Supporting Information

The biosynthetic implications of late-stage condensation domain selectivity during glycopeptide antibiotic biosynthesis

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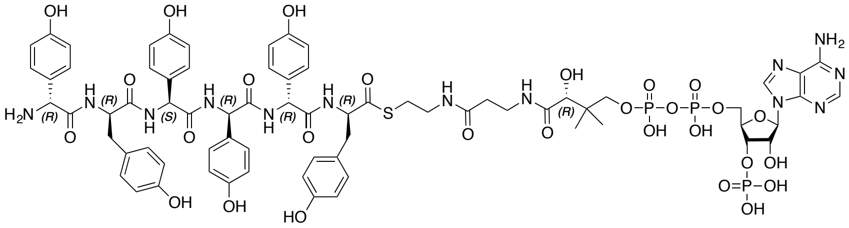
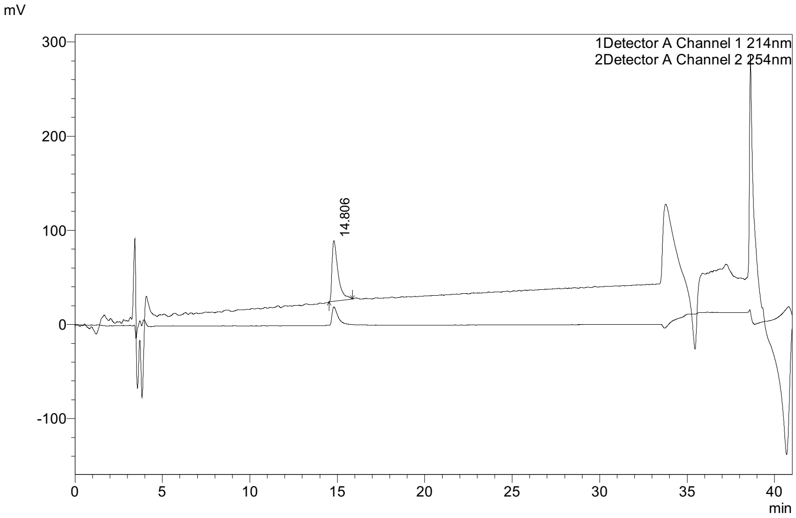
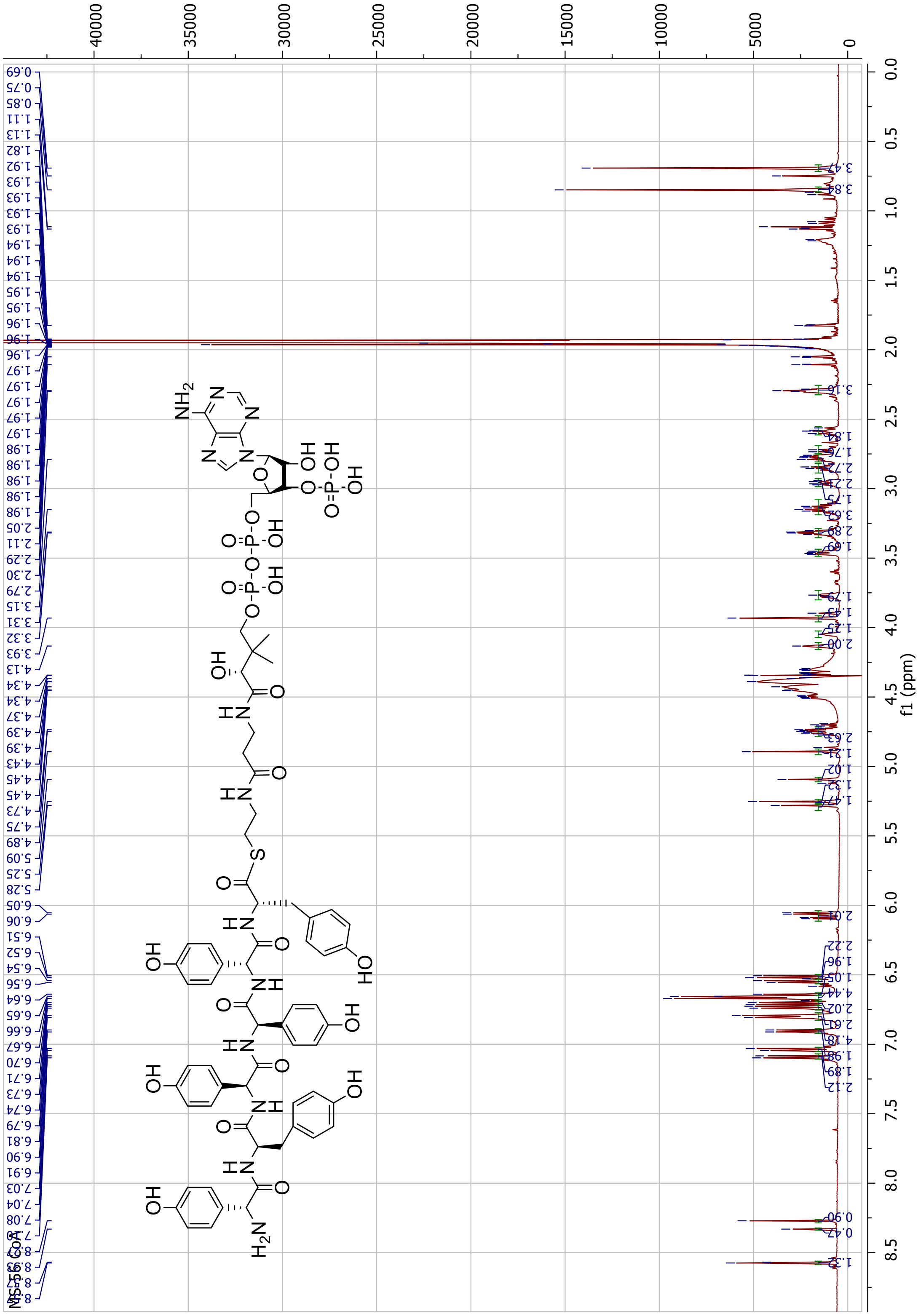
Characterisation of teicoplanin-type hexapeptide (1)



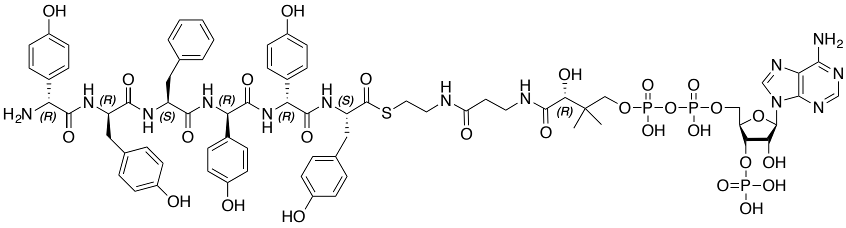
HPLC trace



^{1}H NMR spectra



Characterisation of actinoidin-type hexapeptide (4)



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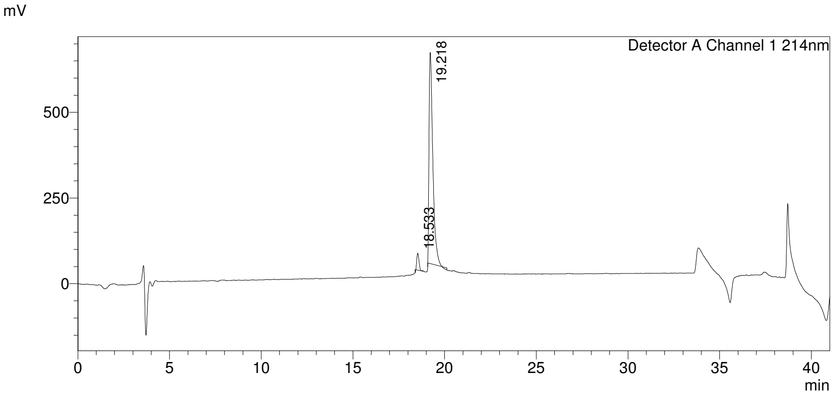
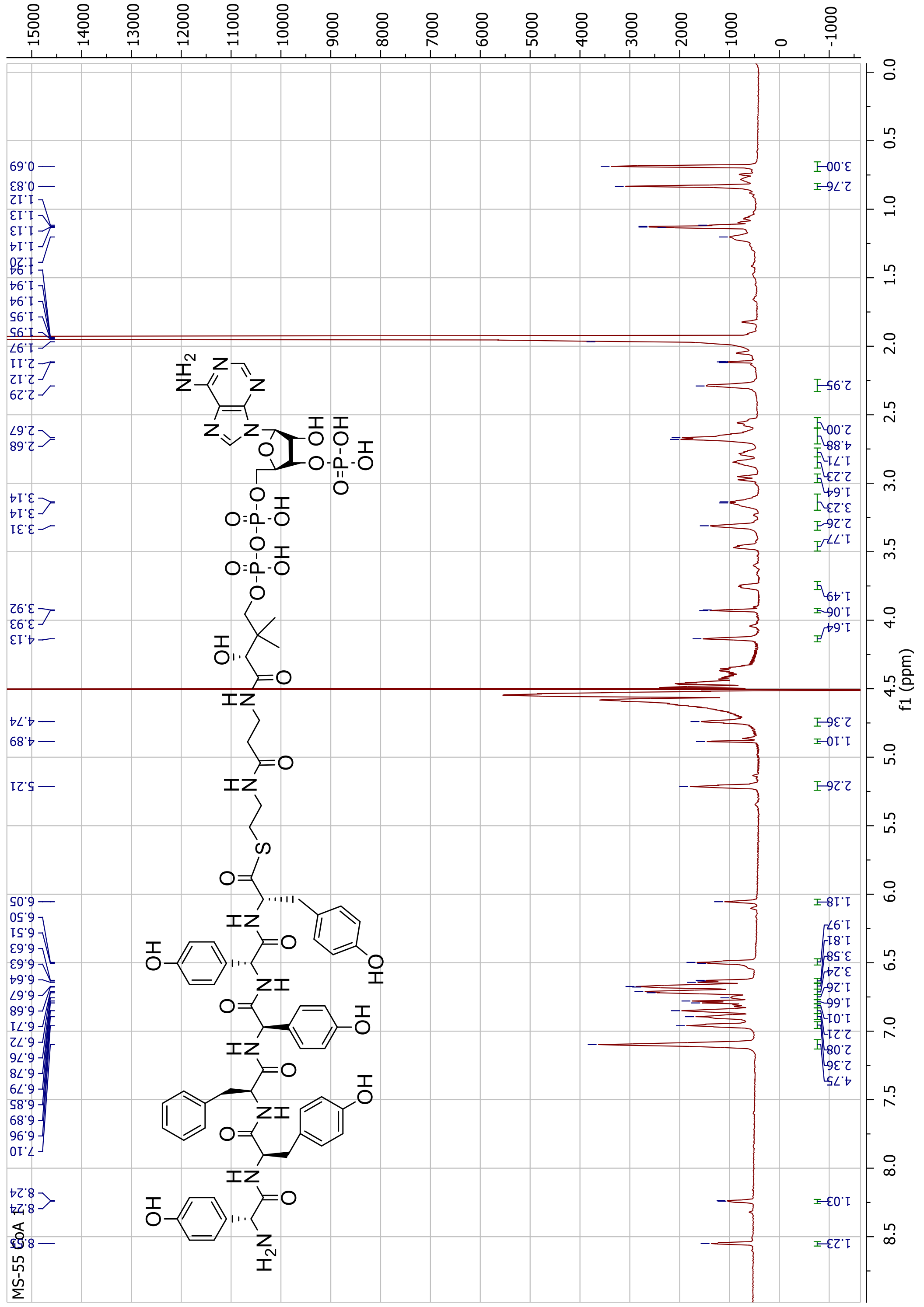
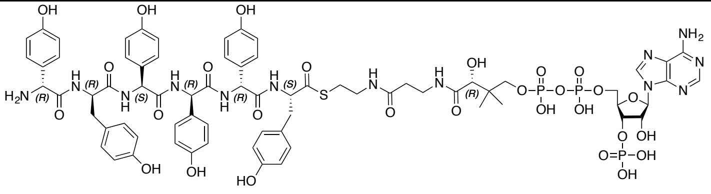
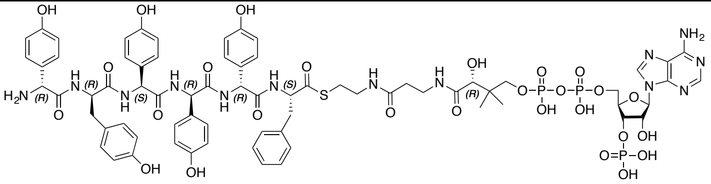
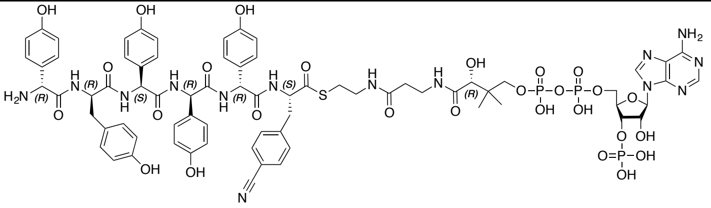
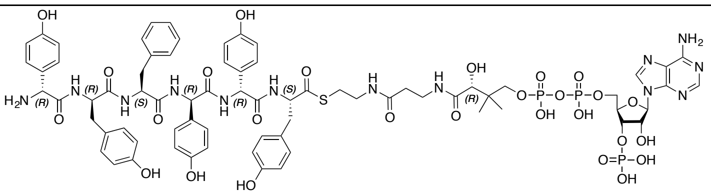
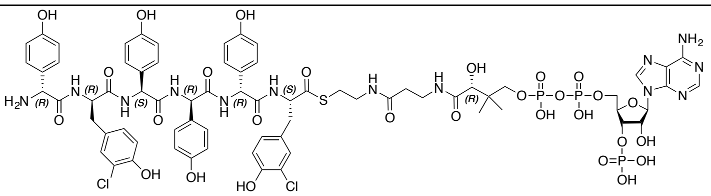
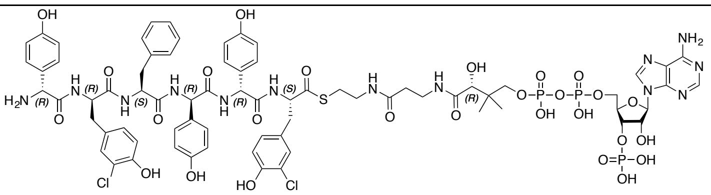
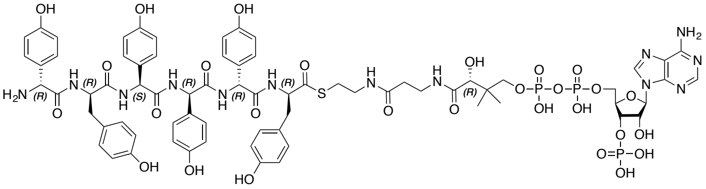


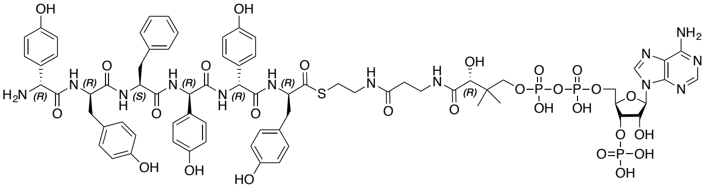
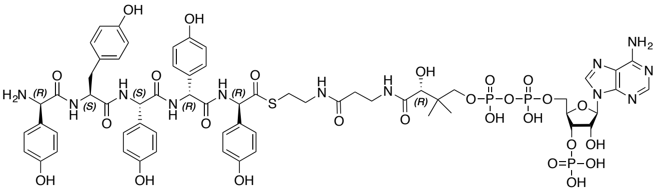
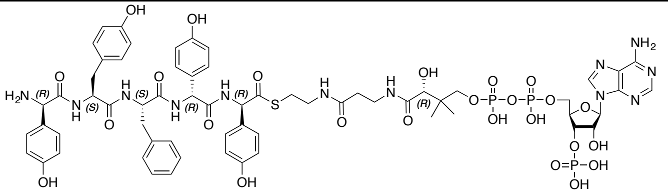
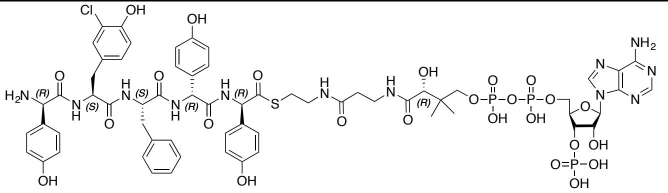
^{1}H NMR spectra



List of all peptide-CoA thioesters

Table SI1: Structure and chemical formula of all peptide-CoA thioesters synthesised in this study.

1 **Chemical Formula:** $C_{71}H_{82}N_{13}O_{28}P_3S$	
2 **Chemical Formula:** $C_{71}H_{82}N_{13}O_{27}P_3S$	
3 **Chemical Formula:** $C_{72}H_{81}N_{14}O_{27}P_3S$	
4 **Chemical Formula:** $C_{72}H_{84}N_{13}O_{27}P_3S$	
5 **Chemical Formula:** $C_{71}H_{80}Cl_2N_{13}O_{28}P_3S$	
6 **Chemical Formula:** $C_{72}H_{82}Cl_2N_{13}O_{27}P_3S$	
D-1* **Chemical Formula:** $C_{71}H_{82}N_{13}O_{28}P_3S$	

D-4* **Chemical Formula:** $C_{72}H_{84}N_{13}O_{27}P_3S$	
7 **Chemical Formula:** $C_{62}H_{73}N_{12}O_{26}P_3S$	
8 **Chemical Formula:** $C_{63}H_{75}N_{12}O_{25}P_3S$	
9 **Chemical Formula:** $C_{63}H_{74}ClN_{12}O_{25}P_3S$	

*D indicates a D-amino acid residue in position six of the peptides instead of the natural L-amino acid.

Species identified based on mass spectral analysis of balhimycin producer strains

***A. balhimycina*_ΔbpsCX**	Theoretical	Observed	Error (ppm)
Bal linear heptapeptide (SP-1135, **2-OH**)	1135.32132	1135.31909	1.96
Bal C-*O*-D crosslinked heptapeptide (SP-1133, $\mathbf{2}_{\mathrm{mono}}$**-OH**)	1133.30567	1133.30347	1.94
Bal linear hexapeptide (SP-970, **1-OH**)	970.27873	970.27529	3.55
Bal C-*O*-D crosslinked hexapeptide (SP-968, $\mathbf{1}_{\mathrm{mono}}$**-OH**)	968.26308	968.26044	2.73
Bal linear pentapeptide (SP-757)	757.25946	757.25913	0.44

***A. balhimycina*_ΔbpsCC**	Theoretical	Observed	Error (ppm)
Bal linear hexapeptide (SP-970, **1-OH**)	970.27873	970.27563	3.19
Bal C-*O*-D crosslinked hexapeptide (SP-968, $\mathbf{1}_{\mathrm{mono}}$**-OH**)	968.26308	968.26058	2.58
Bal linear pentapeptide (SP-757)	757.25946	757.25891	0.73

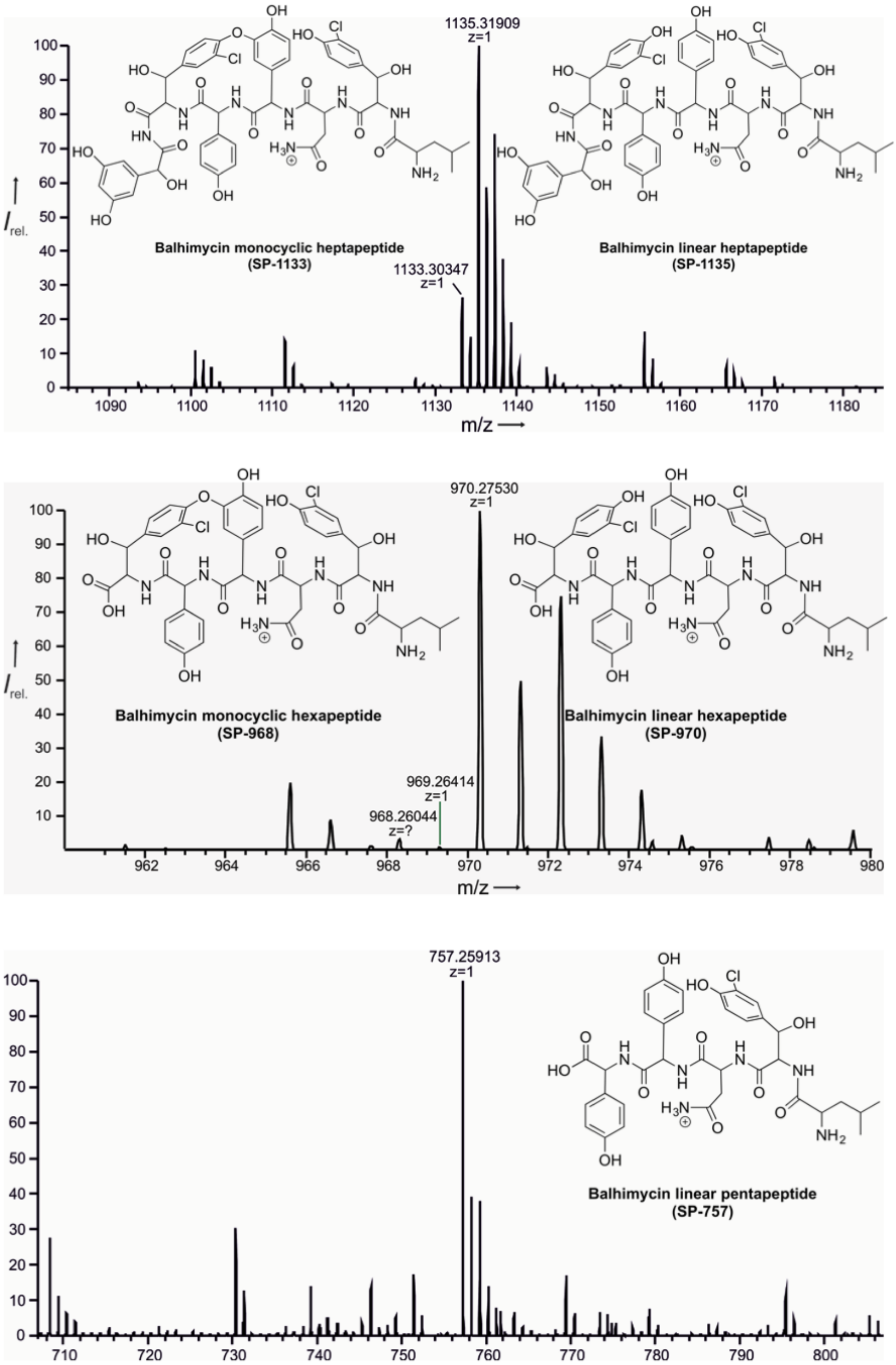


Figure SI1: Major products identified from the extract of strain ***A. balhimycina*_ΔbpsCX**. Results indicate the formation of hepta-, hexa- and pentapeptides, with monocrosslinked species identified for both hepta- and hexapeptides. All peptide species are chlorinated due to the halogenation of specific aminoacyl-PCPs that occurs during NRPS-mediated peptide synthesis.

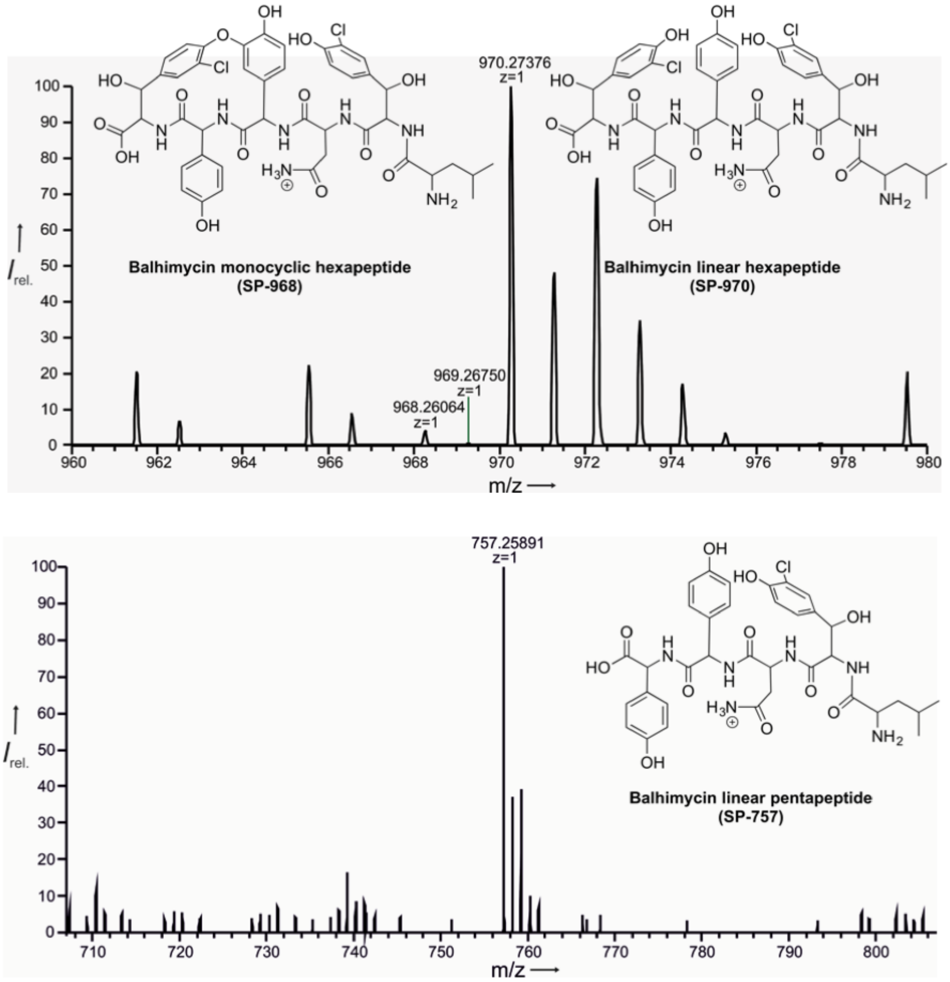


Figure SI2: Major products identified from the extract of strain ***A. balhimycina*_ΔbpsCC**. Results indicate the formation of hexa- and pentapeptides, with monocrosslinked species identified for the hexapeptide species. All peptide species are chlorinated due to the halogenation of specific aminoacyl-PCPs that occurs during NRPS-mediated peptide synthesis.

Condensation domain assay using monocyclic hexapeptidyl-PCP substrate (Mono-4)

Hexapeptide **4** was loaded onto the stand-alone PCP_6 using Sfp as already described and enzymatic transformation with $OxyB_{bal}$ (plus competent redox system) was performed for 1 h, after which a sample was taken and analysed to assess cyclisation progress to produce **Mono-4**. Subsequently, $Tcp12\Delta TE_2$, ATP and Dpg were added and time points were taken after 5 min, 10 min, 30 min, 60 min and 180 min (single experiments). Results indicate that peptide bond formation using the monocyclic hexapeptide (**Mono-4-PCP$_6$**) is possible, although at a significantly reduced rate over that of the linear hexapeptide.

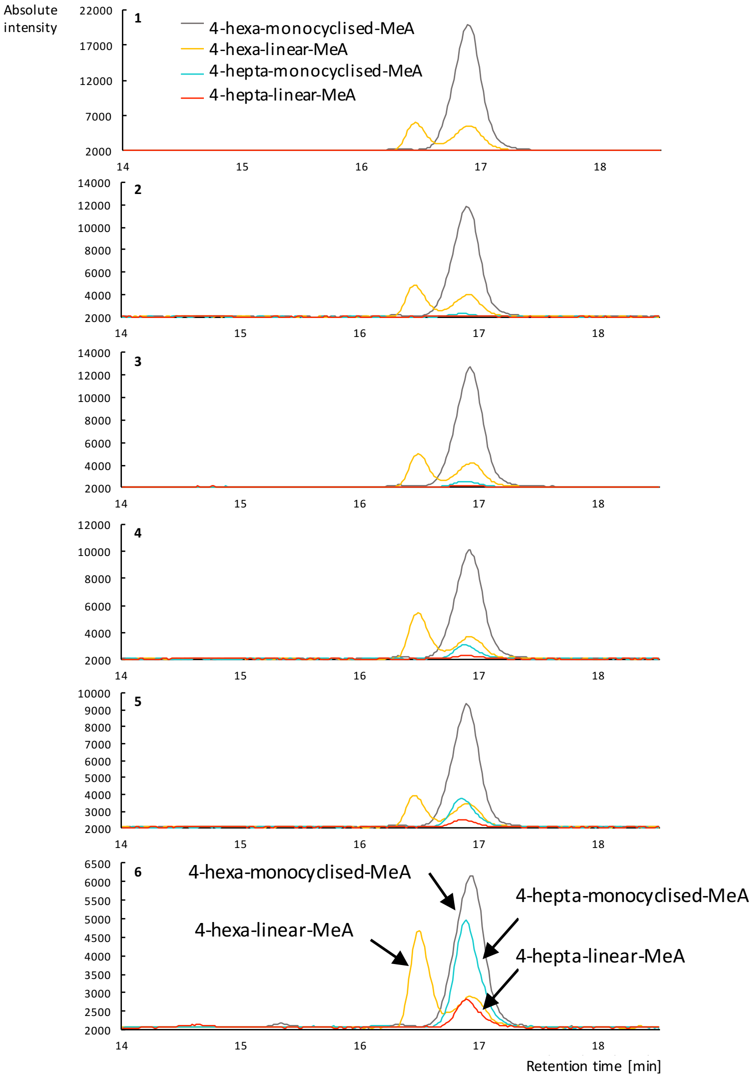


Figure SI3: Comparison of the MS traces (negative mode, SIM) after different time points. Trace 1 incubation of hexapeptide **4-PCP$_6$** with $OxyB_{van}$ for 1 h; Trace 2 addition of $Tcp12\Delta TE_{2l}$ and Dpg after 5 min; Trace 3 after 10 min; Trace 4 after 30 min; Trace 5 after 60 min; Trace 6 after 180 min.