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Supporting Information

A High-Resolution Crystal Structure that Reveals Molecular Details of Target Recognition by the Calcium-Dependent Lipopeptide Antibiotic Laspartomycin C

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Experimental Procedures

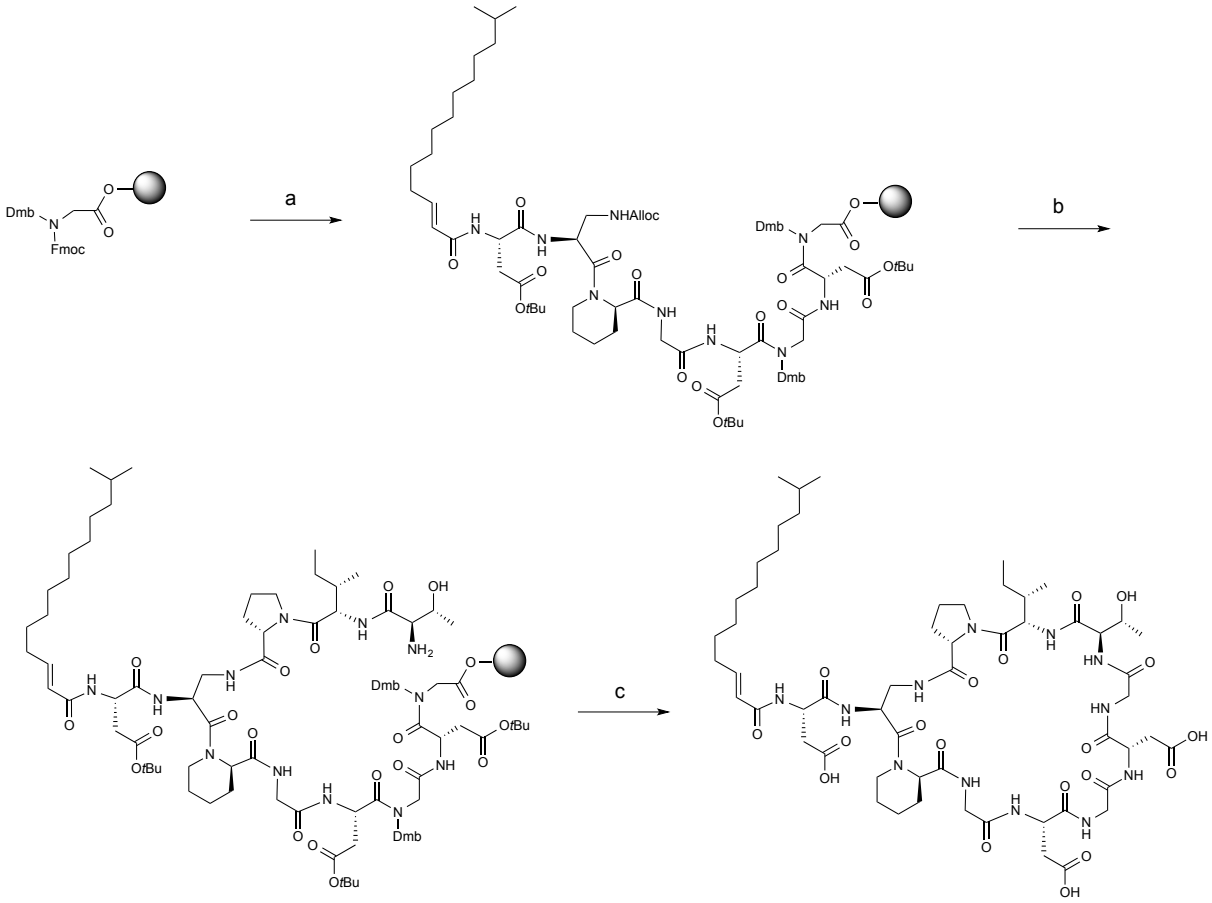
Reagents and general methods

All reagents employed were of American Chemical Society (ACS) grade or finer and were used without further purification unless otherwise stated. D-amino acids and 2-chlorotrityl resin was obtained from Iris Biotech GmbH, Egg PG and 0:6 PA was obtained from INstruchemie BV. C_{10}-P lithium salt was obtained from Sigma Aldrich and lyophilized from warm ^{t}BuOH:H_2O (1:1) to obtain a white powder with increased aqueous solubility. Additional biologically relevant phosphate monoesters including adenosine 5'-monophosphate, *rac*-glycerol-1-phosphate, dihydroxyacetone phosphate, glucose 6-phosphate, *O*-phospho-L-serine, and γ,γ-dimethylallyl phosphate where purchased from Sigma Aldrich.

Instrumentation for compound characterization

High-resolution mass spectrometry (HRMS) analysis was performed using a Bruker micrOTOF instrument. 2D NMR experiments (TOCSY, HSQC, and NOESY) were performed on a 500 MHz instrument and Circular Dichroism spectra were recorded on a Jasco J-810 CD spectrometer.

Synthetic procedures and compound characterization



Scheme S-1. Synthesis of *ent*-Laspartomycin C in accordance with previously published methodology [ref]. (a) Fmoc SPPS; (b) (i) $Pd[(C_6H_5)_3P]_4$, $C_6H_5SiH_3$, CH_2Cl_2, 1 h; (ii) Fmoc SPPS; (c) (i) $(CF_3)_2CHOH$, CH_2Cl_2, 1 h; (ii) BOP, DIPEA, CH_2Cl_2, 16 h; (iii) TFA, TIS, H_2O, 1 h.

Ent-laspartomycin C was synthesized and purified using the methodology established for the synthesis of laspartomycin C using the appropriate enantiomeric building blocks in 14.0 mg (11.2 μmol) quantity.[1] L-*allo*-Thr was introduced as the side chain protected species Fmoc-L-*allo*-Thr(tBu)-OH where in the synthesis of laspartomycin C the unprotected building block Fmoc-D-*allo*-Thr-OH had been used.

Analytical HPLC was performed by applying a gradient from H_2O:MeCN:TFA (95:5:0.1) to H_2O:MeCN:TFA (5:95:0.1) over 44 minutes using a Dr. Maisch C18 column (250 x 4.6 mm, 300 Å, 10μm). **HRESI-MS**: $[M+H]^+$ calculated: 1247.6518; *ent*-laspartomycin C found: 1247.6529; laspartomycin C found: 1247.6507.

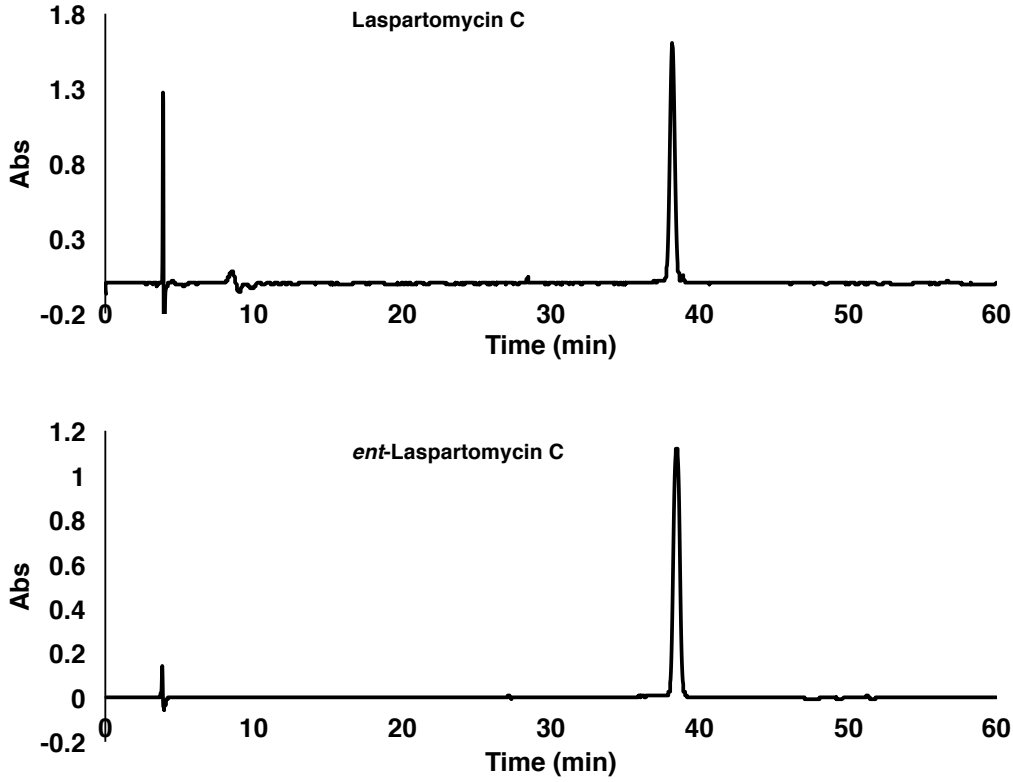


Figure S-1. Analytical HPLC trace of laspartomycin C (top) and *ent*-laspartomycin C (bottom).

NMR

TOCSY, HSQC and NOESY spectra were recorded for laspartomycin C and *ent*-laspartomycin C (5 mM in $DMSO_{d6}$) resulting in matching spectra in all cases.[1] The NOESY spectrum of laspartomycin C is included here to allow for direct comparison.

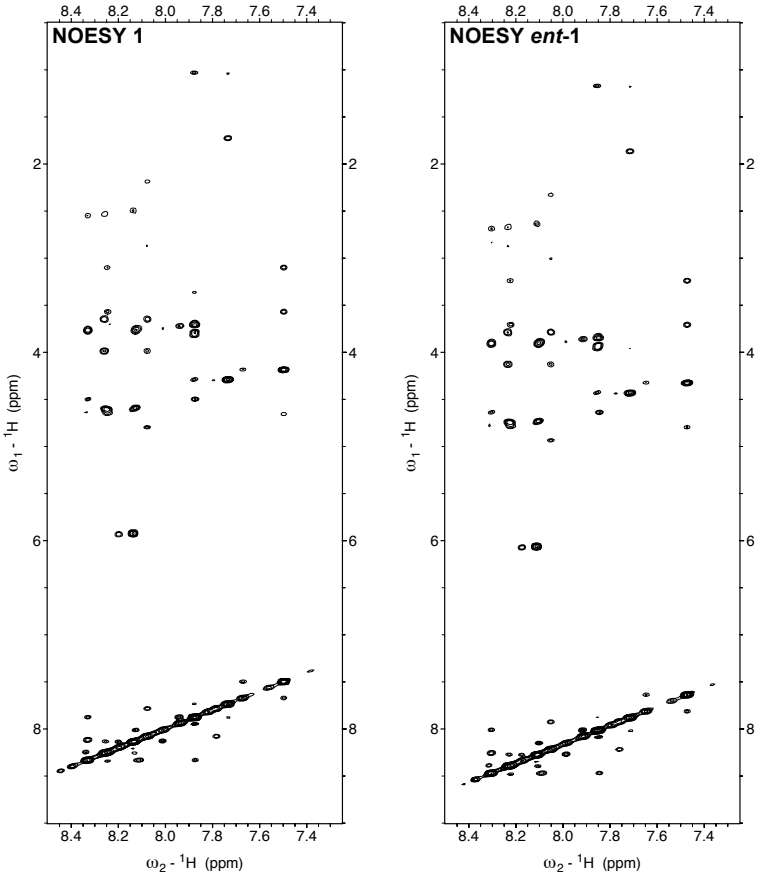


Figure S-2 The NOESY spectrum for *ent*-laspartomycin C (right) matches the previously reported NOESY spectrum of laspartomycin C (left).1 1

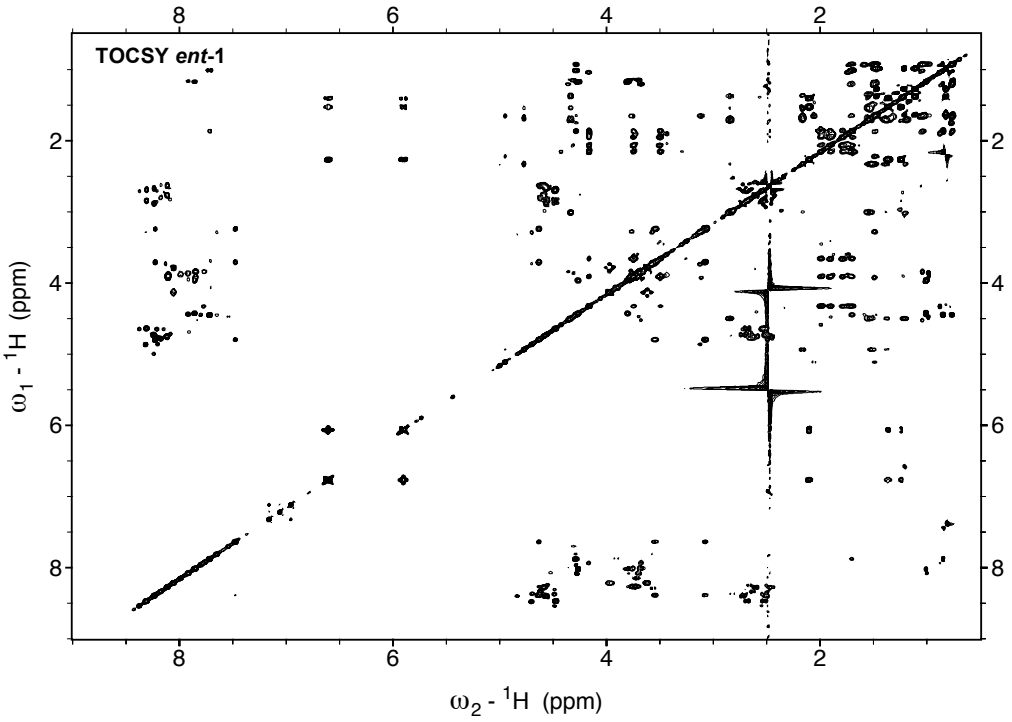


Figure S-3. TOCSY spectrum for *ent*-laspartomycin C

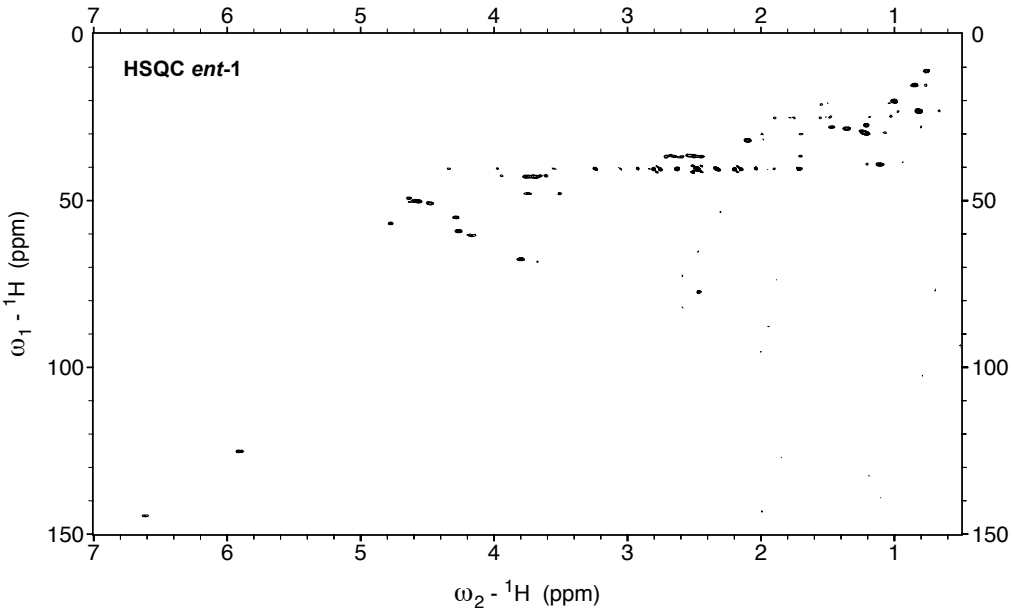


Figure S-4. HSQC spectrum for *ent*-laspartomycin C

Antibacterial Assays

Minimum inhibitory concentrations were determined in accordance with CLSI guidelines.[2] Antibiotic stocks in DMSO were diluted 50x in cation-adjusted Mueller Hinton broth (CAMHB; 10 mg.L^{-1} Mg^{2+}, 50 mg.L^{-1} Ca^{2+}, 0.002 v/v% TWEEN 80) and serially diluted in polypropylene 96-well plates to reach a volume of 50 μL per well. Bacterial cells were cultured in TSB until the exponential growth phase (OD_{600} = 0.5) before dilution in CAMHB and addition to the wells (50 μL) to reach a final CFU concentration of $5x10^5$ mL^{-1}. After overnight incubation (35°C, 250 RPM) the plates were inspected visually for growth. MIC determinations against *enterococci* were carried out in cation-adjusted LB medium (10 mg.L^{-1} Mg^{2+}, 50 mg.L^{-1} Ca^{2+}, TWEEN 80 0.002 v/v %) to ensure reliable bacterial growth (**Supplementary Table 1**).

Supplementary Table S-1. MICs for laspartomycin C, ent-laspartomycin C and daptomycin against a panel of Gram-positive pathogens in μg.mL^{-1}.

compound	**MRSA USA300**	**S. *aureus* 29213**	**B. *subtilis***	**VRE *faecalis* E4125**	**VRE *faecium* E155**
lasparomycin C	8	8	8	32	16
ent-laspartomycin C	8	8	8	32	16
daptomycin	0.5	0.5	1	4-8	4

Circular Dichroism

CD spectra were recorded for 60 µM laspartomycin C and *ent*-laspartomycin C solutions in 20 mM HEPES (pH=7.4) buffer in the presence and absence of 5.0 mM $CaCl_2$ and 0-4.0 eq. C_{10}-P. Data collection was limited to the 210-260 nm range because extensive scattering occurs below 210 nm under these conditions. The experiments were performed using a 1.0 mm cuvet, a bandwith of 1 nm and a scan speed of 20 nm min^{-1}. The average of 10 scans was baseline corrected by subtracting the average elipticity over 255-260 nm and units were converted to mean residue molar elipticities (deg cm^2 $dmol^{-1}$) (**Supplementary Fig. 5-6**).

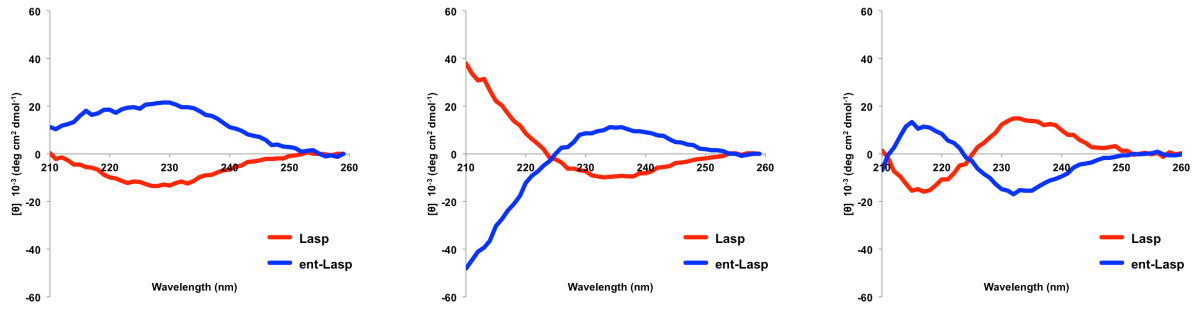


Figure S-5. CD spectra. **Left**: laspartomycin C and *ent*-laspartomycin C in the absence of $CaCl_2$. **Middle**: laspartomycin C and *ent*-laspartomycin C in the presence of 5.0 mM $CaCl_2$. **Right**: laspartomycin C and *ent*-laspartomycin C in the presence of 5.0 mM $CaCl_2$ and 1.0 eq. C_{10}-P.

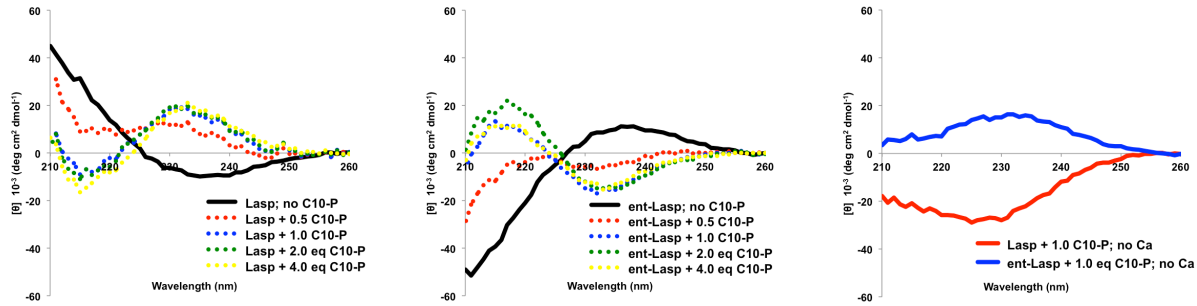


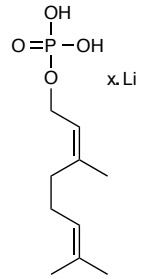
Figure S-6. CD spectra. **Left**: laspartomycin C in the presence of 5.0 mM $CaCl_2$ and 0-4.0 eq. C_{10}-P. **Middle**: *ent*-laspartomycin C in the presence of 5.0 mM $CaCl_2$ and 0-4.0 eq. C_{10}-P. **Right**: control measurement with laspartomycin C and *ent*-laspartomycin C in the absence of $CaCl_2$ mixed with 1.0 eq. C_{10}-P.

Antagonization of antibiotic activity

Antagonists were dissolved in MHB before serial dilution on a polypropylene 96-well plate to reach a volume of 50 μL per well. Unadjusted MHB was used to prevent the formation of C_{10}-P:Ca^{2+} species that show poor aqueous solubility in the absence of laspartomycin C. Linear aliphatic phosphate monoesters were dissolved in 20 mM HEPES:MHB (1:3) before serial dilution with MHB. Antibiotics in CAMHB were added (25 μL) to reach a final concentration of 8xMIC with antagonists present in up to 32 molar equivalents relative to the antibiotics. Na_2HPO_4 was applied at a fixed concentration of 1.25 mM corresponding to 24 and 500 molar eq. for laspartomycin C and daptomycin respectively. The antibiotic stocks contained extra cations and TWEEN 80 to achieve CAMHB medium composition (vide supra). After mixing for a minimum of 30 minutes, MRSA USA300 in CAMHB (25 μL) was added to reach a final concentration of $5x10^5$ CFU.mL^{-1}. The plates were incubated overnight (35°C, 250 RPM) and inspected visually for growth. A volume of 10 μL was transferred from each well onto blood agar plates and incubated overnight at 37°C to confirm antagonization of antibiotic activity (**Supplementary Fig. 7-9**).

The tables show antagonization of laspartomycin C and daptomycin by selected antagonists C_{10}-P, C_6-PA and PG. Inorganic phosphate ($Na_2HPO_4^{3-}$) did not antagonize activity of laspartomycin C and daptomycin at 1.25 mM (no separate table). Tables: antibiotic activity was antagonized (+), unaffected (-) or largely antagonized (+/-). Plates: bacterial growth was observed on blood-agar plates (seen here as circle) when antibiotic activity was antagonized. Table S-2 contains an assessment of the antibiotic activity of laspartomycin C in the presence of an extended panel of biologically relevant phosphate monoesters including: adenosine 5'-monophosphate, *rac*-glycerol-1-phosphate, dihydroxyacetone phosphate, glucose 6-phosphate, *O*-phospho-L-serine, and γ,γ-dimethylallyl phosphate. Also included in Table S-2 is a panel of linear aliphatic phosphate monoesters (C1, C3, C6, C10) that were prepared according to literature protocols.[3,4]

	no C_{10}-P	0.5 eq.	1.0 eq.	2.0 eq.	4.0 eq.	8.0 eq
Laspartomycin C	-	-	+ / -	+	+	+
Daptomycin	-	-	-	-	-	-

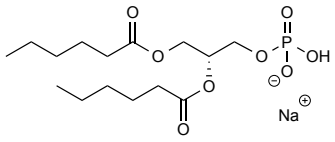


C_{10}-P

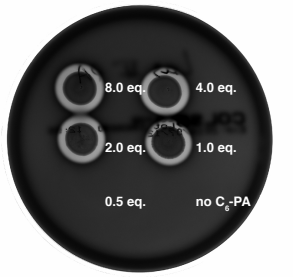
Laspartomycin C

Figure S-7. Antagonization by C_{10}-P

	no C_6-PA	0.5 eq.	1.0 eq.	2.0 eq.	4.0 eq.	8.0 eq
Laspartomycin C	-	-	+	+	+	+
Daptomycin	-	-	-	-	-	-



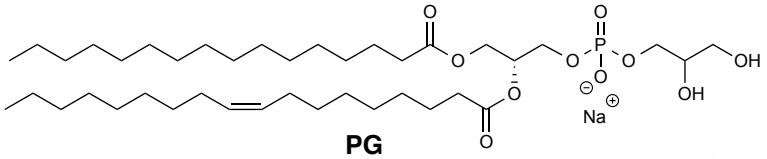
C_6-PA



Laspartomycin C

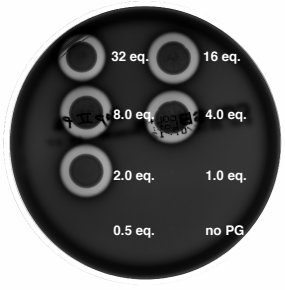
Figure S-8 Antagonization by C_6-PA

	no PG	0.5 eq.	1.0 eq.	2.0 eq.	4.0 eq.	8.0 eq	16 eq.	32 eq
Laspartomycin C	-	-	-	-	-	+	+	+
Daptomycin	-	-	-	+	+	+	+	+



PG

Laspartomycin C

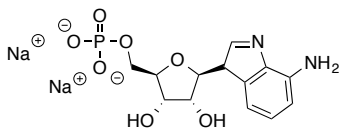


Daptomycin

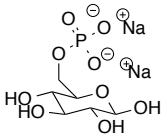
Figure S-9. Antagonization by PG

Supplementary Table S-2. Laspartomycin C antagonization study with expanded panel of biologically relevant (non-lipid) phosphate monoesters and aliphatic linear phosphate monoesters.

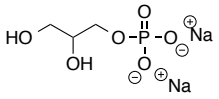
	no antagonist	0.5 eq.	1.0 eq.	2.0 eq.	4.0 eq.	8.0 eq
Adenosine 5'-monophosphate	-	-	-	-	-	-
D-glucose 6-phosphate	-	-	-	-	-	-
Rac-glycerol-1-phosphate	-	-	-	-	-	-
Dihydroxyacetone phosphate hemimagnesium salt	-	-	-	-	-	-
O-phospho-L-serine	-	-	-	-	-	-
methyl dihydrogen phosphate	-	-	-	-	-	-
propyl dihydrogen phosphate	-	-	-	-	-	-
hexyl dihydrogen phosphate	-	-	-	-	-	-
decyl dihydrogen phosphate	-	-	+	+	+	+
γ,γ-dimethylallyl phosphate	-	-	-	-	-	-



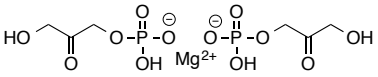
Adenosine 5'-monophosphate disodium salt



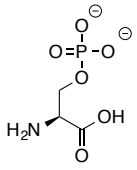
D-glucose 6-phosphate sodium salt



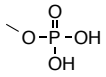
Rac-glycerol-1-phosphate sodium salt hydrate



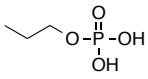
Dihydroxyacetone phosphate hemimagnesium salt hydrate



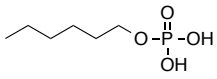
O-phospho-L-serine



methyl dihydrogen phosphate



propyl dihydrogen phosphate



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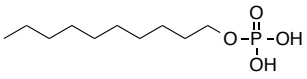
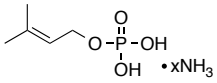


decyl dihydrogen phosphate



gamma,gamma-dimethylallyl phosphate ammonium salt

Mass Spectrometric Characterization of Laspartomcyin C/Ca^{2+}/C_{10}-P Complex

Laspartomycin C samples 1-3 (vide infra) were prepared in water and analyzed with capillary electrophoresis - time-of-flight mass spectrometry (CE - TOF-MS). CE was performed on a Beckman PA800 (Sciex, Framingham, MA, USA) with a background electrolyte (BGE) consisting of 20 mM formic acid and 22 mM ammonium hydroxide (NH_4OH; pH = 8.3) for analysis of sample 1, and with the same BGE with an additional 1.25 mM $CaCl_2$ for the analysis of sample 2 and 3. Samples were injected hydrodynamically into a 90 cm bare fused-silica capillary with 50 μm internal diameter by applying a pressure of 1 psi for 12 s. A 30-kV separation voltage with an additional pressure of 2 psi was used for separation, resulting in a constant 16 μA current for sample A and 18 μA for sample B and C. The temperature of the capillary was maintained at 25 °C during analysis. Detection was performed on a Bruker TOF-MS (Bruker Daltonics, Bremen, Germany) equipped with a co-axial sheath liquid sprayer (Agilent Technologies, Waldbronn, Germany). The sheath liquid consisted of MeOH:H_2O 1:1 (v/v) containing 10 mM NH_4OH and was used at a flow of 5 μL.min^{-1}. The MS settings were optimized for laspartomycin C signal intensity. The capillary voltage of the MS was 3.5 kV, the nebulizer gas pressure 5.8 psi, the dry gas flow 4.0 L.min^{-1}, the dry temperature 180 °C and the scan range *m/z* 50-3000 at a spectral rate of 0.5 Hz. Sample 1**:** laspartomycin C (100 µM); sample 2: laspartomycin C (100 µM) + $CaCl_2$ (1.25 mM); 3: Laspartomycin C (100 µM) + $CaCl_2$ (1.25 mM) + C_{10}-P (100 µM) (**Supplementary Fig. 10-12**).

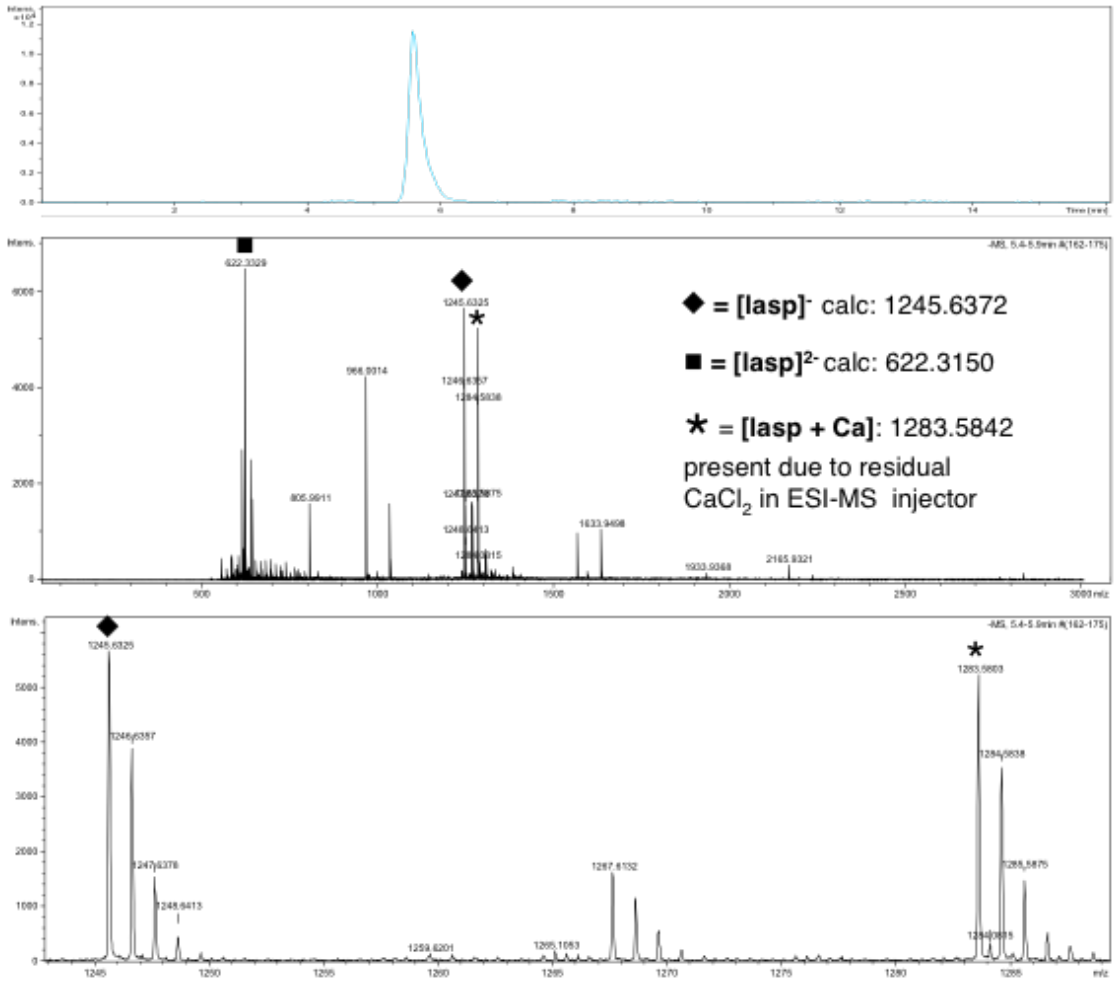


Figure S-10. CE-MS electropherogram and spectra of sample **1**: unbound laspartomycin C. A: CE-MS electropherogram selected for 1245.6 m/z; B: full mass spectrum of the peak present in the electropherogram at 5.4-5.9 min; C: zoomed mass spectrum of the peak present in the electropherogram at 5.4-5.9 min.

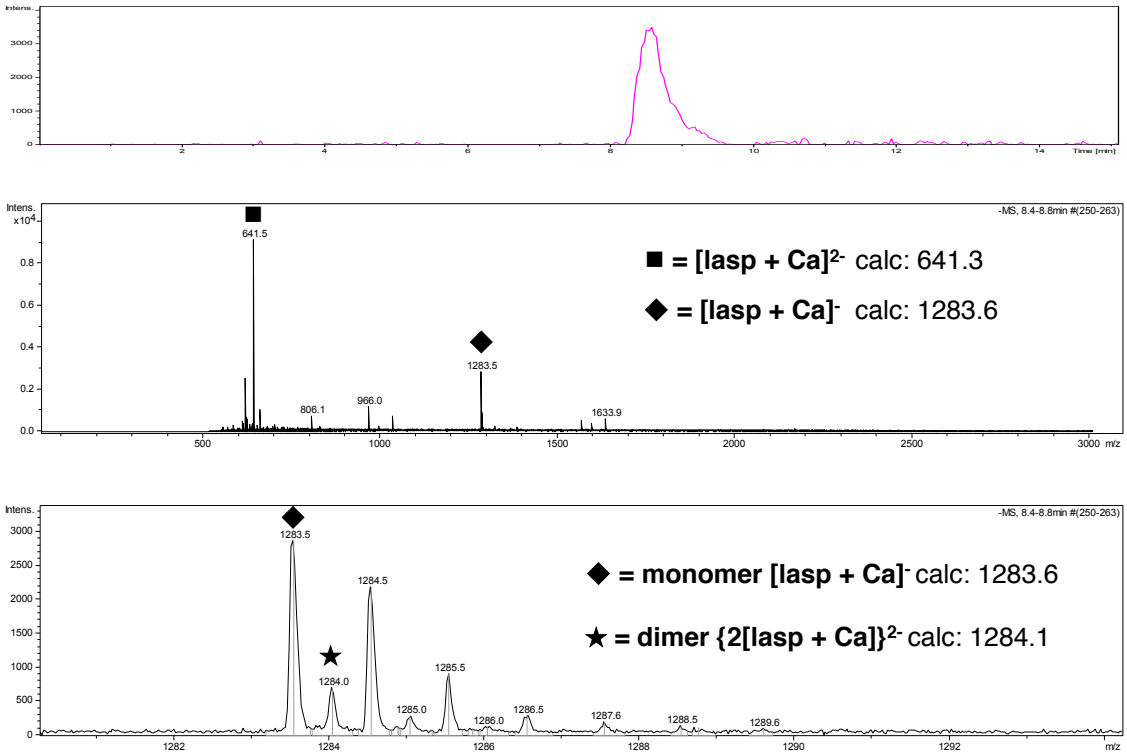


Figure S-11. CE-MS electropherogram and spectra of sample **2**: The Laspartomycin C:Ca^{2+} complex, predominantly present in 1:1 rather than 1:2 stochiometry, is observed both as monomer (■&◆) and as dimeric species (★). A: CE-MS electropherogram selected for 1285.5; B: full mass spectrum of the peak present in the electropherogram at 8.4-8.8 min; C: zoomed mass spectrum of the peak present in the electropherogram at 8.4-8.8 min.

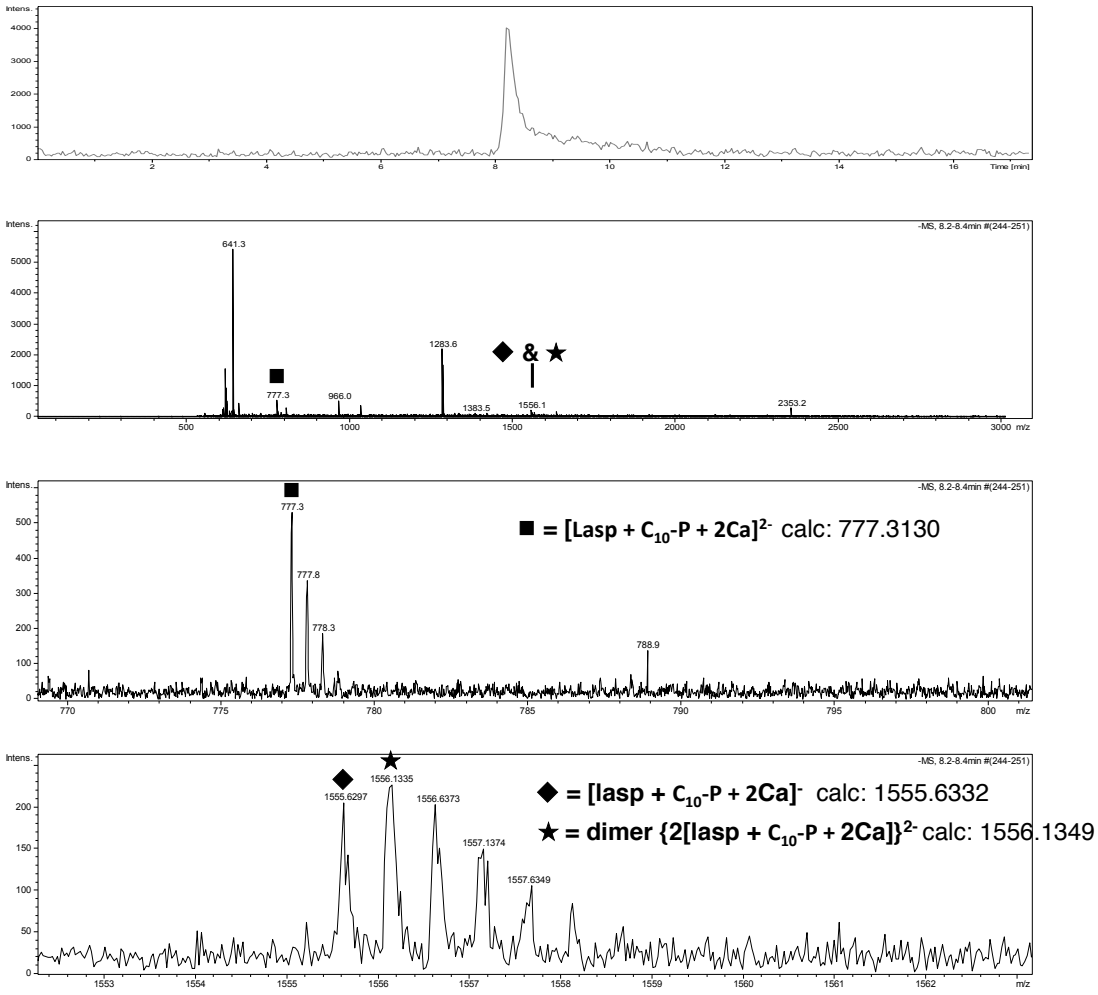


Figure S-12. CE-MS electropherogram and spectra of sample **3**: In addition to the Laspartomycin C:Ca^{2+} complex, the Laspartomycin C:C_{10}-P:$2Ca^{2}$ complex is now observed both as monomer (■&◆) and as dimer (★). A: CE-MS electropherogram selected for 1283.6 m/z; B: full mass spectrum of the peak present in the electropherogram at 8.2-8.4 min; C: zoomed mass spectra of peak present in electropherogram at 8.2-8.4 min.

Supplementary Segment for Crystallographic Studies

Crystallization and data collection

C_{10}-P was solubilized in PEG-200 and mixed with a solution of laspartomycin C in HEPES buffer (5 mM, pH 7.5, 10 mM $CaCl_2$) to achieve a 1:2 peptide:ligand molar ratio (7.2 mM:14.4 mM) with a final PEG-200 concentration of 10% (v/v). This solution was contact dispensed in 100 nL drops by a Gryphon LCP robot (Art Robbins Instruments) and 200 nL of reservoir solution, supplemented with 10% v/v PEG-200, was added on top. Crystals were grown at 20 °C under sitting drop vapor diffusion conditions against a reservoir solution containing either 20 mM $CaCl_2$, 0.10 M NaAc pH 4.6 and 40% v/v MPD or 20 mM $CaCl_2$ and 37.5% v/v MPD. Datasets were collected at the ERSF beamline ID23-2 (wavelength 0.873 Å, temperature 100 K) to 1.60 Å and 1.28 Å resolution respectively. Crystals for anomalous data collection were grown against a reservoir solution of 0.20 M $CdCl_2$ and 40% v/v MPD and a dataset was collected in-house using a rotating anode X-ray source (Bruker, wavelength 1.541 Å).

Structure solution and refinement

The data for the native Ca^{2+} datasets were integrated in iMOSFLM followed by scaling and merging using AIMLESS.[5,6] The anomalous data for the Cd^{2+} derivative was processed using EVAL.[7] The laspartomycin C ternary complex structure was solved by single isomorphous replacement with anomalous scattering using the Cd^{2+} derivative and the native Ca^{2+} dataset in SHELX c/d/e and autobuilding in ARP/wARP.[8,9] The structure was further refined to high resolution against the 1.28 Å dataset in REFMAC and manual building in Coot.[10,11] Custom restraints for the non-standard amino acids and C_{10}-P were generated by adapting existing restraints from the CCP4 monomer library. Restraints for the laspartomycin C fatty acid tail were generated using Ghemical and eLBOW.[12,13] Peptide bonds that were not automatically recognized by the refinement software were defined as trans-peptide bonds. No non-crystallographic symmetry restraints were used during the refinement. Figures were prepared using PyMOL Molecular Graphics System (DeLano Scientific LLC) and the structure has been deposited to the Protein Data Bank with accession code 5O0Z (**Supplementary Table 3-6; Supplementary Fig. 13-17**).

Table. S-3. Crystallographic data collection and refinement statistics

	Native	Anomalous (Cd^{2+})	High resolution
Data collection			
Space group	$P\,4_3\,3\,2$	$P\,4_3\,3\,2$	$P\,4_3\,3\,2$
Cell dimensions			
a, *b*, *c* (Å)	56.88, 56.88, 56.88	56.79, 56.79, 56.79	56.93, 56.93, 56.93
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90
Resolution (Å)	32.84-1.60 (1.64-1.60)	32.79-1.76 (1.82-1.76)	28.47-1.28 (1.30-1.28)
R_{merge}	0.172 (1.691)	0.106 (0.497)	0.067 (0.699)
$I / \sigma I$	15.6 (3.3)	4.91 (0.91)	15.0 (2.9)
Completeness (%)	100 (100)	96.97 (71.60)	100 (100)
Redundancy	22.3 (22.5)	87.89 (5.81)	9.4 (9.6)
Refinement			
Resolution (Å)			1.28
No. reflections			8650 (438)
No. free reflections			435
R_{work} / R_{free}			0.1450 / 0.1651
No. atoms			213
Protein			144
Ligand/ion			52
Water			17
B-factors (Å^2)			22.5
Protein			18.2
Ligand/ion			31.7
Water			30.7
R.m.s. deviations			
Bond lengths (Å)			0.019
Bond angles (°)			1.86

Table S-4. C_{10}-P coordination distances – from high resolution structure monomer 'A'

Ligand	Distance, Å
Head group oxygens	
LaspC: Gly^8 backbone amine	3.4
LaspC: Gly^8 backbone amine	3.2
Central calcium	2.3
Peripheral calcium	2.2
Connecting oxygen	
LaspC: Dap^2 backbone amine	3.3
LaspC: Dap^2 amine in ring closing bond	3.0

Table S-5. Ca^{2+} coordination distances – from high resolution structure monomer 'A'

Ligand	Distance, Å
Central calcium coordination	
LaspC: Dap^2 backbone carbonyl	2.3
LaspC: Asp^5 side chain oxygen	2.3
LaspC: Gly^6 backbone carbonyl	2.3
LaspC: Gly^8 backbone carbonyl	2.3
LaspC: Ile^{10} backbone carbonyl	2.3
C10-P: head group oxygen	2.3
Peripheral calcium coordination	
LaspC: lipid tail amide bond carbonyl	2.3
LaspC: Asp^1 side chain oxygen	2.3
LaspC: Asp^7 side chain oxygen	2.3
C10-P: Head group oxygen	2.2
Water	2.4
Water	2.4

Table S-6. Dimer interface*

Atom monomer 'A'	Atom monomer 'B'	Distance, Å
Phosphate headgroup	Phosphate headgroup	2.5
Thr^9 carbonyl	Coordinated water**	2.8
Thr^9 amide	Asp^7 side chain	2.9

* The interactions at the dimer interface are symmetrical; each unique interaction is listed only once.

** The Thr^9 carbonyl interacts with two coordinated waters, both are 2.8 Å.

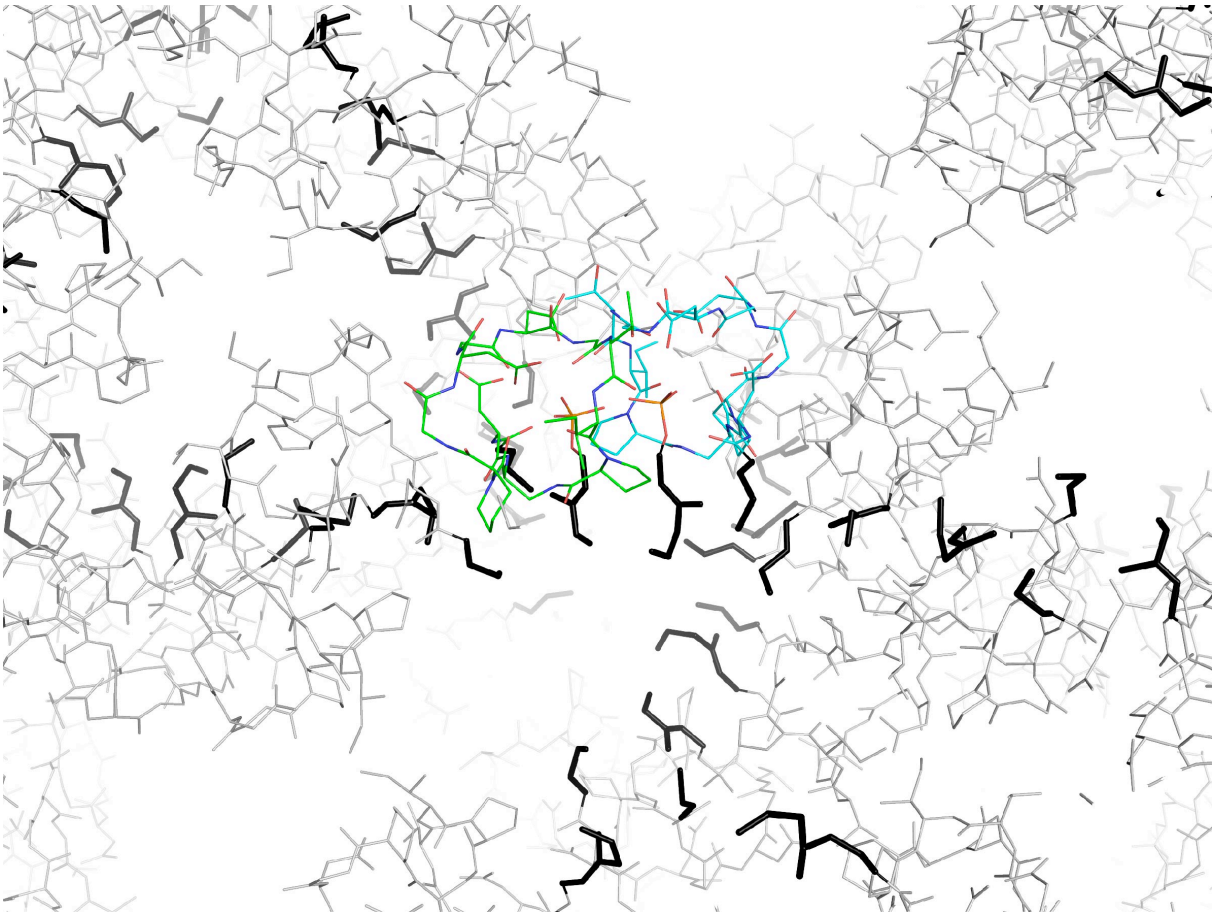


Figure S-13. Packing in the crystal. The hydrophobic tails of the laspartomycin C fatty acid and C_{10}-P hydrocarbon tail (both in fat black lines) pack together in the three-dimensional crystal lattice. The two laspartomycin C monomers present in one asymmetric unit are colored green and cyan while the symmetry related chains are shown in grey.

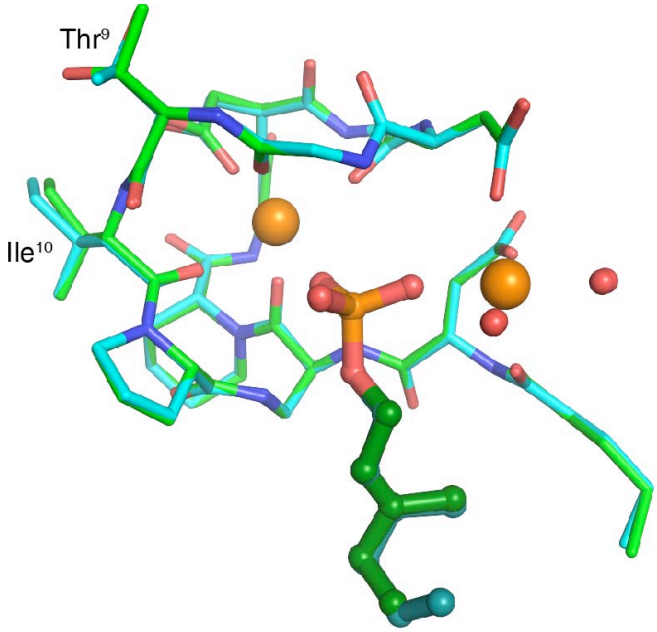


Figure S-14. Superposition of the two laspartomycin C ternary complexes present in the asymmetric unit. Monomer A (green) and B (cyan) are superposed. The bound Ca-ions (orange spheres), coordinated water molecules (red spheres) and C_{10}-P ligand (dark green for monomer A and dark teal for monomer B) are also shown.

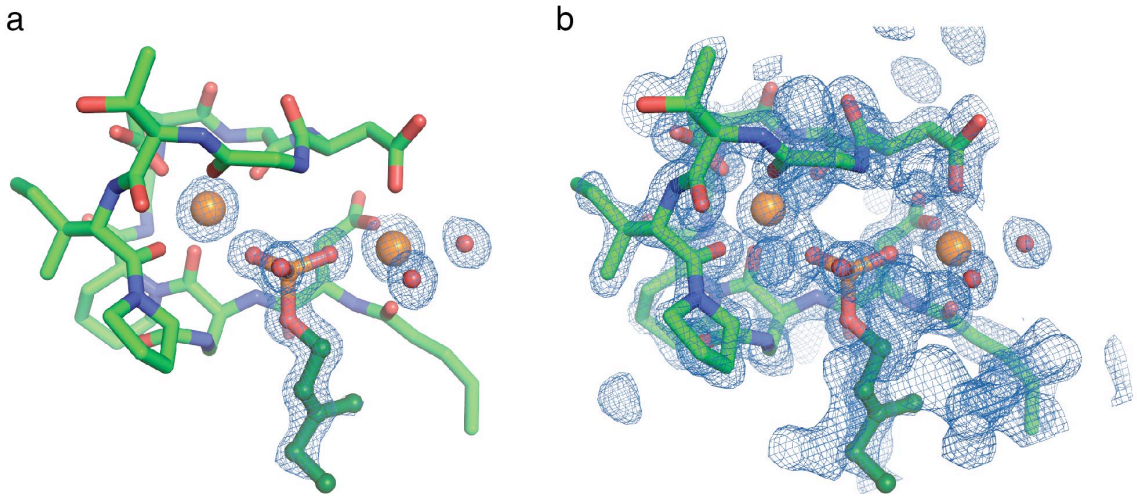


Figure S-15. Electron density. The map was refined to 1.28Å and is shown as a dark blue mesh at contour level 1.2σ (0.467 e/Å^3). (a) Map carved to show the density for the C_{10}-P ligand, coordinated Ca-ions and water molecules. (b) Uncarved map, note the density for a second C_{10}-P ligand in the foreground.

[1]

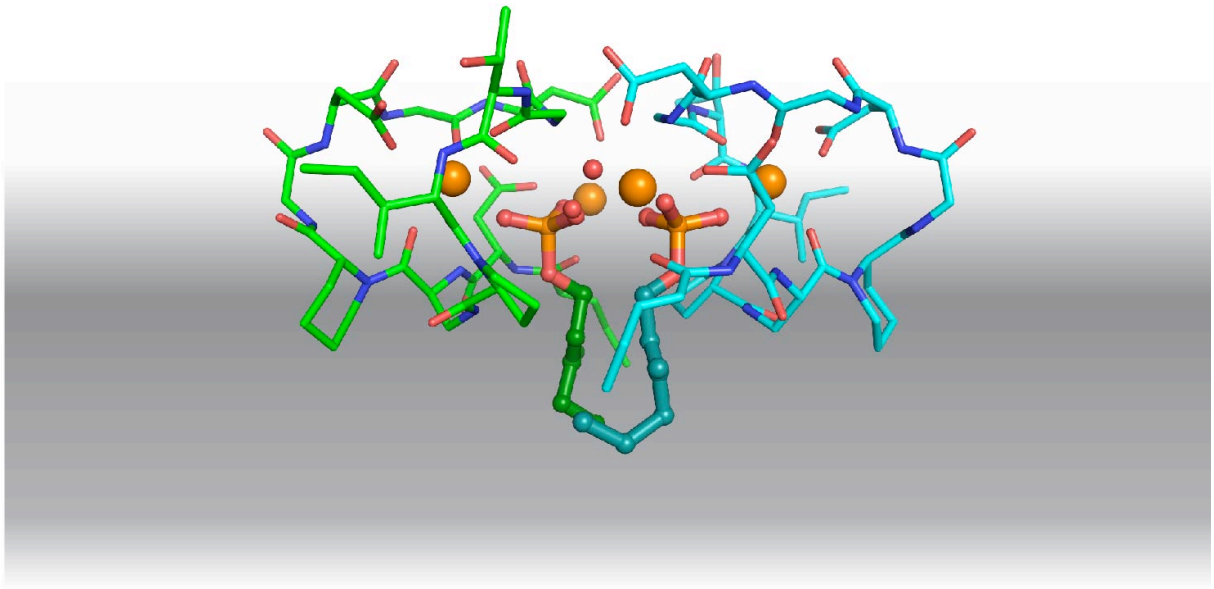
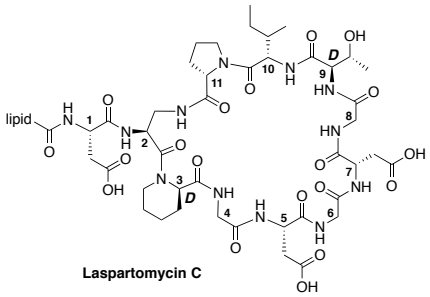


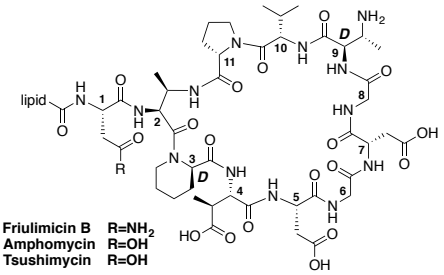
Figure S-16. Proposed orientation of the laspartomycin C dimer in the membrane. The dimer of two laspartomycin ternary complexes is positioned such that the hydrophobic part points towards the membrane (indicated with a grey gradient) and the hydrophobic tails of laspartomycin C and the ligand are inserted in the membrane. The dimer is proposed to be partially embedded within the membrane bilayer.

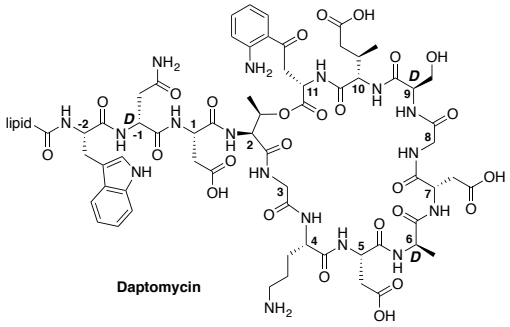
Table S-7 Conservation of residues/residue properties across different lipopeptide families.

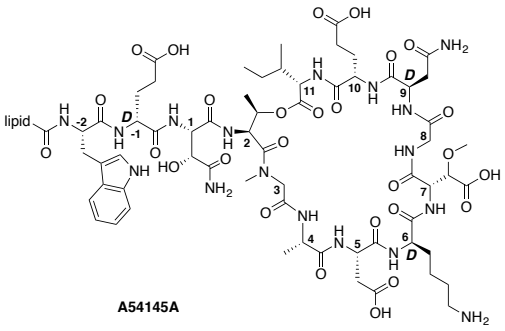
The calcium dependent lipopeptides			The calcium dependent lipodepsipeptides			Restrictions based on the Laspartomycin C dimer
Residue	**Laspartomycin C**	**Friulimicin B/ Amphomycin/ Tsushimycin**	**Daptomycin**	**A54145A**	**CDA1b**	
-3			lipid	lipid		-
-2			Trp	Trp		-
-1	lipid	lipid	D-Asn	D-Glu	lipid	First carbon should be C=O
1	Asp	Asn/Asp	Asp	hAsn	D-Ser	Asp or Asn
2	Dap	Dab	Thr	Thr	Thr	L-aa closing the cycle
3	D-Pip	D-Pip	Gly	NMe-Gly	D-Trp	D-aa or Gly
4	Gly	MeAsp	Orn	Ala	Asp	L-aa; sidechain allowed
5	Asp	Asp	Asp	Asp	Asp	Asp or Asn
6	Gly	Gly	D-Ala	D-Lys	D-Hpg	D-aa or Gly
7	Asp	Asp	Asp	MeOAsp	Asp	Asp
8	Gly	Gly	Gly	Gly	Gly	Gly, sidechain not allowed
9	D-*allo*-Thr	D-Dab	D-Ser	D-Asn	D-pAsn	D-aa or Gly, polar functionality
10	Ile	Val	MeGlu	Glu	Glu	L-aa or Gly
11	Pro	Pro	Kyn	Ile	Trp	Should be hydrophobic

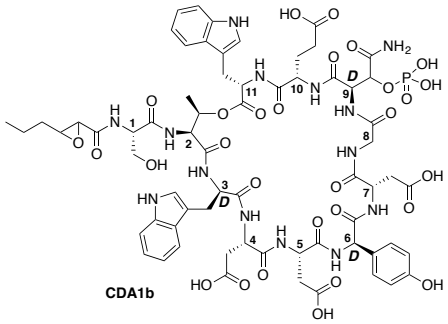
Structural features that are important for dimer formation and Ca^{2+} binding in the laspartomycin C dimer are conserved (green) among the calcium dependent lipopeptides and lipodepsipeptides. Residue D-Ser[1] (grey) in the lipodepsipeptide CDA1b, in place of an Asp or Asn residue, represents the single exception.











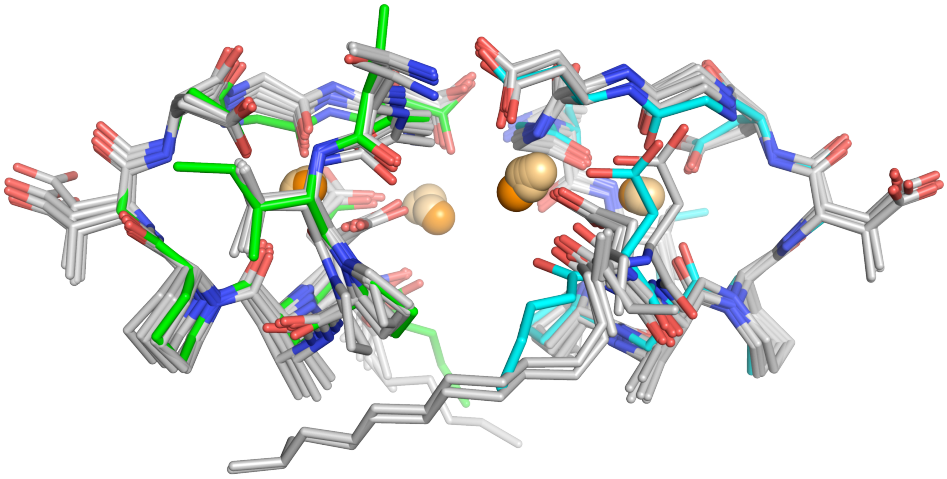


Figure S-17. The laspartomycin C dimer superposed onto dimers of tsushimycin. The structure of the ternary laspartomycin C/Ca2+/C10-P dimer (green and cyan) is very similar to the calcium-bound unliganded tsushimycin dimer solved previously[14] (grey). Ca-ions bound to the laspartomycin dimer are shown as bright orange spheres, those bound inside the tsushimycin dimer are shown as light orange spheres. When comparing the laspartomycin dimer with the six tsushimycin dimer structures the maximum root mean square deviation (rmsd) of all 22 Cα atoms is 0.57 Å, this is less than the maximum rmsd of the tsushimycin dimers compared with each other. The superposition was created using the 'align' command in PYMOL with default settings, i.e. non-standard amino acids were not used to determine the superposition.

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