
Supplementary information

A new antibiotic traps lipopolysaccharide in its intermembrane transporter

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Supplementary material for

A new antibiotic traps lipopolysaccharide in its intermembrane transporter

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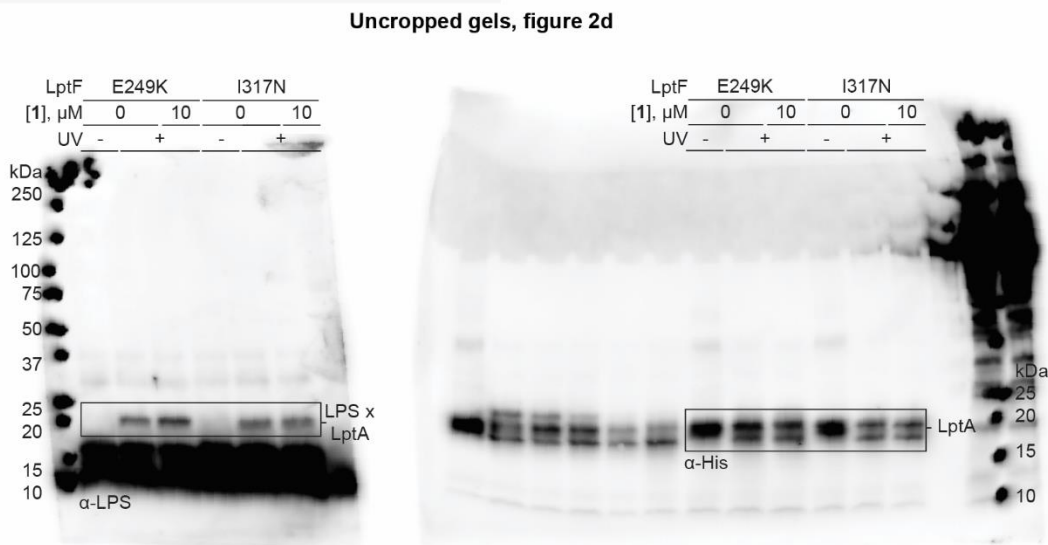
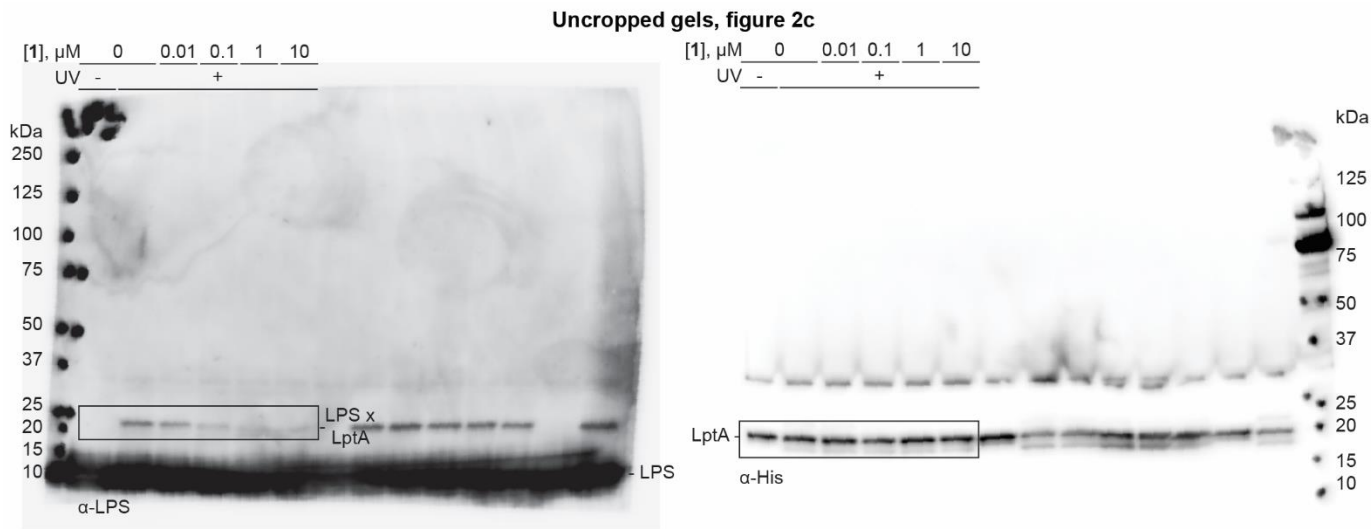
This PDF file includes:

Supplementary Figure 1

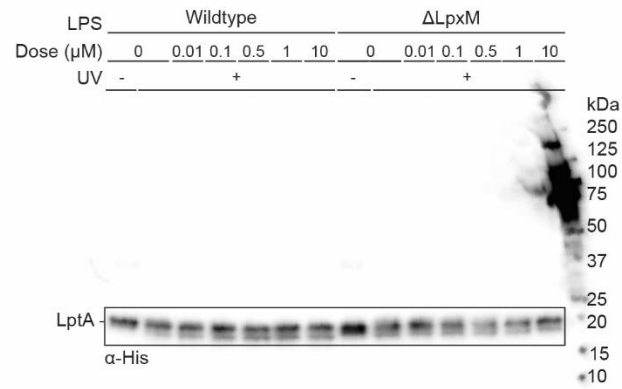
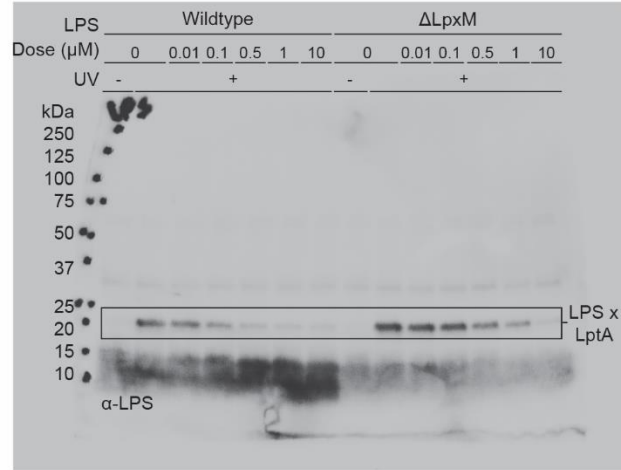
Supplementary Tables 1-5

Supplementary methods – Synthetic methods and sequences of unique plasmids

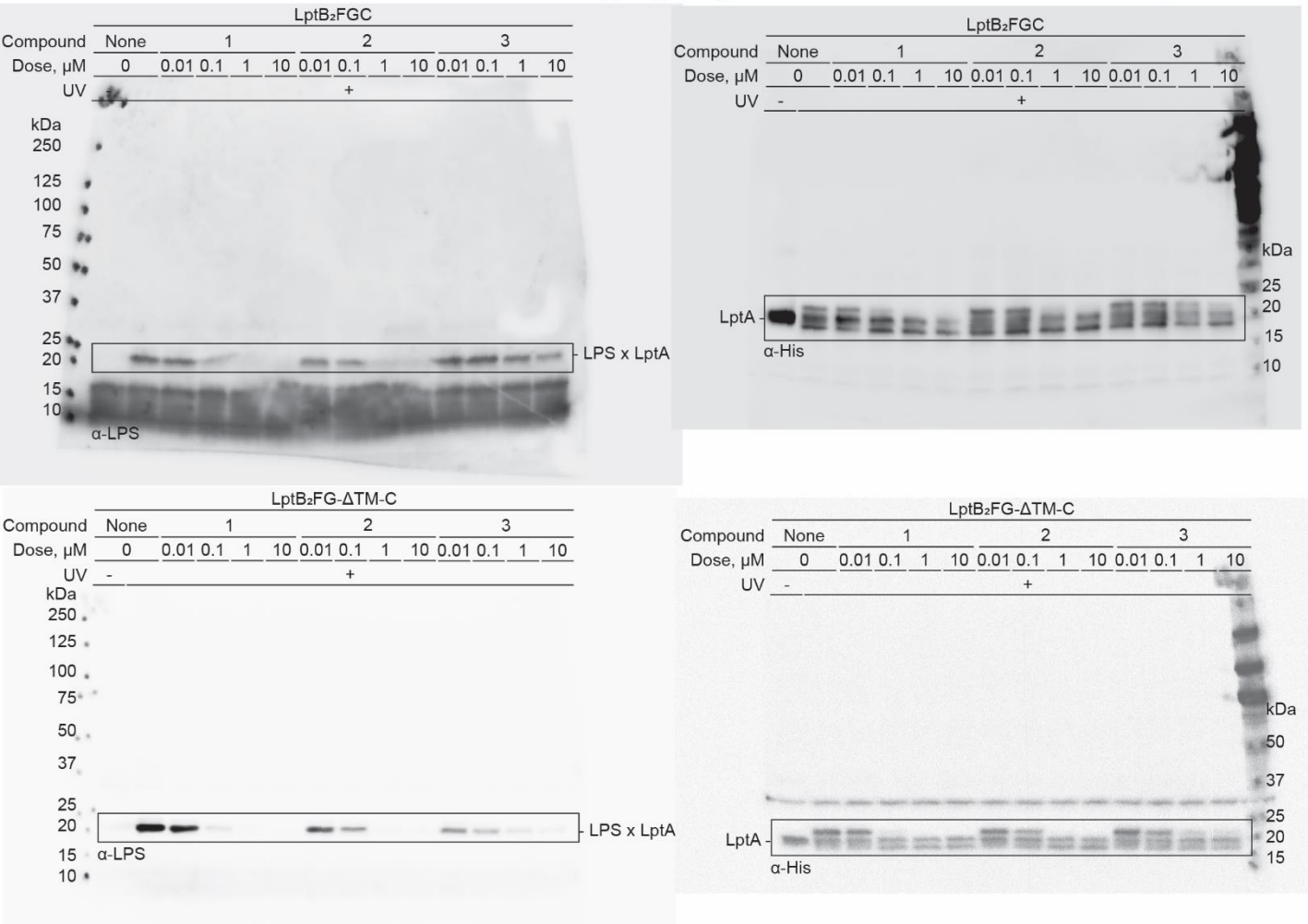
Supplementary figure 1 Uncropped gels and blots for data shown in main-text and extended data figures. For each figure, the cropped regions are denoted by boxes.



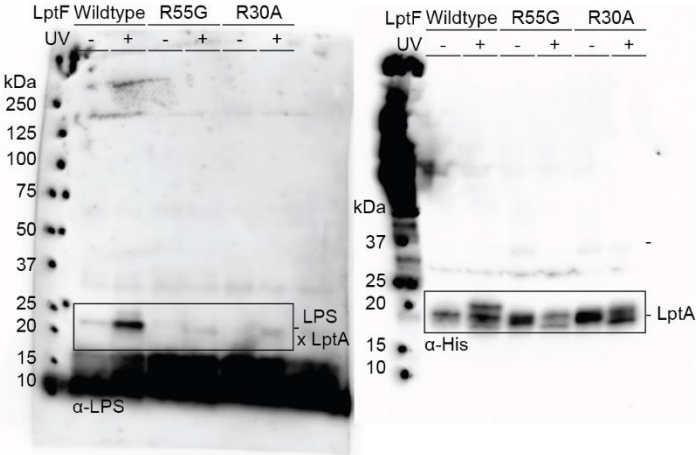
Uncropped gels, figure 3b



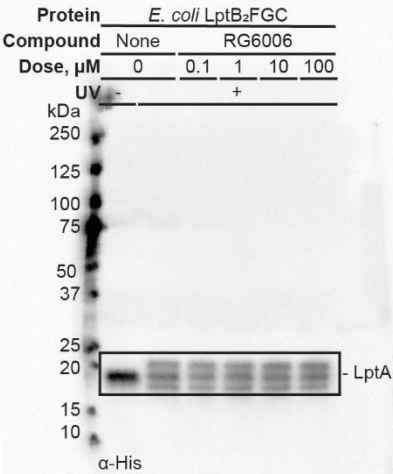
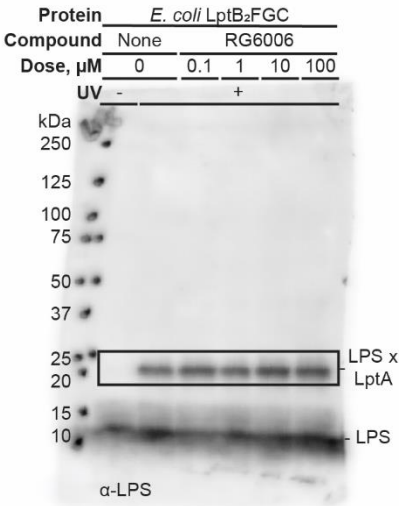
Uncropped gels, figure 4d



Uncropped gels, Extended data figure 4l



Uncropped gels, Extended data figure 8d



Compound	MIC, μM ($\mu\text{g/mL}$)		
	1	2	3
Wildtype	0.01 (0.008)	0.06 (0.05)	0.04 (0.03)
LptG(L36Q)	0.07 (0.06)	0.44 (0.35)	0.44 (0.33)
LptF(E58V)	1.2 (0.96)	3.4 (2.7)	14 (10)
LptF(E249K)	1.2 (0.96)	17 (13)	>50 (>37)
LptF(W271R)	0.02 (0.02)	0.1 (0.08)	0.1 (0.07)
LptF(V314L 315del)	0.07 (0.06)	3 (2)	0.24 (0.18)
LptF(I317N)	50 (40)	9 (7)	>50 (>37)
LptF(R320T)	0.04 (0.03)	1.4 (1.1)	0.24 (0.18)

Supplementary table 1. Mutations in LptFG renders *Acinetobacter baylyi* broadly resistant to compounds **1-3**. MIC values of *Acinetobacter baylyi* containing the indicated point mutants in LptF or LptG against compounds **1-3** are shown. MIC values were consistent across three cultures started from individual colonies.

	MIC, μM ($\mu\text{g/mL}$)	
	Wildtype	ΔLpxM
Novobiocin	4 (2)	0.6 (0.4)
Coumermycin A1	5 (6)	0.16 (0.18)
Bacitracin	500 (700)	20 (30)
Rifampicin	1.6 (1.3)	0.6 (0.5)
Vancomycin	500 (700)	17 (25)
Triclosan	0.126 (0.036)	0.03 (0.009)

Supplementary table 2. The MICs of wildtype and ΔLpxM *Acinetobacter baylyi* against various antibiotics with diverse mechanisms of actions.

Strain	MIC, μM ($\mu\text{g/mL}$)	
	Wildtype	ΔLpxC
1	0.01 (0.008)	>50 (>40)

Supplementary table 3. Knockout of LpxC renders *Acinetobacter baylyi* resistant to **1**. MIC values of wildtype and ΔLpxC *Acinetobacter baylyi* compound **1** are shown. MICs values were consistent across three cultures started from individual colonies.

Strains	Genotype	Source
NovaBlue	<i>endA1 hsdR17</i> (r_K^- , m_K^+) <i>supE44 thi-1 recA1 gyrA96 relA1 lac F'</i> [<i>proA</i> ⁺ <i>B</i> ⁺ <i>lacI</i> ^q $\Delta\text{M15::Tn10}$]	Novagen
BL21(DE3)	<i>fhuA2 [lon] ompT gal</i> (λ DE3) [<i>dcm</i>] ΔhsdS	Novagen
C43(DE3)	<i>F – ompT hsdSB</i> (<i>rB- mB-</i>) <i>gal dcm</i> (DE3)	Lucigen
<i>A. baylyi</i>	ADP1	ATCC
<i>A. baylyi</i>	Single deletion mutant in <i>lpxC</i> in the background of WT ADP1	33
$\Delta\text{lpxC}::\text{kan}$		

Supplementary table 4. Bacterial strains used in this work

Plasmid	Description	Source
pET22/42-LptC-thrombin-His ₇	pET22/42 encoding full-length <i>Acinetobacter baylyi</i> LptC with a C-terminal cleavable His ₇ tag	Companion paper
pET22/42-LptCΔTM-thrombin-His ₇	pET22/42 encoding <i>Acinetobacter baylyi</i> LptC residues 24-182 with a C-terminal cleavable His ₇ tag	This work
pCDFduet-LptB-LptFG	pCDFduet encoding full-length <i>Acinetobacter baylyi</i> LptB at the first site and <i>Acinetobacter baylyi</i> LptFG at the second site.	Companion paper
pCDFduet-HisLptB-LptFG	pCDFduet encoding full-length <i>Acinetobacter baylyi</i> LptB with an N-terminal His ₇ tag at the first site and <i>Acinetobacter baylyi</i> LptFG at the second site.	This work
pTRAB-FLAG-LptB-LptFG	pBR322WH1266 spectinomycinR plasmid encoding full-length <i>Acinetobacter baylyi</i> LptB with an N-terminal FLAG tag and LptFG under a TRC promoter.	This work
pCDFduet-LptB-LptF ^{E249K} G	pCDFduet-LptB-LptFG with LptF(E249K)	Companion paper
pCDFduet-LptB-LptF ^{I317N} G	pCDFduet-LptB-LptFG with LptF(I317N)	Companion paper
pCDFduet-LptB-LptF ^{E249K,I317N} G	pCDFduet-LptB-LptFG with LptF(E249K, I317N)	Companion paper
pCDFduet-HisLptB-LptF ^{R30A} G	pCDFduet-HisLptB-LptFG with LptF(R30A)	This work
pCDFduet-HisLptB-LptF ^{R55G} G	pCDFduet-HisLptB-LptFG with LptF(R55G)	This work
pTRAB-FLAG-LptB-LptFG	The gDNA amplicons of the LptBFG from <i>Acinetobacter baylyi</i> incorporating a linker-less FLAG tag at the N-terminus of LptB, a modified trp promoter of <i>E. coli</i> and adjacent regions from pTRC99a, a hybrid pBR322WH1266 replicon, and a spectinomycin resistance cassette from pCDFduet	This work
pET22b-LptA(I36Am)	pET22b encoding LptA(I36Am) with a His tag.	14

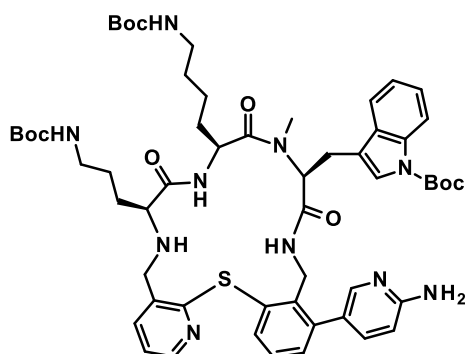
Supplementary table 5. Bacterial plasmids used in this work

Supplementary methods

Synthetic methods

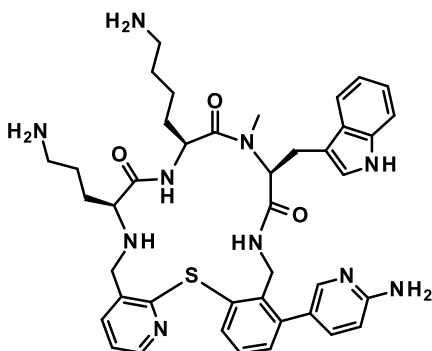
Synthesis of (11S,14S,17S)-14-(4-aminobutyl)-11-(3-aminopropyl)-22-(6-amino-3-pyridyl)-25-chloro-17-(1H-indol-3-ylmethyl)-16-methyl-2-thia-4,10,13,16,19-pentazatricyclo[19.4.0.0^{3,8}]pentacosa-1(25),3(8),4,6,21,23-hexaene-12,15,18-trione (**1**):

a) tert-Butyl 3-[[[(11S,14S,17S)-22-(6-amino-3-pyridyl)-14-[4-(tert-butoxycarbonylamino)butyl]-11-[3-(tert-butoxycarbonylamino)propyl]-25-chloro-16-methyl-12,15,18-trioxo-2-thia-4,10,13, 16,19-pentazatricyclo[19.4.0.0^{3,8}]pentacosa-1(25),3(8),4,6,21, 23-hexaen-17-yl]methyl] indole-1-carboxylate



To a solution of tert-butyl 3-(((7S,10S,13S)-17-bromo-10-(4-((tert-butoxycarbonyl) amino) butyl)-7-(3-((tert-butoxycarbonyl)amino)propyl)-20-chloro-12-methyl-8,11,14-trioxo-5,6,7,8, 9,10,11,12,13,14,15,16-dodecahydrobenzo[b]pyrido[3,2-p][1]thia[5,8,11,14]tetraazacyclo heptadecin-13-yl)methyl)-1H-indole-1-carboxylate (190 mg, 0.18 mmol, 1.0 eq) and 6-aminopyridine-3-boronic acid (48.3 mg, 0.35 mmol, 2.0 eq) in 1,4-dioxane (2 mL) and water (0.2 mL) was added sodium carbonate (46.4 mg, 0.44 mmol, 2.5 eq) and cataCXium-A-PdG3 (CAS-RN [1651823-59-4]; 25.5 mg, 0.04 mmol, 0.2 eq) in glove box in a microwave tube. After addition, the mixture was stirred at 100 °C for 16 h. The mixture was then concentrated to give a residue, which was purified by preparative thin-layer chromatography (petroleum ether / ethyl acetate 1:3) to produce the title compound (75 mg, 39%). White solid, MS: 1097.5 [M+H]⁺.

b) (11S,14S,17S)-14-(4-Aminobutyl)-11-(3-aminopropyl)-22-(6-amino-3-pyridyl)-25-chloro-17-(1H-indol-3-ylmethyl)-16-methyl-2-thia-4,10,13,16,19-pentazatricyclo[19.4.0.0^{3,8}]pentacosa-1(25),3(8),4,6,21,23-hexaene-12,15,18-trione (**1**)



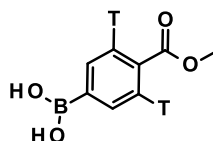
To a solution of tert-butyl 3-[[[(11S,14S,17S)-22-(6-amino-3-pyridyl)-14-[4-(tert-butoxycarbonylamino)butyl]-11-[3-(tert-butoxycarbonylamino)propyl]-25-chloro-16-methyl-12,15,18-trioxo-2-thia-4,10,13, 16,19-pentazatricyclo[19.4.0.0^{3,8}]pentacosa-1(25),3(8),4,6,21, 23-hexaen-17-yl]methyl] indole-1-carboxylate (65.0 mg, 0.06 mmol, 1.0 eq) in 1,4-Dioxane (1 mL) was added hydrogen chloride solution (4 M in 1,4-dioxane, 3.25 mL, 13.0 mmol). After addition, the mixture was stirred at 25 °C for 2 h to give a white suspension. The solid was collected by filtration and washed with 1,4-dioxane (2 mL). The filter cake was collected and dissolved in water (2 mL), then the solution was

purified by preparative HPLC (column: YMC Triart C18 150*25mm particle size: 5 µm; mobile phase: A: water (0.05 % conc. HCl in water (v/v)), B: MeCN; gradient: B%: 10%-40%, 8.5 min; flow rate: 29 mL/min) and freeze drying to give the title compound as the dihydrochloride salt (25 mg, 48%).

Yellow solid, MS: 797.5 [M+H]⁺. ¹H NMR (400 MHz, D₂O) δ ppm 8.77 (s, 1H), 8.38 (br s, 1H), 7.96 (d, J = 9.3 Hz, 1H), 7.91 (s, 1H), 7.72 (d, J = 6.7 Hz, 1H), 7.68 (d, J = 8.2 Hz, 1H), 7.58 (d, J = 7.1 Hz, 1H), 7.45 (d, J = 8.2 Hz, 2H), 7.31-7.18 (m, 2H), 7.10 (d, J = 9.0 Hz, 1H), 7.07-6.97 (m, 2H), 4.68-4.51 (m, 3H), 4.37-4.23 (m, 1H), 4.14-3.91 (m, 2H), 3.22-3.11 (m, 1H), 3.04-2.85 (m, 3H), 2.77-2.55 (m, 5H), 2.42-2.23 (m, 1H), 1.97-1.84 (m, 1H), 1.83-1.71 (m, 1H), 1.68-1.50 (m, 2H), 1.10-0.79 (m, 2H), 0.73-0.42 (m, 2H), -0.55--1.08 (m, 2H).

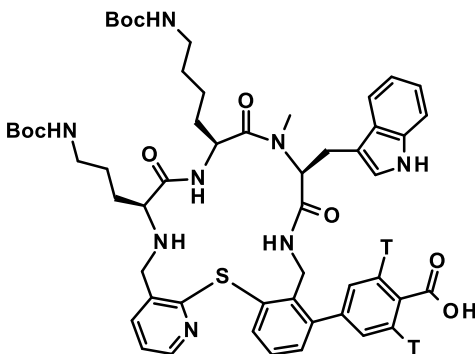
Synthesis of [³H]-RG6006

a) (4-Methoxycarbonyl-3,5-ditritio-phenyl)boronic acid



In a 2 ml tritiation flask, methyl 2,6-dichloro-4-(4,4,5,5-tetramethyl-1,2,3-dioxoborolan-2-yl)benzoate (10.0 mg, 30.2 µmol, 1.00 eq.) Pd/C (10%) (9.65 mg, 9.06 µmol, 0.30 eq.), and diisopropylethylamine (14.8 mg, 20.1 µL, 115 µmol, 3.80 eq.) were suspended in dimethylformamide (0.8 mL). The flask was attached to the tritium manifold (RC-TRITEC) and degassed by 3 freeze-pump-thaws. Tritium gas was introduced, and the black suspension was vigorously stirred for 20 hours under an atmosphere of tritium at an initial pressure of 600 mbar. The solution was cooled by liquid nitrogen and the excess tritium gas in the reaction vessel was reabsorbed on a uranium-trap for waste-tritium. The solvent was lyophilized off and labile tritium was removed by lyophilization with methanol (3 x 1.0 mL). The remaining black residue was suspended in ethanol (15 mL) and filtered over a 17 mm Titan HPLC filter (0.45 µm, PTFE). The crude product was concentrated, deprotected and purified by preparative HPLC (Gilson PLC2050, Sunfire C18 OBD, 5 µm, 10 x 250 mm, detection: UV at 254 nm) using 1% trifluoroacetic acid in water [A] acetonitrile [B] and 5% acetonitrile in water [C] as eluent (gradient: 10% [A], 20% [B], 70% [C] to 10% [A], 90% [B] in 12 min, then back to initial conditions for 3 min at a flow rate of 6 mL/min). The pure fractions were lyophilized and dissolved in ethanol (20 mL). 19.7 GBq (533 mCi) were obtained of the title compound with a radiochemical purity of 98% and a molar activity of 1150 GBq/mmol (31 Ci/mmol), determined by MS spectrometry. The compound was stored as an ethanolic solution. MS m/z: 225.2 [M+HCOO]⁻ (23%), 227.2 [M(³H)+HCOO]⁻ (46%), 229.1 [M(³H₂)+HCOO]⁻ (31%). The identity was confirmed by co-injection with a cold reference sample.

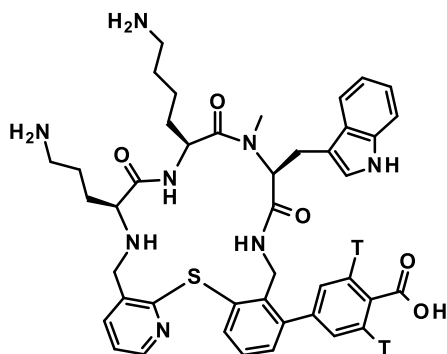
b) 4-(11S,14S,17S)-14-[4-(tert-Butoxycarbonylamino)butyl]-11-[3-(tert-butoxycarbonylamino)propyl]-17-(1H-indol-3-ylmethyl)-16-methyl-12,15,18-trioxo-2-thia-4,10,13,16,19-pentazatricyclo[19.4.0.0^{3,8}]pentacosa-1(21),3,5,7,22,24-hexaen-22-yl]-2,6-ditritio-benzoic acid



In a 1-mL reaction-vial (4-methoxycarbonyl-3,5-ditritio-phenyl)boronic acid (9.88 GBq (267 mCi), 1.58 mg, 8.6 µmol, 1.00 eq.), *tert*-butyl 3-(((7*R*,10*R*,13*R*)-17-bromo-10-(4-((*tert*-butoxycarbonyl)amino)butyl)-7-(3-((*tert*-

butoxycarbonyl)amino)propyl)-12-methyl-8,11,14-trioxo-5,6,7,8,9,10,11,12,13,14,15,16-dodecahydrobenzo[b]pyrido[3,2-p][1]thia[5,8,11,14]tetraazacycloheptadecin-13-yl)methyl)-1*H*-indole-1-carboxylate (9.03 mg, 2.15 μ mol, 1.00 eq.) and CPhos Pd G3 (1.73 mg, 2.15 μ mol, 0.25 eq.) were added to toluene (0.4 mL). Then a solution of potassium phosphate hydrate (3.11 mg, 13.5 μ mol, 1.57 eq.) in water (79.1 μ L) were added. Upon heating to 85 °C, the reaction mixture turned yellow and was stirred at that temperature for 18 h. The yellow emulsion was cooled down to room temperature, and toluene was removed under a stream of argon. To the remaining solid was added methanol (0.3 mL), water (47.5 μ L), and aqueous sodium hydroxide solution (32%) (8.0 μ L, 86 μ mol, 10 eq.). The yellow reaction mixture was heated to 55 °C, stirred for 4 hours, and cooled down to room temperature. The mixture was added to dichloromethane (20 mL) and water (20 mL). Under strong stirring aqueous citric acid (5%) (0.8 μ L) was slowly added to reach pH 5 (checked by pH paper). The layers were separated and the aqueous layer was washed with dichloromethane (20 mL). The combined organic phases were dried over sodium sulfate, filtered, and concentrated under reduced pressure at 40 °C. The product could not be separated by extraction, therefore, also the aqueous layer was concentrated. Both layers were dissolved in acetonitrile:water:trifluoroacetic acid 50:50:1 and purified by preparative HPLC (Gilson PLC2050, Sunfire C18 OBD, 5 μ m, 10 x 250 mm, detection: UV at 254 nm, using 1% trifluoroacetic acid in water[A] acetonitrile [B] and 5% acetonitrile in water [C] as eluent (gradient: 20% [A], 30% [B], 50% [C] to 20 % [A], 80 % [B] in 12 min, hold for 2 min, then back to initial conditions for 3 min at a flow rate of 6 mL/min). The pure fraction was lyophilized and dissolved in ethanol (15 mL). 1.80 GBq (48.7 mCi) (18% RCY) of the desired compound was obtained with a radiochemical purity of 99% and a molar activity of 1147 GBq/mmol (31 Ci/mmol), as determined by MS spectrometry. MS *m/z*: 991.5 [$M(^3H)+H$]⁺ (24%), 993.5 [$M(^3H)+H$]⁺ (44%), 995.5 [$M(^3H)_2+H$]⁺ (32%). The identity was confirmed by co-injection with a cold reference sample.

c) 4-[(11*S*,14*S*,17*S*)-14-(4-Aminobutyl)-11-(3-aminopropyl)-17-(1*H*-indol-3-ylmethyl)-16-methyl-12,15,18-trioxo-2-thia-4,10,13,16,19-pentazatricyclo[19.4.0.0^{3,8}]pentacosa-1(25),3(8),4,6,21,23-hexaen-22-yl]-2,6-ditritio-benzoic acid (³H]-RG6006)



4-(11*S*,14*S*,17*S*)-14-[4-(*tert*-Butoxycarbonylamino)butyl]-11-[3-(*tert*-butoxycarbonylamino)propyl]-17-(1*H*-indol-3-ylmethyl)-16-methyl-12,15,18-trioxo-2-thia-4,10,13,16,19-pentazatricyclo[19.4.0.0^{3,8}]pentacosa-1(21),3,5,7,22,24-hexaen-22-yl]-2,6-ditritio-benzoic acid (1590 MBq (43.0 mCi), 1.39 μ mol, 1.00 eq.) was dissolved in 1,4-dioxane (5.5 mL) and HCl in 1,4-dioxane (4 Molar) (347 μ L, 1.39 mmol, 1000 eq.) were added, and the clear solution was stirred at 40 °C for 5 h. The light turbid suspension was evaporated and dried under vacuum. The crude product was dissolved in water:acetonitrile:TFA 50:50:1 and purified by preparative HPLC (Gilson PLC2050, Sunfire C18 OBD, 5 μ m, 10 x 250 mm, detection: UV at 254 nm, using 1% trifluoroacetic acid in water[A] acetonitrile [B] and 5% acetonitrile in water [C] as eluent (start at 15% [A], 10% [B], 75% [C] for 1 min, then a gradient to 15 % [A], 50 % [B], 35% [C] in 14 min, to 15 % [A], 75 % [B], 10% [C] in 3 min, hold for 2 min, then back to initial conditions for 5 min at a flow rate of 6 mL/min). The product containing fraction was lyophilized and dissolved in ethanol/water (10 mL). 407 MBq (11.0 mCi) (26% RCY) of the desired compound as TFA salt was obtained with a radiochemical purity of 99% and a molar activity of 1151 GBq/mmol (31.1 Ci/mmol), as determined by MS spectrometry. MS *m/z*: 791.3 [$M(^3H)+H$]⁺ (23%), 793.3 [$M(^3H)+H$]⁺ (45%), 795.4 [$M(^3H)_2+H$]⁺ (32%). The identity was confirmed by co-injection with a cold reference sample.

Sequence of unique plasmids

Sequence of the plasmid pET22/42-LptC-thrombin-His₇

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Sequence of the plasmid pCDFduet-HisLptB-LptFG

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Sequence of the plasmid pTRAB-FLAGLptB-LptFG

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Sequence of the plasmid pCDFduet-LptB-LptF(E249K)G

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