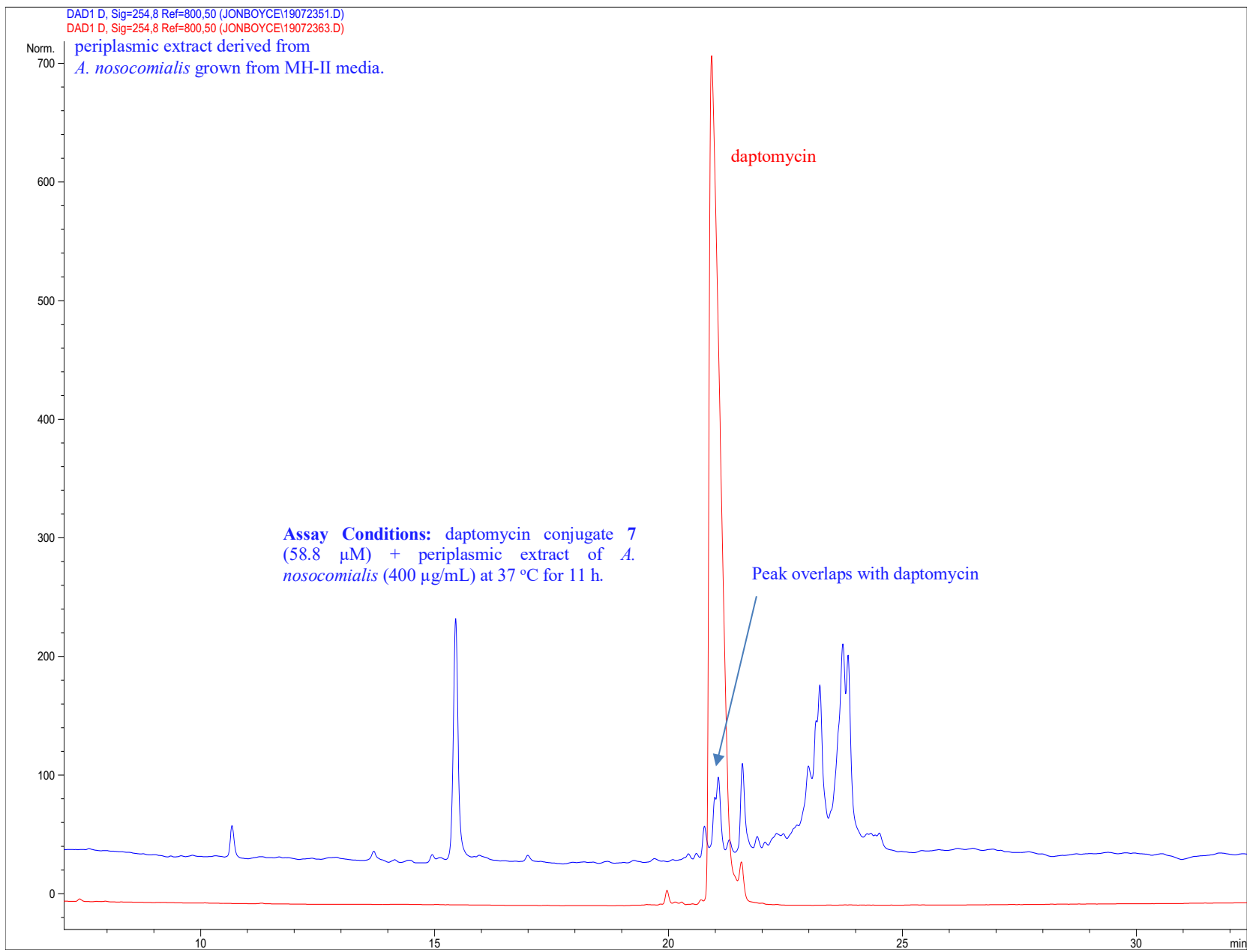


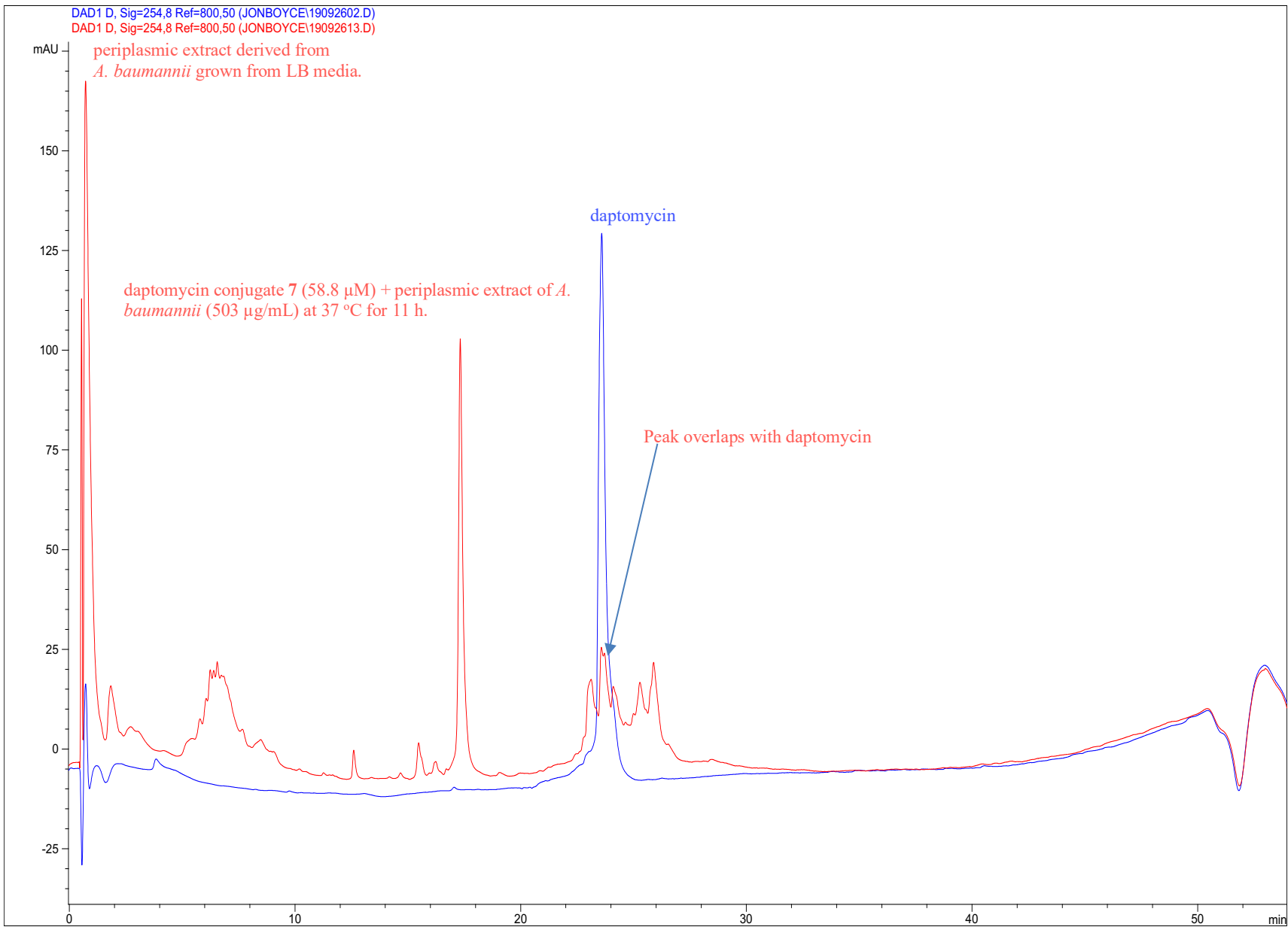
## VIII: HPLC TRACES FOR CLEAVAGE IN PERIPLASMIC EXTRACT

### Cleavage Analysis of Daptomycin Conjugate 7 (58.8 $\mu\text{M}$ ) in Periplasmic Extract:

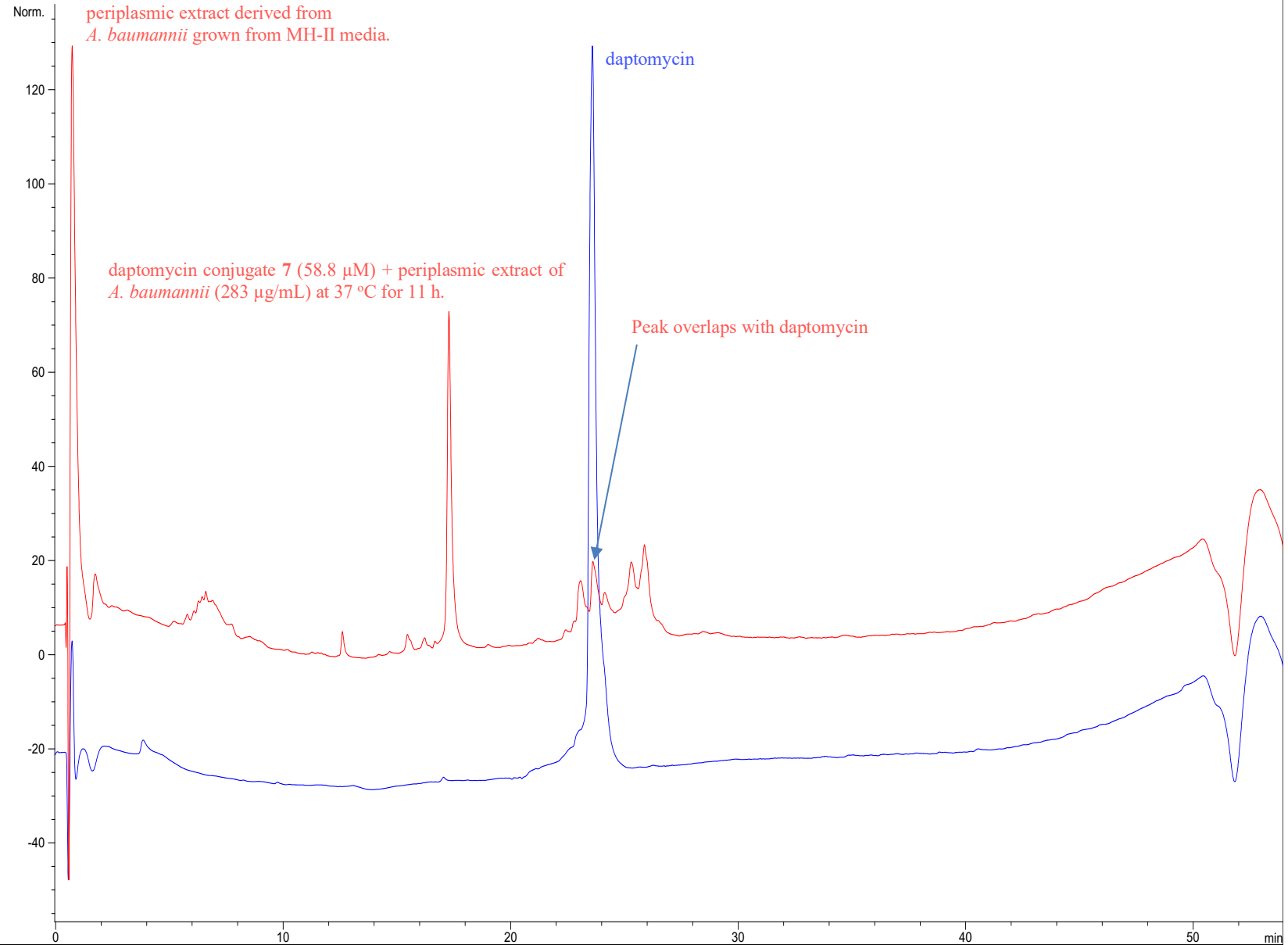


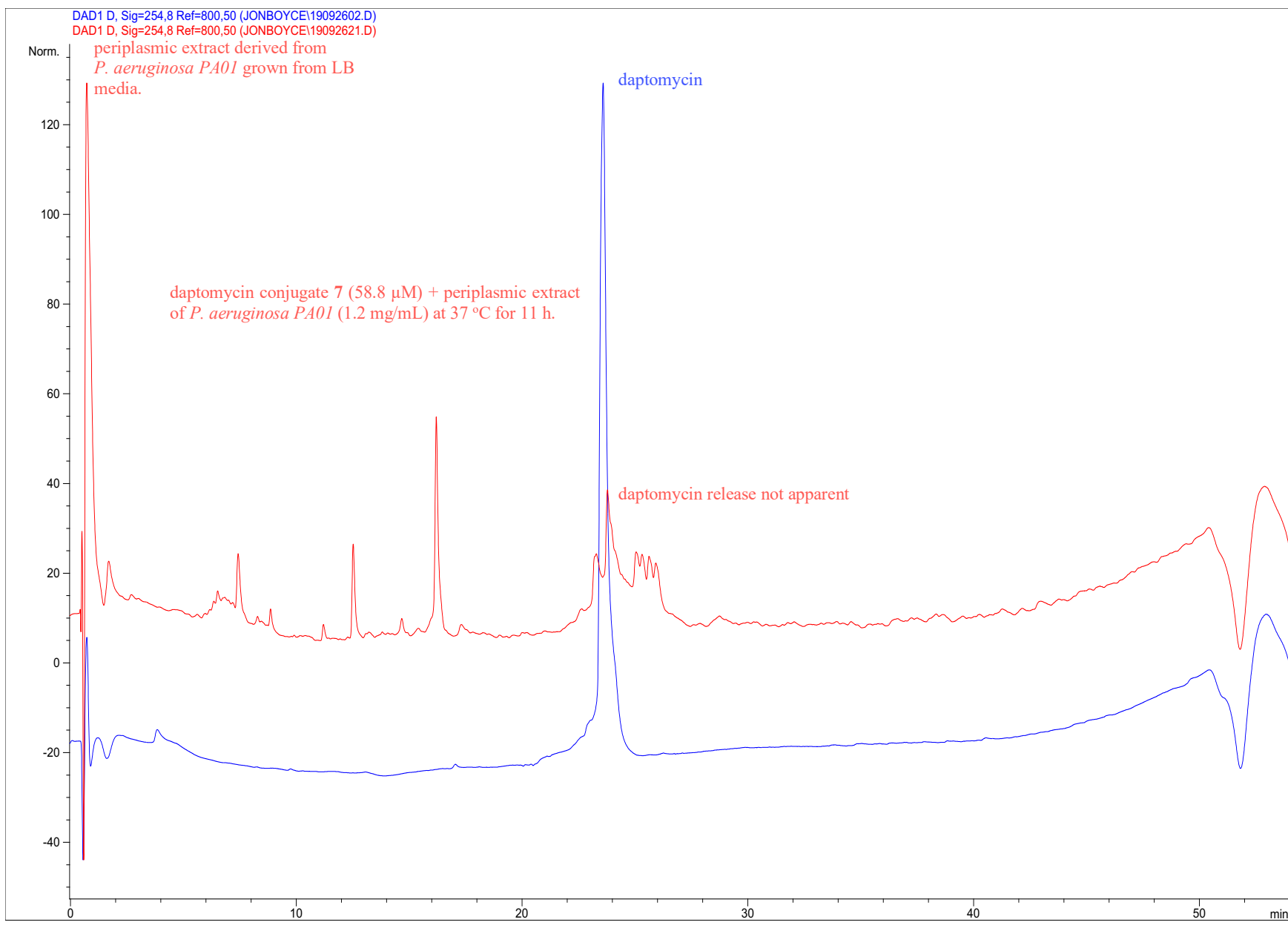




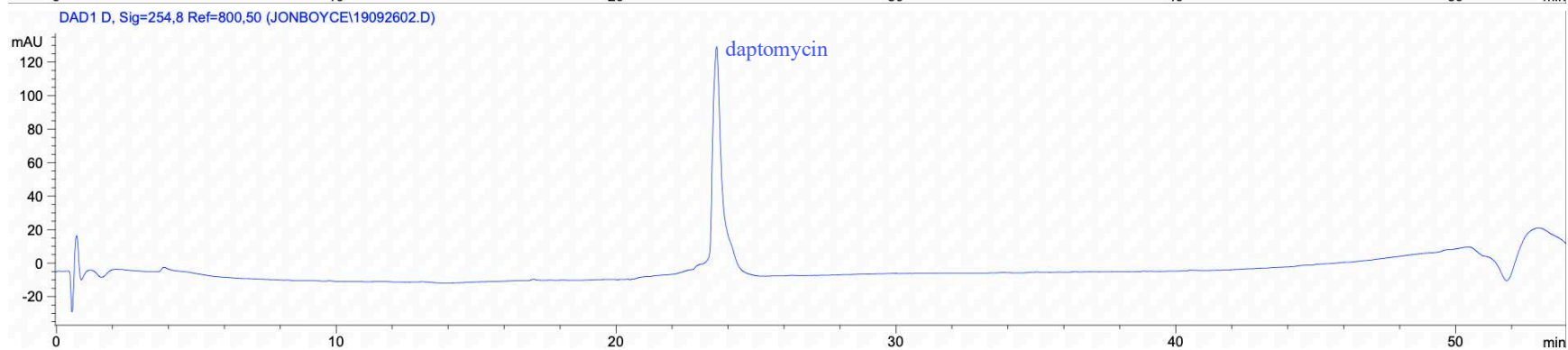
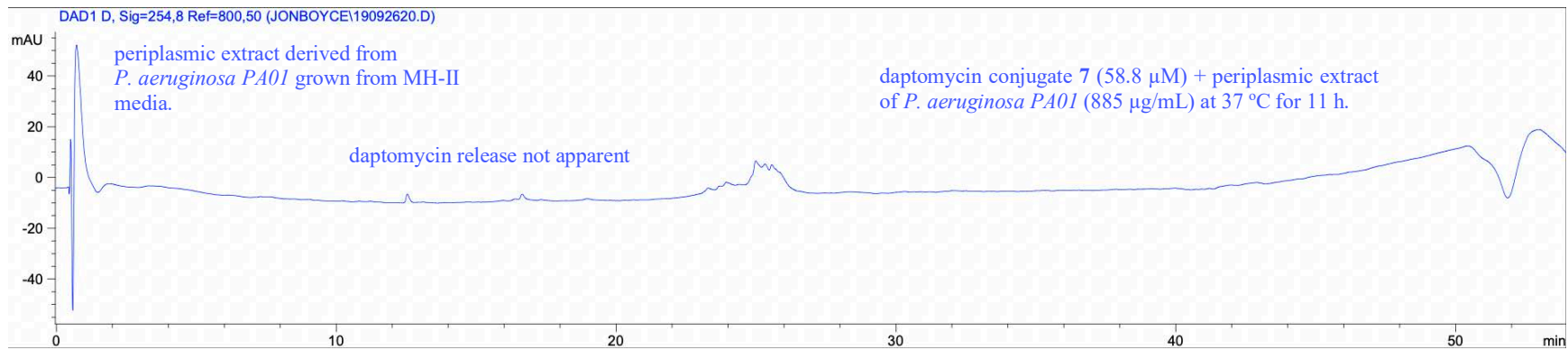


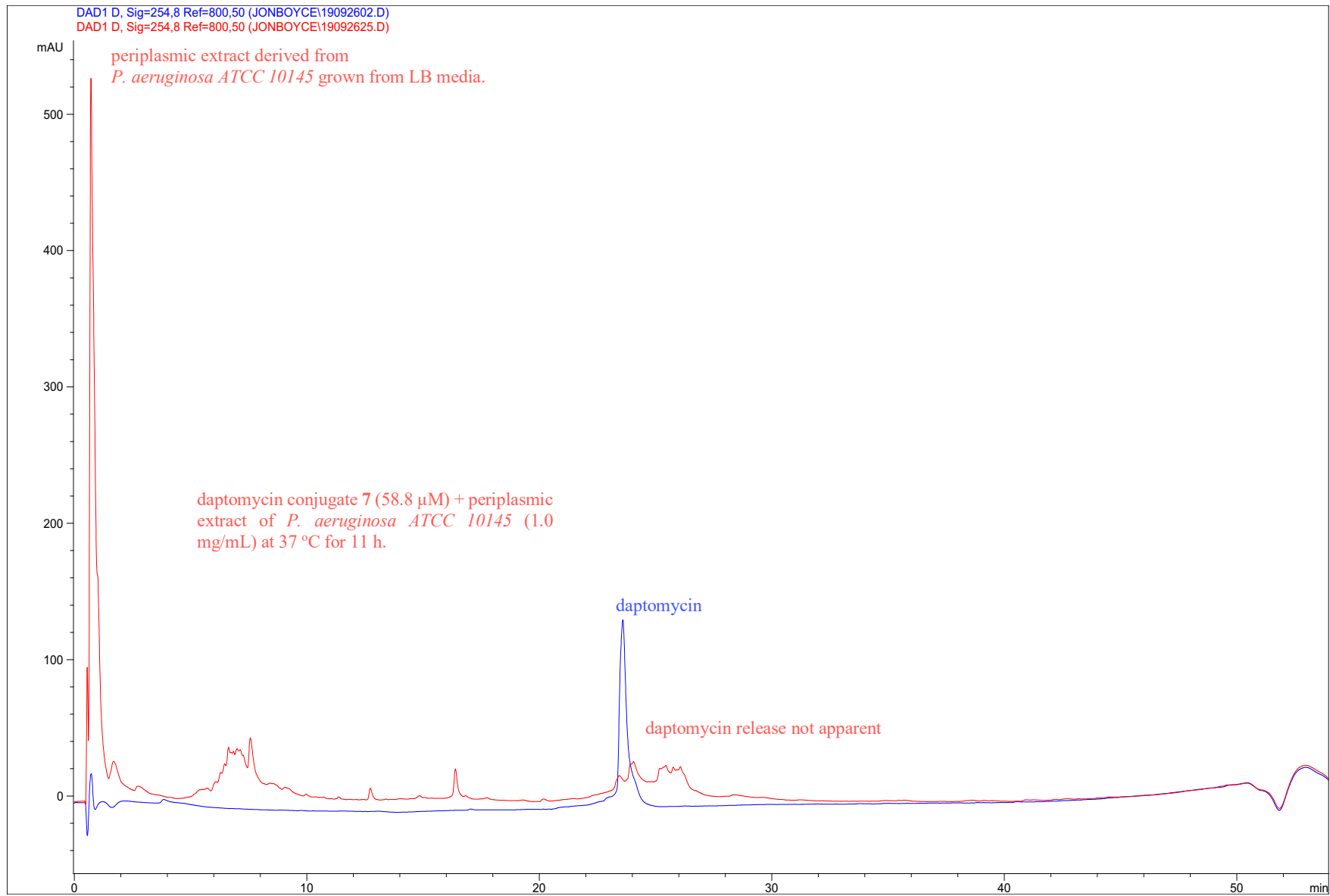
DAD1 D, Sig=254,8 Ref=800,50 (JONBOYCE\19092602.D)  
DAD1 D, Sig=254,8 Ref=800,50 (JONBOYCE\19092612.D)

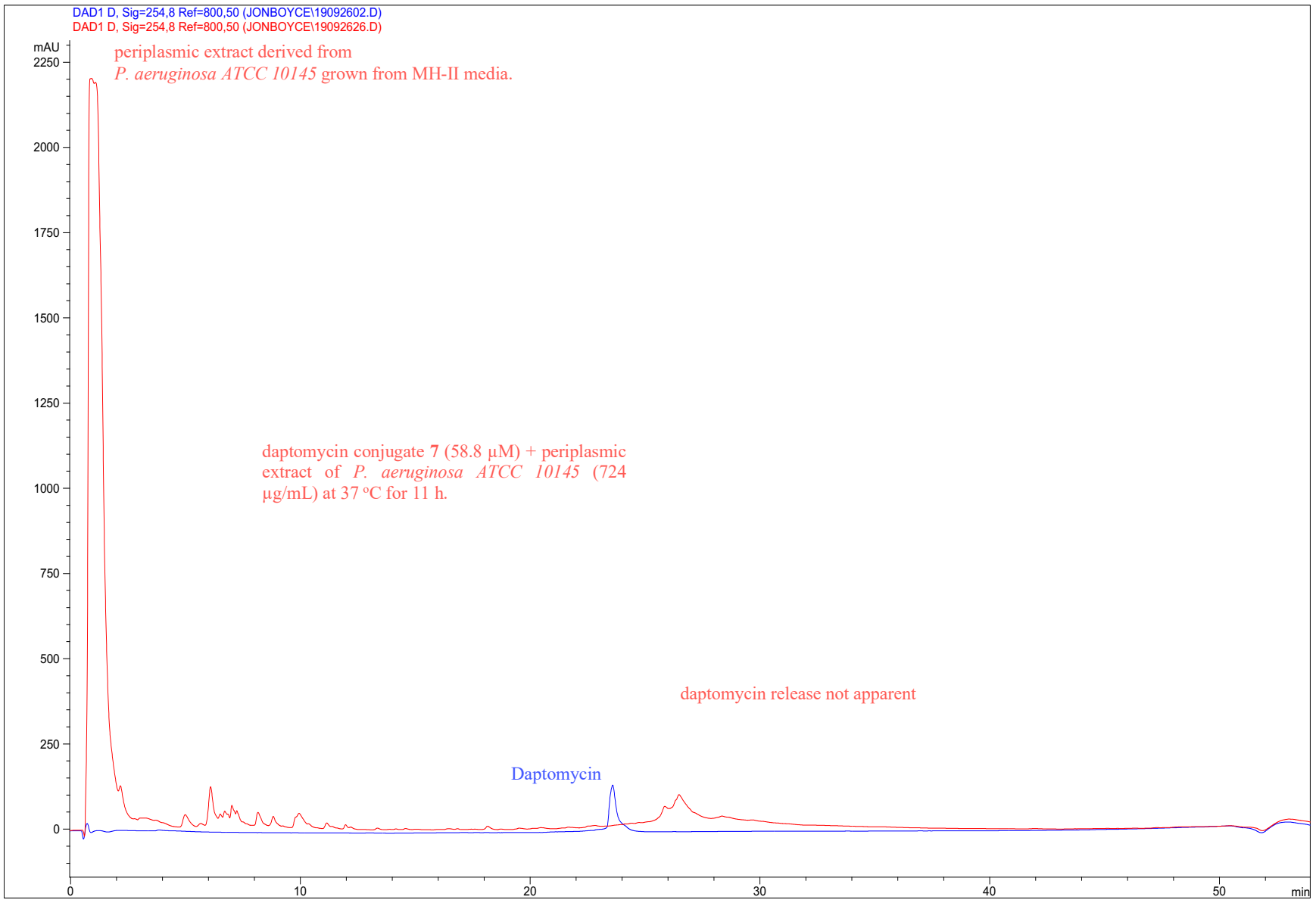


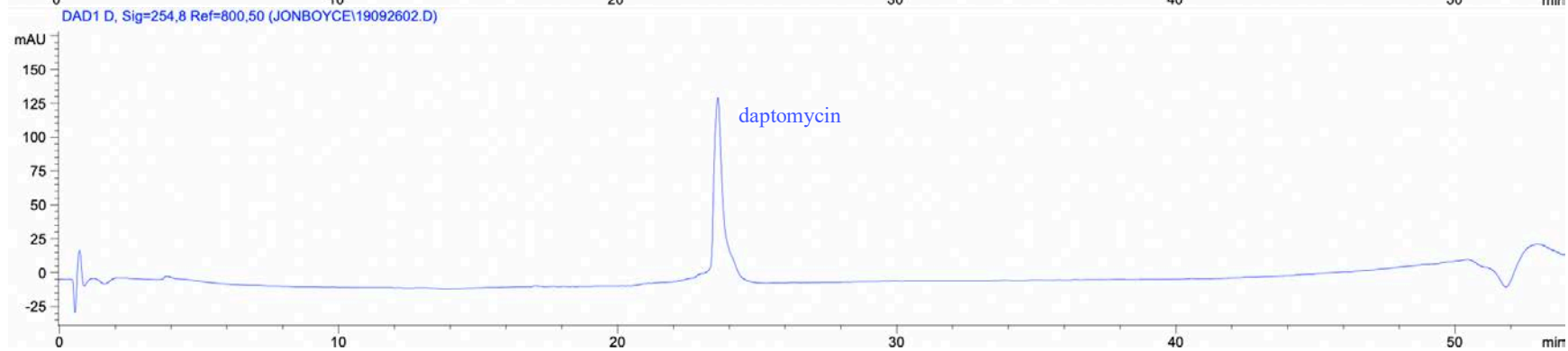
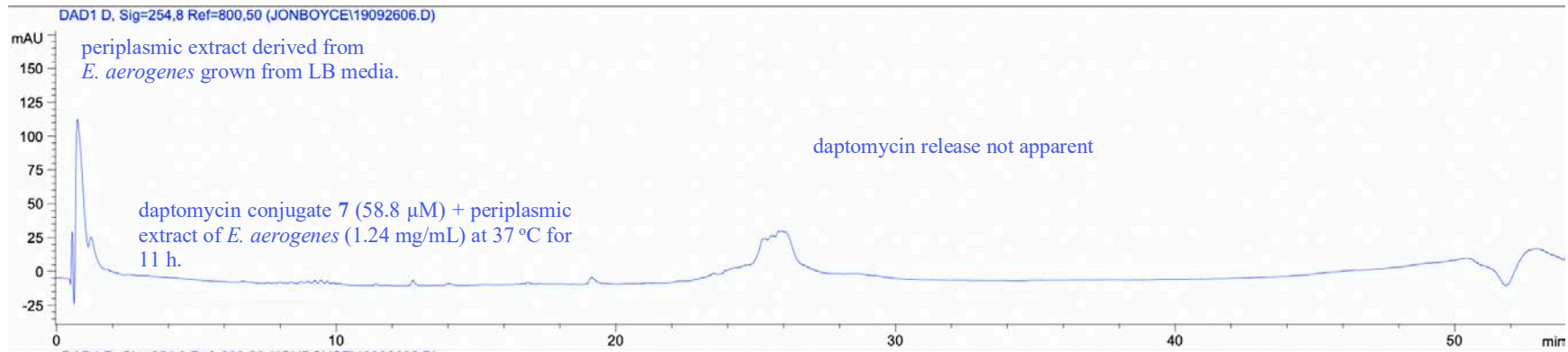


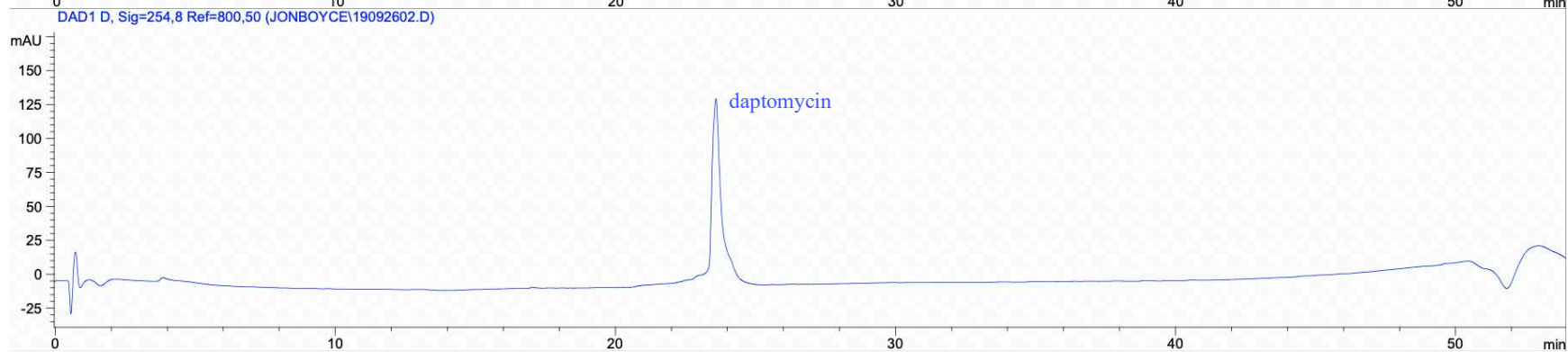
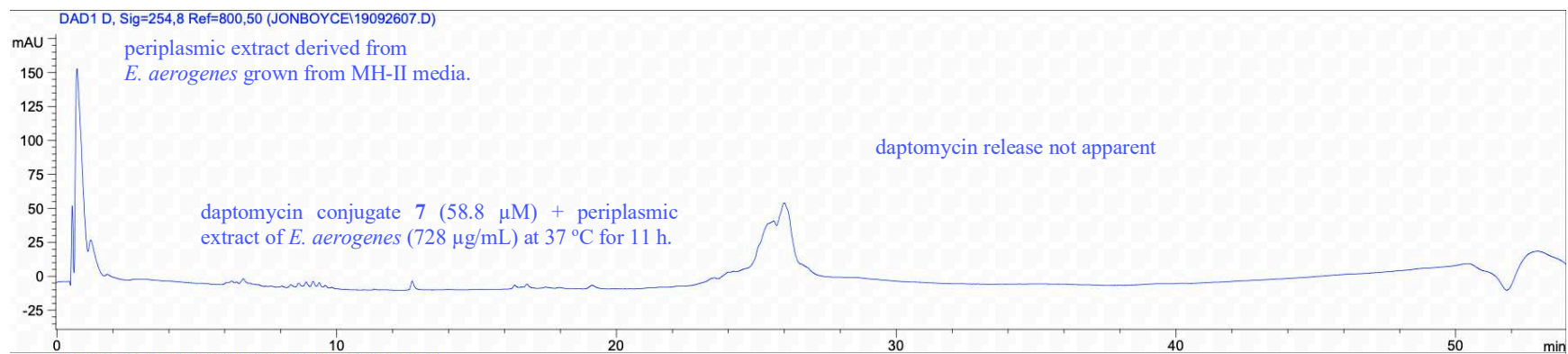


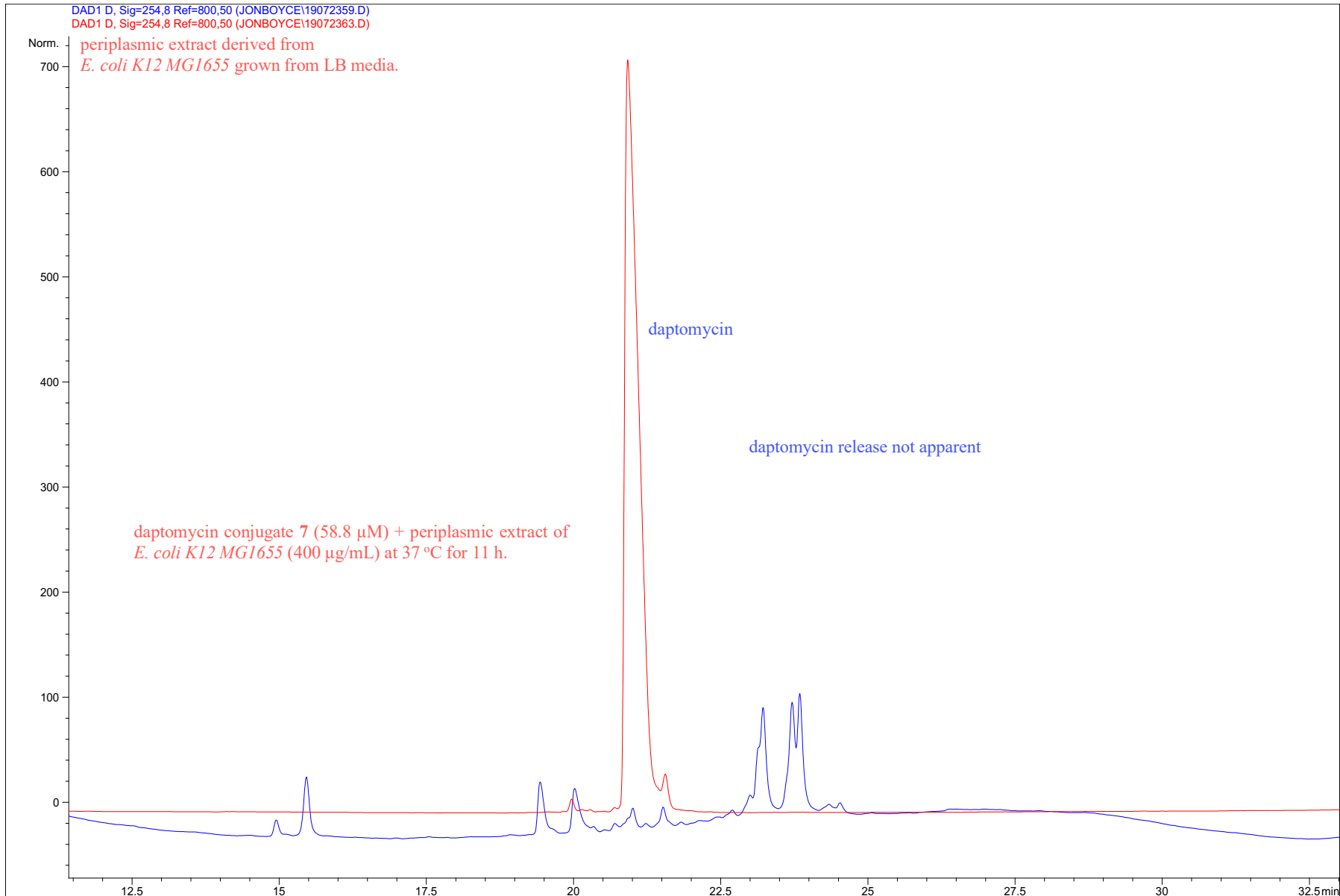


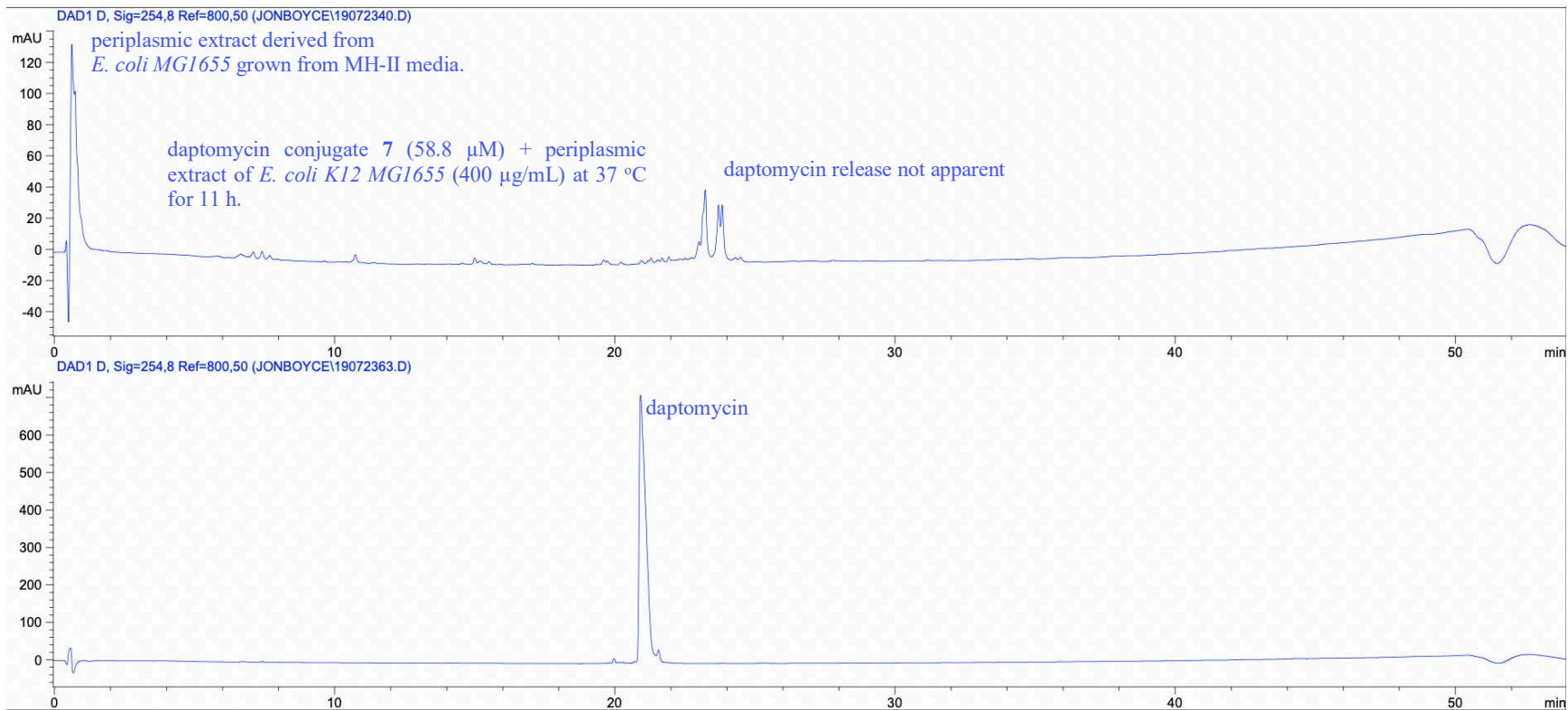


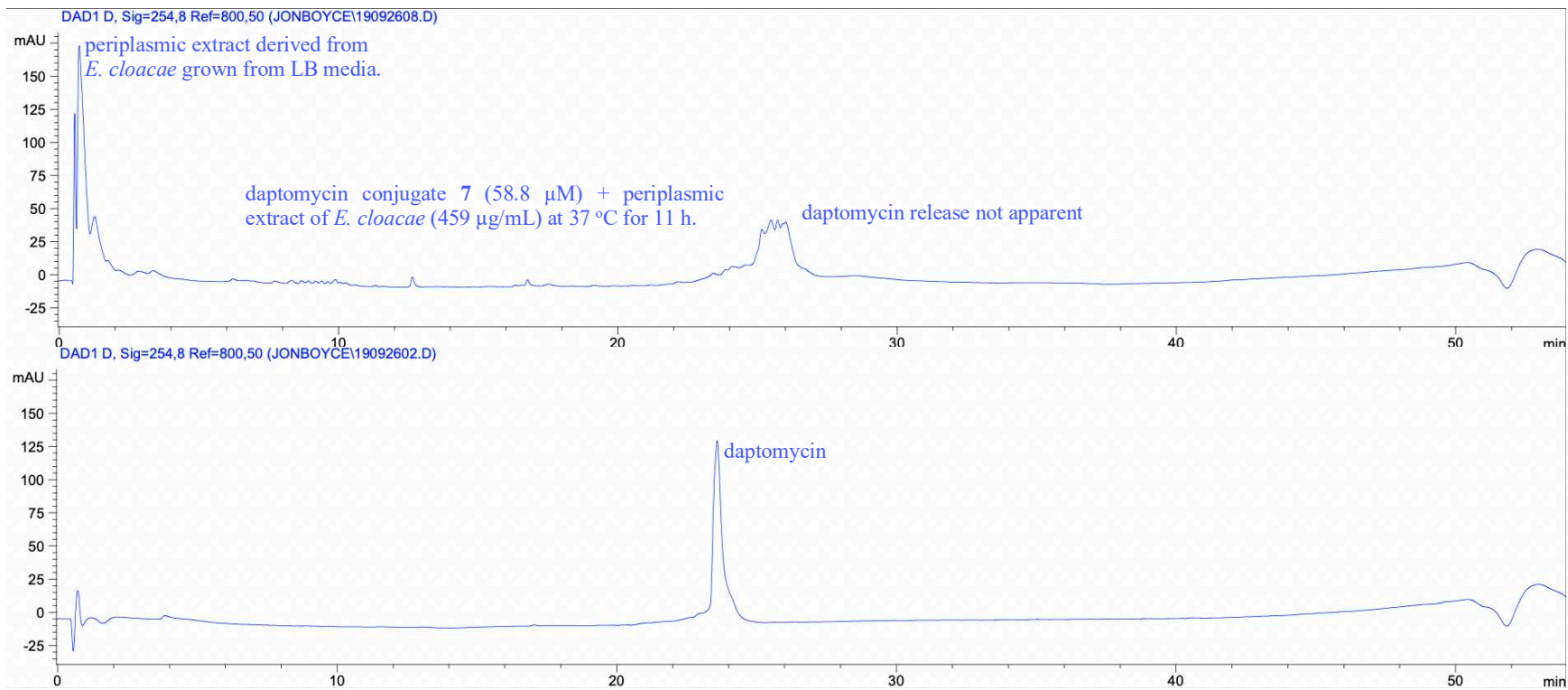




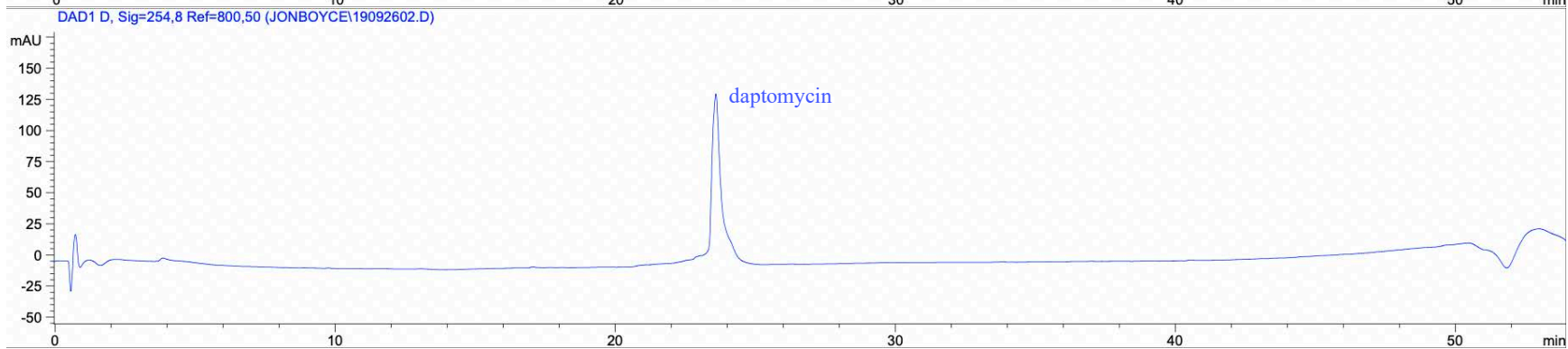
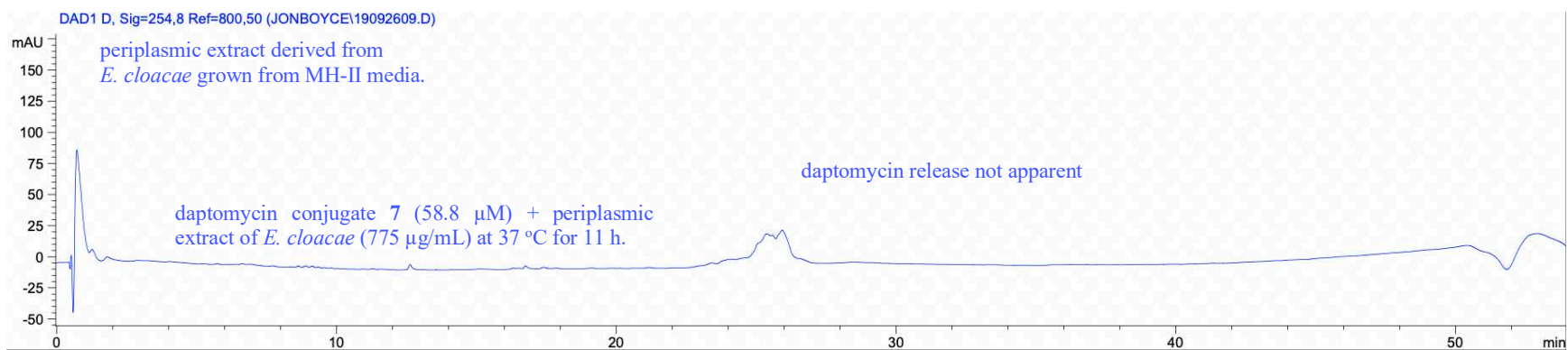


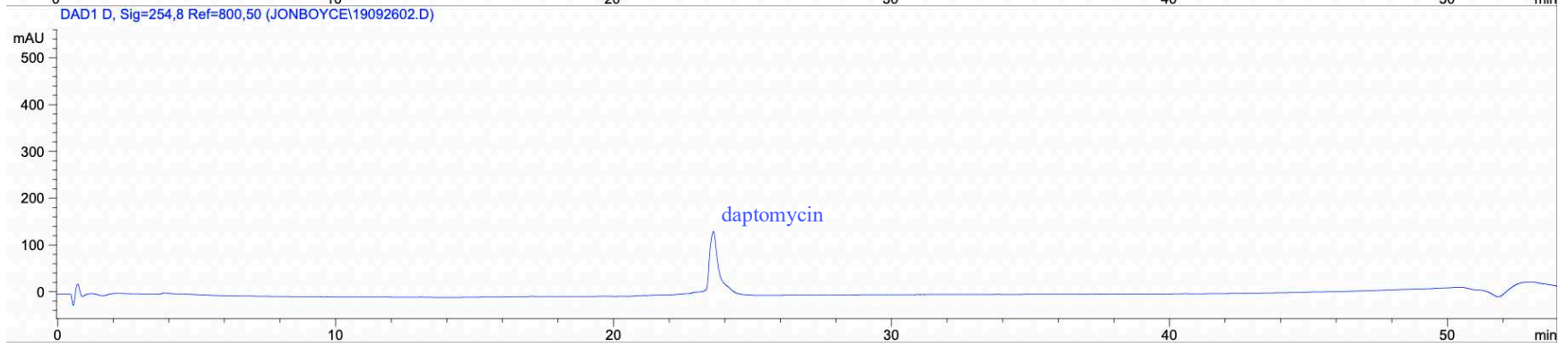
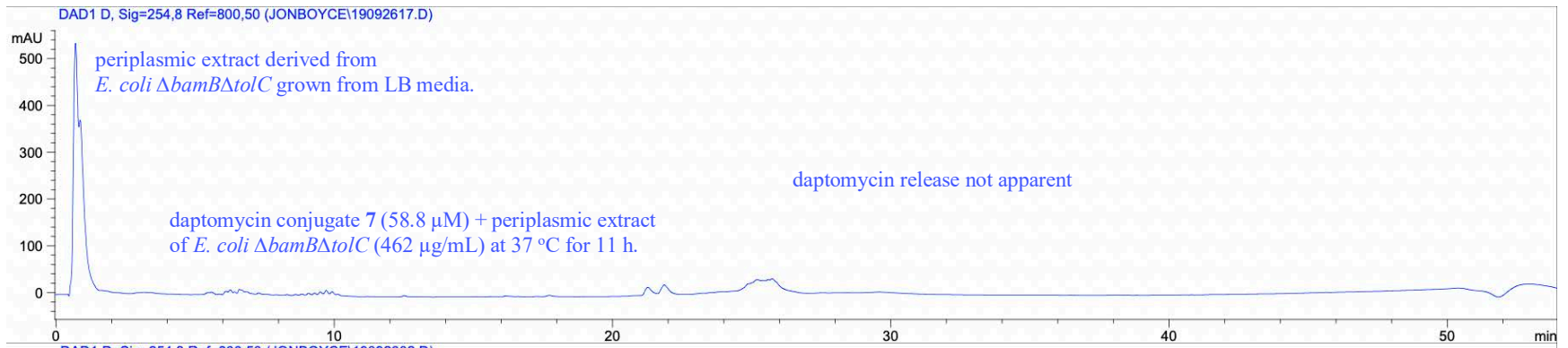


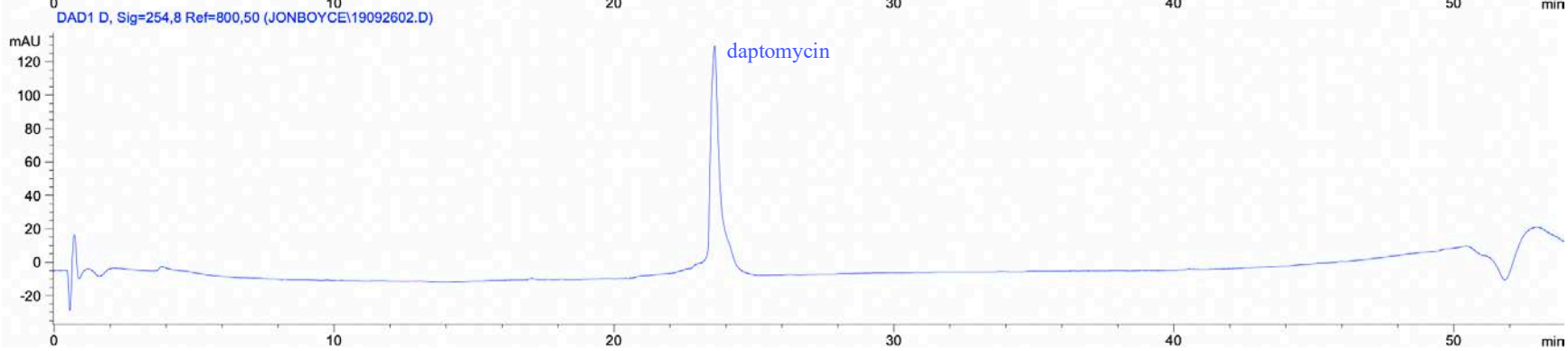
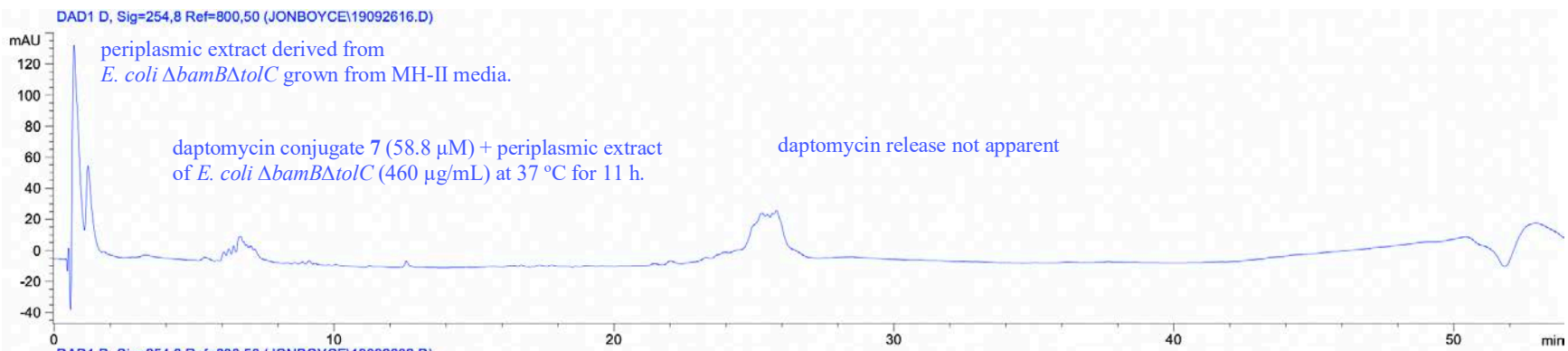


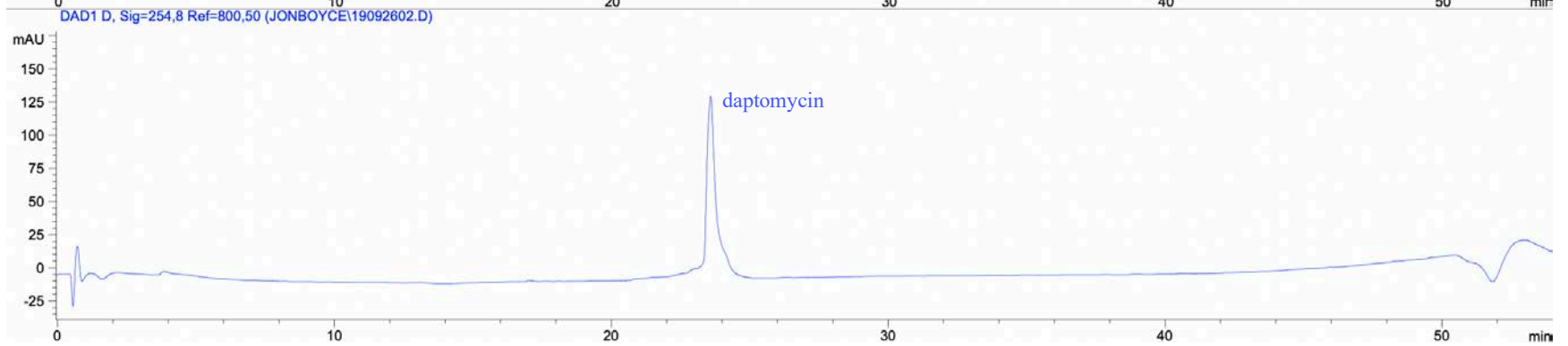
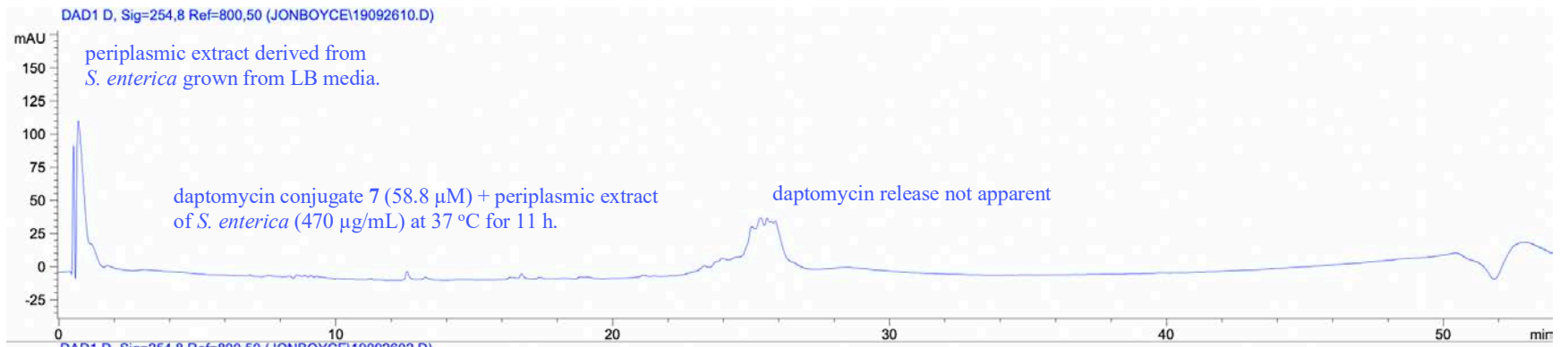


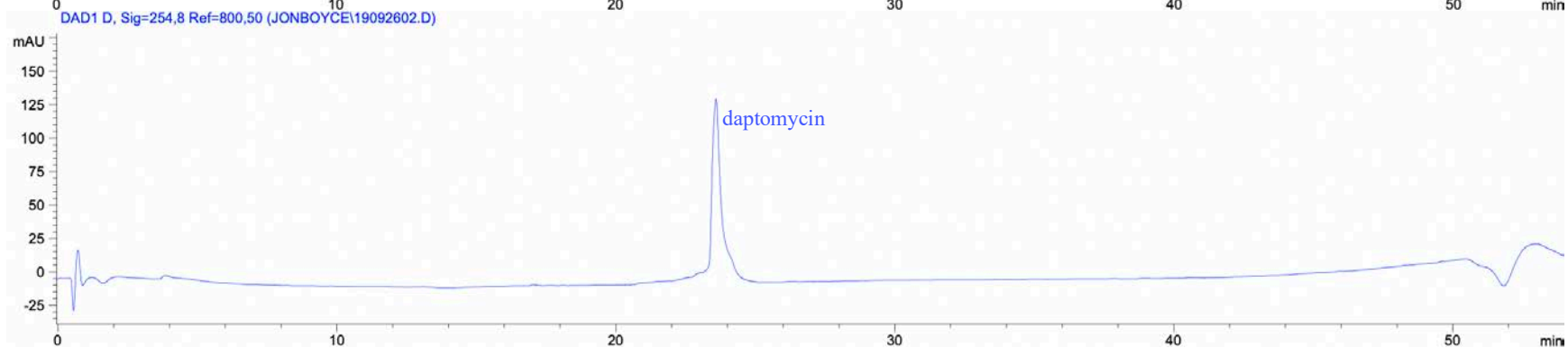
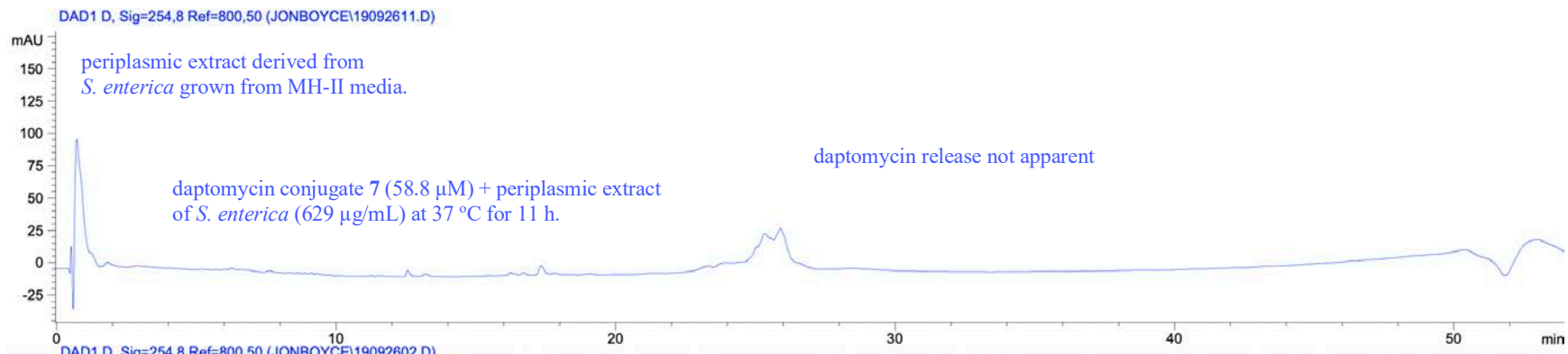


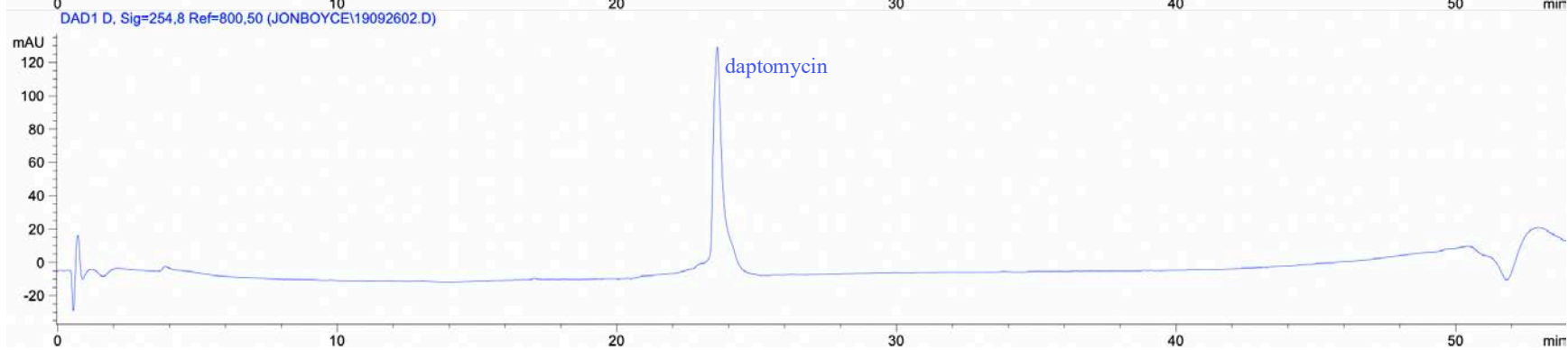
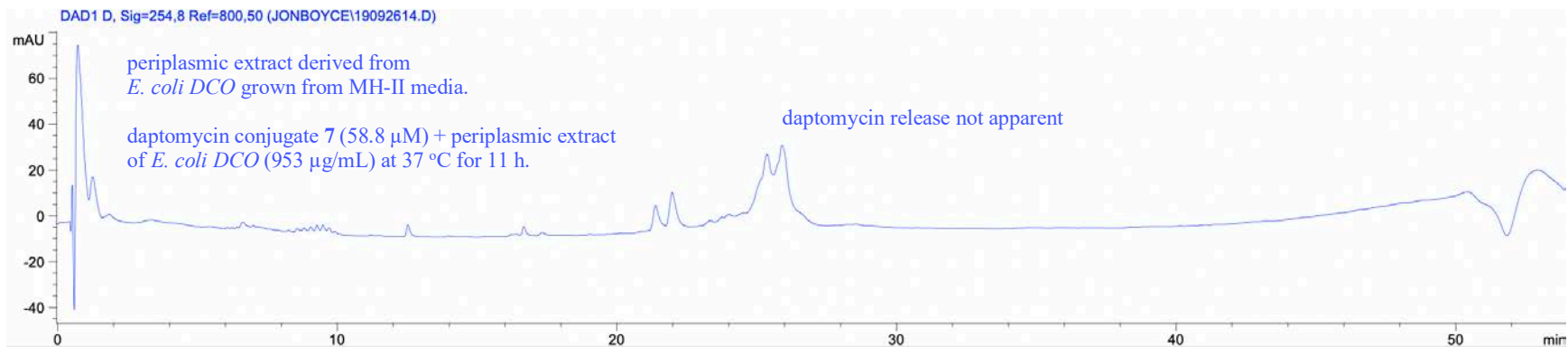




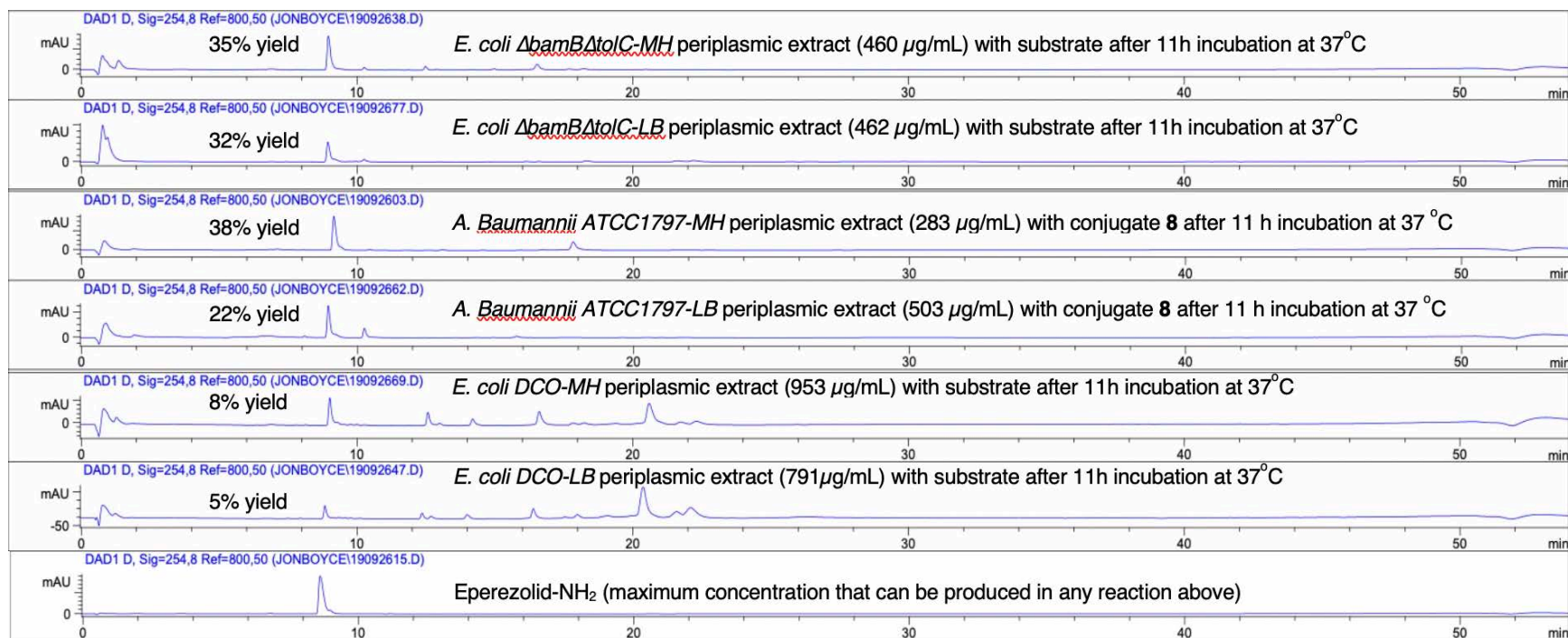


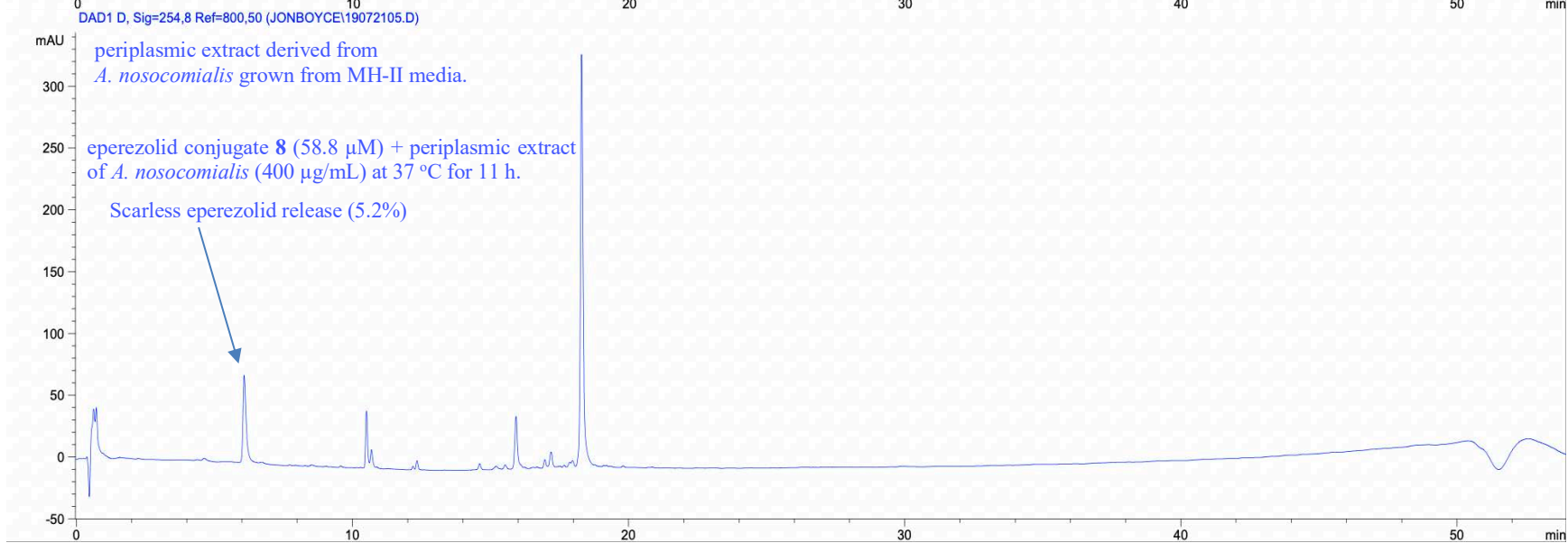
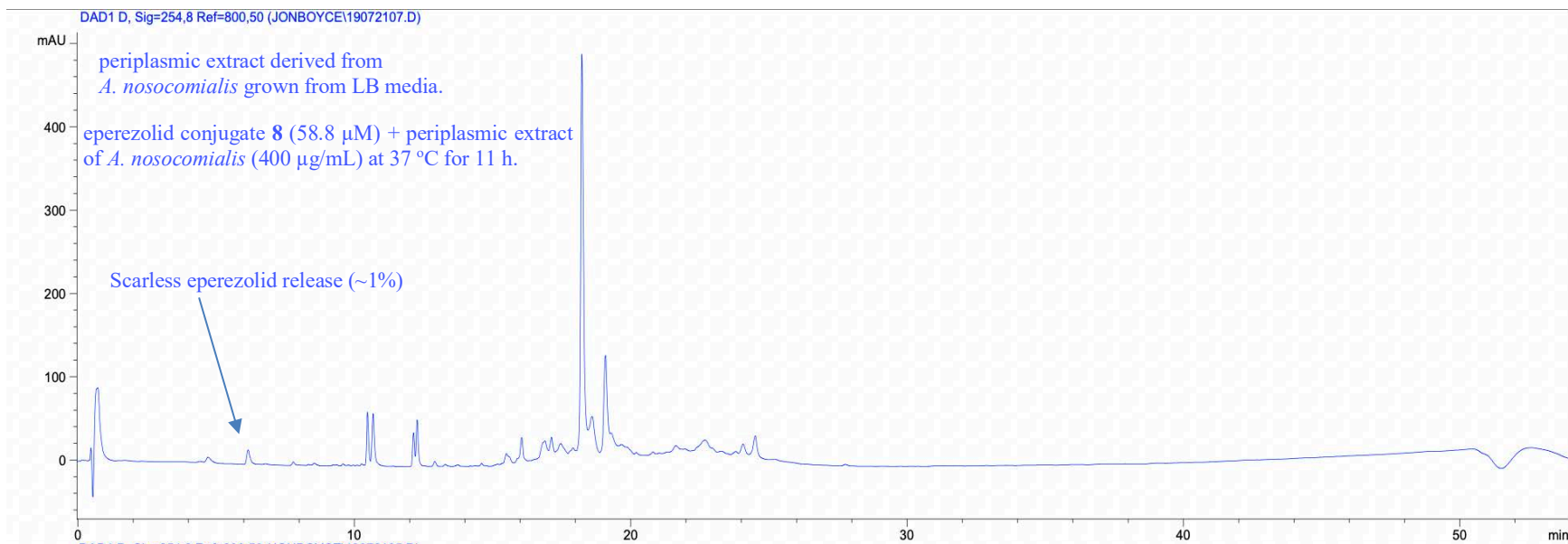




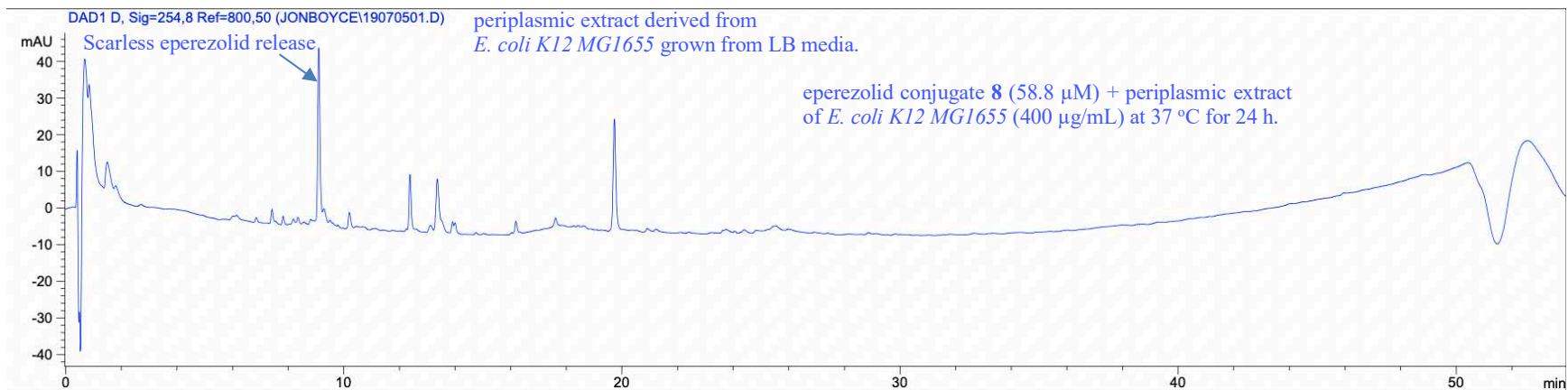


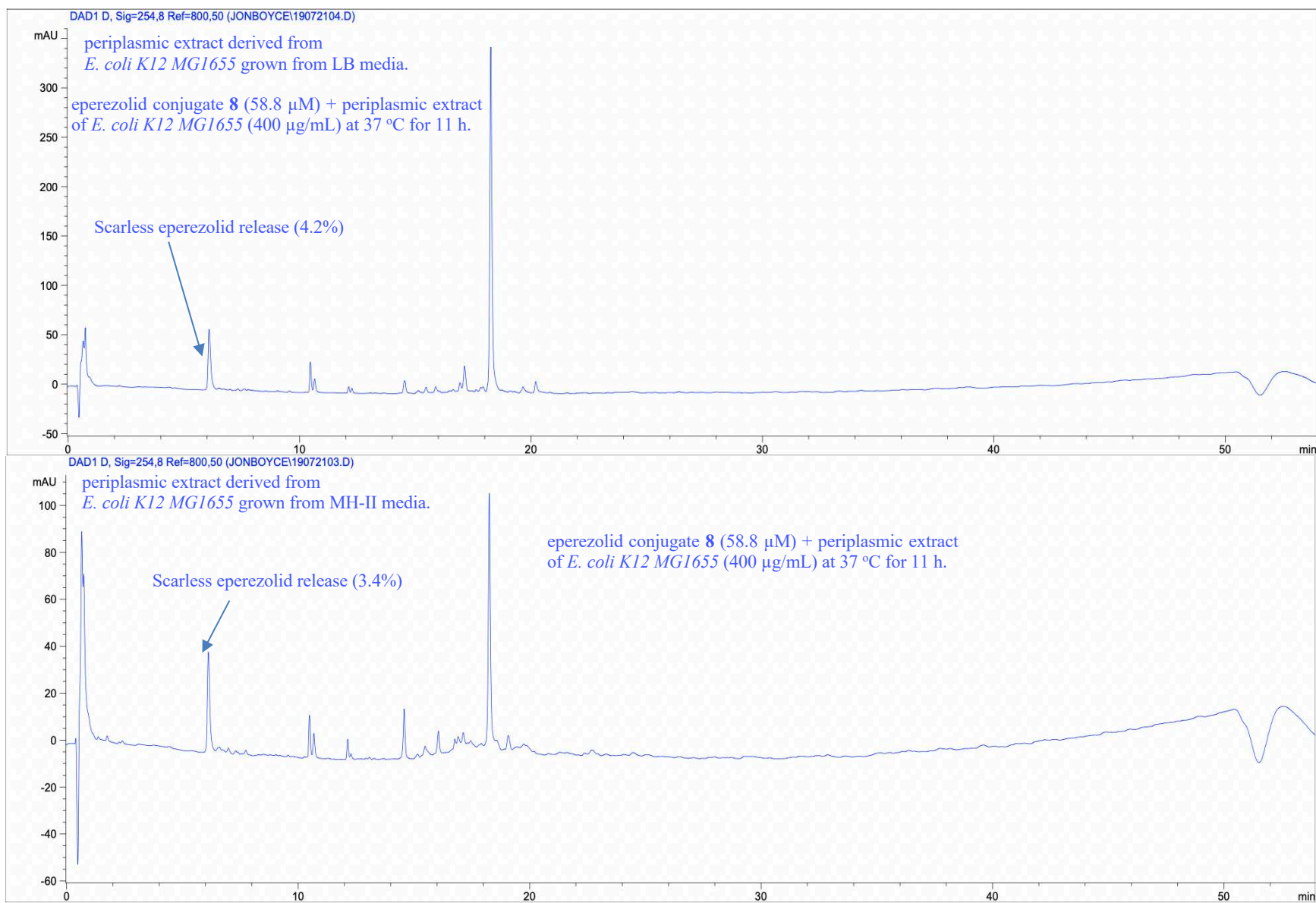
**Cleavage Analysis of Oxazolidinone Conjugate 8 (58.8  $\mu$ M) in Periplasmic Extract.** The yields represent the amount of eperezolid-NH<sub>2</sub> (5) released in each reaction divided by the maximal amount of 5 that can be produced in each reaction, which was determined by the peak areas. **The release of eperezolid-NH<sub>2</sub> (5) from conjugate 8 was confirmed by Mass Spec and *via* comparison of overlapping retention times with synthesized eperezolid-NH<sub>2</sub> (5) (see below).**



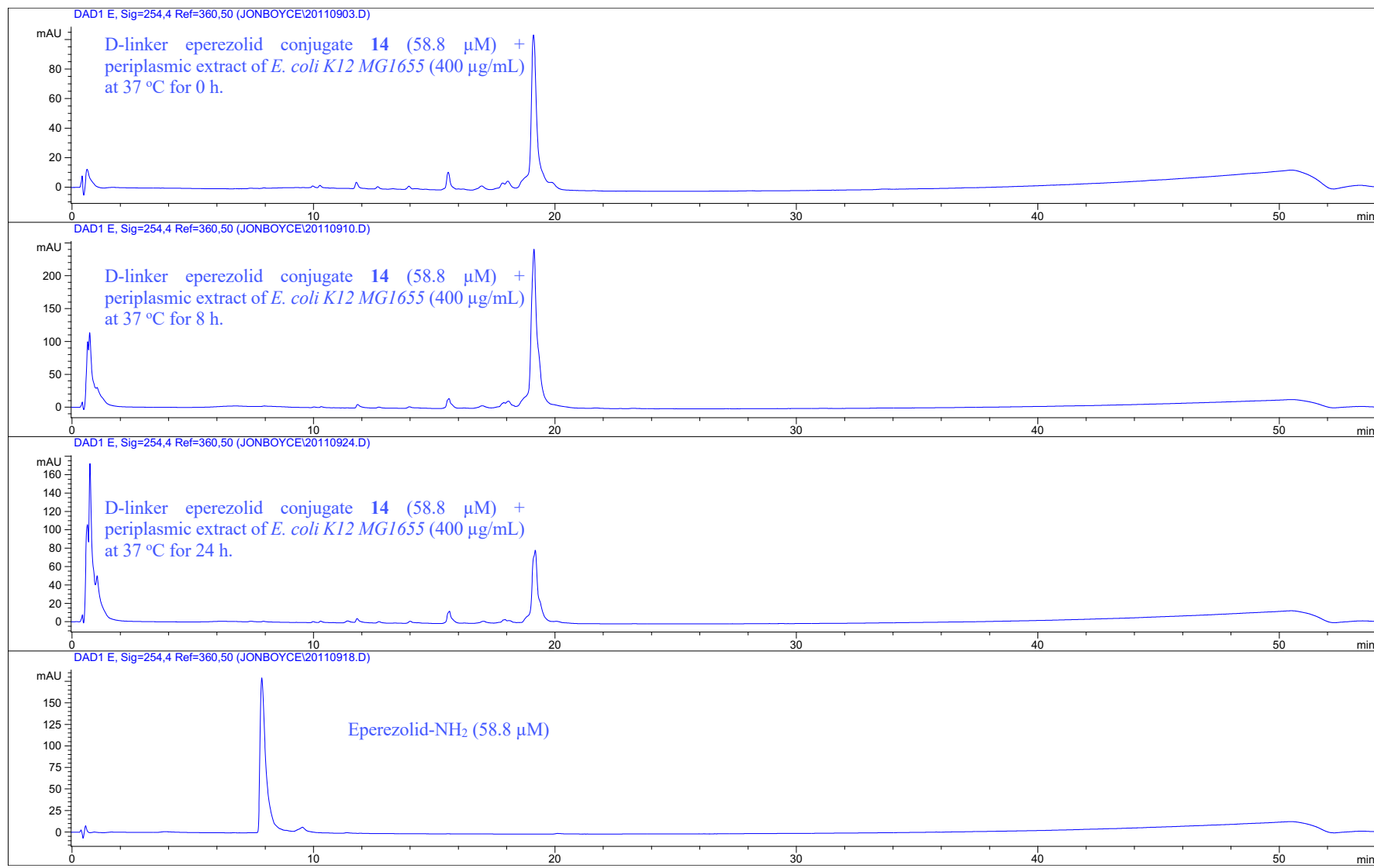




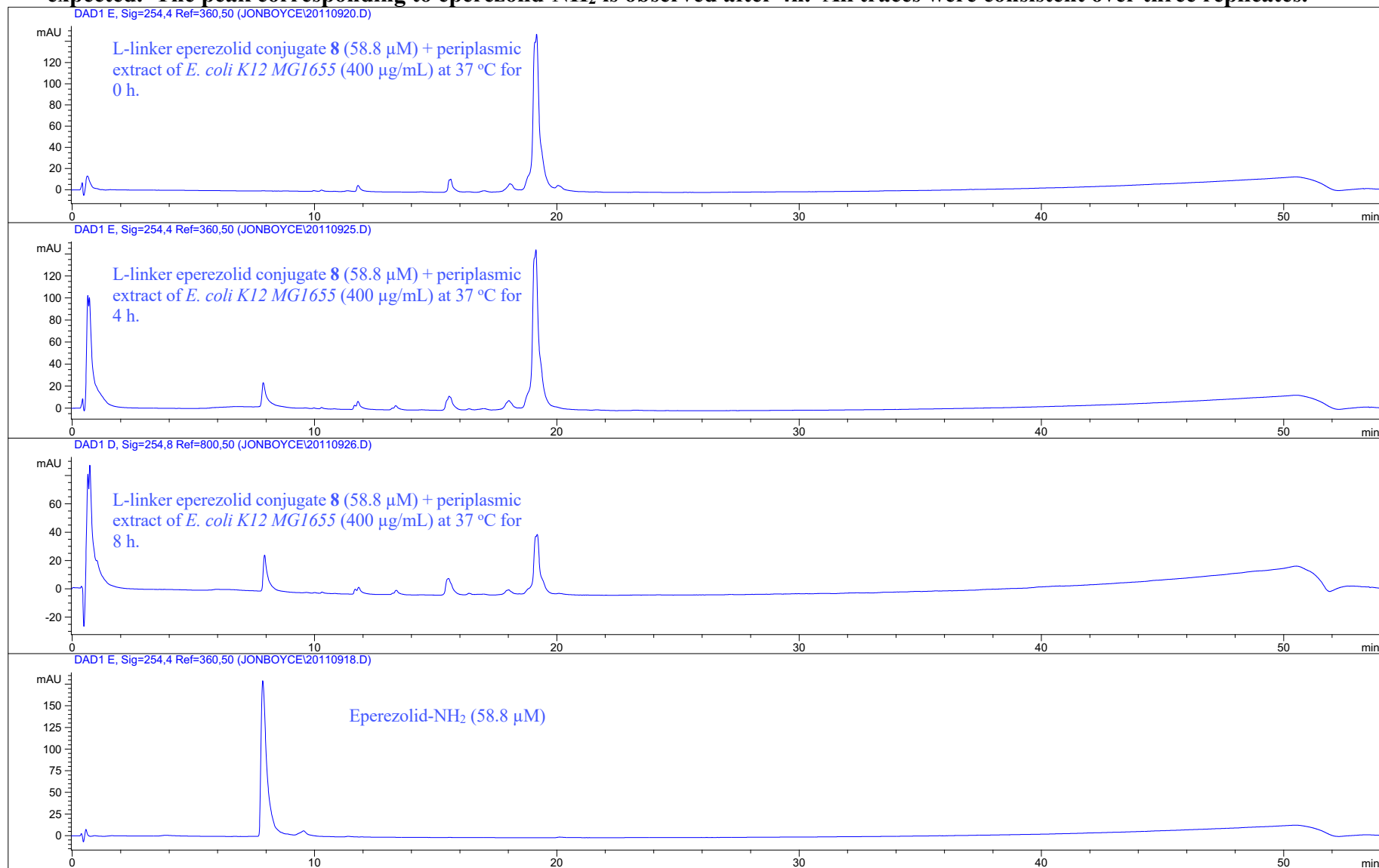




**Stability of D-linker Oxazolidinone Conjugate 14 (58.8  $\mu$ M) in *E. coli* K12 Periplasmic Extract. See page S140 for comparison of the L-linker conjugate 8. In contrast to the D-linker, the L-linker is significantly more degraded and releases eperezolid-NH<sub>2</sub> after 4 h. All traces were consistent over three replicates.**



**Stability of L-linker Oxazolidinone Conjugate 8 (58.8  $\mu$ M) in *E. coli* K12 Periplasmic Extract over 4 hours and 8 hours. See page S139 (above) for comparison of the D-linker variant 14. The D-linker is significantly more stable than the L-linker as expected. The peak corresponding to eperezolid-NH<sub>2</sub> is observed after 4h. All traces were consistent over three replicates.**



**Stability of L- and D-linker Solithromycin Conjugates 9 and 16 (58.8  $\mu\text{M}$ ) in *A. nosocomialis* Periplasmic Extract Over 20 Hours. All traces were consistent over three replicates. Cleavage byproducts of the L-linker solithromycin conjugate 9 are observed in the periplasmic extract of *A. nosocomialis* (see below). Cleavage byproducts of the D-linker variant 16 are not observed. This result agrees with our hypothesis that cleavage of 9 may be occurring in this strain and is in line with our discussion on page S2 concerning Table S1A and Figure S1A.**



**Stability of D- and L-linker Solithromycin Conjugates 16 and 9 (58.8  $\mu\text{M}$ ) in *E. coli* K12 MG1655 Periplasmic Extract at Time  $t = 0$  h and  $t = 8$  h. All traces were consistent over three replicates. Cleavage byproducts of 9 are observed in the periplasmic extract of *A. nosocomialis* (see page S141 above) but not *E. coli* K12 (see below), which is in line with our discussion presented on page S2 concerning Table S1A and Figure S1A.**

