SUPPORTING INFORMATION

Bio-orthogonal chemistry and reloadable biomaterial enable local activation of antibiotic prodrugs and enhance treatments against *Staphylococcus aureus* infections

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Materials and Methods

Vancomycin hydrochloride was purchased from Sigma-Aldrich (Woburn, MA), cat.# 1709007-4VL. All other chemicals were purchased from Krackeler Scientific and used without further purification. Chromatographic purifications were conducted using SiliaSphere™ spherical silica gel 5μm, 60 Å silica gel (Silicycle). Thin layer chromatography (TLC) was performed on SiliaPlate™ silica gel TLC plates (250 μm thickness) purchased from Silicycle. Preparative TLC was performed on SiliaPlate™ silica gel TLC plates (1000 μm thickness). HPLC purification was performed on Shimadzu LC-20 Instrument using Phenomenex Luna 5u C18(2) semi-preparative column (250 x 10 mm) using a gradient of 10-70% CH_3CN (0.01% formic acid) in H_2O (0.01% formic acid). Analytical HPLC was performed on Shimadzu LC-20 Instrument using Phenomenex Luna 5u C18(2) column (250 x 5 mm). COSTAR® Spin-X spin columns (0.22 μm Cellulose Acetate), purchased from Fisher Scientific (cat# 07-200-385), were used for the kinetic experiments. ^{1}H, ^{13}C and 2D NMR spectroscopy was performed on a Bruker 500 and 600 MHz NMR instruments. All ^{13}C NMR spectra were proton decoupled. LC-MS data was acquired using Agilent Technologies 6530 Q-TOF instrument. Ultrapure (UP) MVG sodium alginate, a medium-viscosity (>200 mPa s) sodium alginate where a minimum of 60% of the monomer units are guluronate, was purchased from ProNova BioPharma ASA (Lysaker, Norway).

Bacterial strains and growth conditions

The microcalorimetry experiments were carried out using standard laboratory strains: *S. aureus* methicillin resistant MRSA (ATCC 43300) and *S. aureus* methicillin sensitive MSSA (ATCC 29213). Stocks of each strain were maintained on cryovials (Roth, Karlsruhe, Germany) at -80°C. The strains were cultured on an aerobe blood agar with 5% sheep blood (Becton, Dickinson), and incubated at 37°C with ambient air for 24h. The inoculum was prepared by dilution of a 0.5 McFarland solution to the concentration of $2x10^5$ CFU/ml. When mixed with the incubation reaction of Tryptic Soy Broth (TSB) (Becton, Dickinson), alginate gel and vancomycin the final concentration of the inoculum was $1x10^5$ CFU/ml. This concentration was used for the microcalorimetry studies. The inoculum concentrations were determined using quantitative cultures.

Preparation of samples for microcalorimetry

Synthesis of tetrazine-modified alginate gel (**TAG**) has previously been described (*Acta Biomater.* **2014**,*10*(12), 5099-5105). **TAG** (600 μL) was crosslinked using a supersaturated solution of $CaSO_4$ (150 μL). The crosslinked **TAG** was placed between two glass plates for 30 min and circular pieces, weighing 25 mg, were cut out with a puncher. The **TAG** disks were placed in TSB (400 μL), at room temperature (23°C), without shaking, in the sterile calorimeter ampoules of 4 mL volume capacity. The **TAG** was treated with variable concentrations of antibiotics or pro-drugs, 2-10 [μg/mL], for 2.5 h. Subsequently, 400 μL of $2x10^5$ CFU/ml *S. aureus* inoculum was added. The reaction was mixed gently and the ampule was closed with a rubber cap and sealed by manual crimping. The incubation reaction without the gels was used as a comparison to standard protocol for microcalorimetry.

Susceptibility testing by microcalorimetry.

Ampoules were inserted into the 48-channel batch calorimeter (thermal activity monitor, model 3102 TAM III; TA Instruments, New Castle, DE, USA). The samples remained 15 min in the thermal equilibration position before lowering into the measurement position. Heat flow and total

heat produced by *S. aureus* at 37°C were measured for up to 24 h at 240 sec intervals. The heat was measured continuously and expressed as heat flow over time in microwatts [μW] and total heat expressed in Joules [J]. The peak heat flow is defined as the maximum heat flow during the experiment and the time to reach the peak heat flow is recorded. The total heat is the integration of the area below the heat flow-time curve. Data analysis was performed by employing the manufacturer's software (TAM Assistant; TA Instruments) and GraphPad Prism 6 software (GraphPad Software). The samples with medium, gels and bacteria but without antibiotics were used as a positive control. The unexpected contamination was tested by the samples with medium, gels and without antibiotic and bacteria.

Kinetic study of Vancomycin release from TAG

Crosslinked **TAG** (25 mg), prepared the same way as above was placed inside of amicon 3K spin columns and treated with 5 nmol of TCO-Vanco or TCO-Dapto in 400 μL of PBS. After 2 h, the supernatant fractions were collected centrifugation at 6,000 RPM and **TAG** was resuspended in fresh PBS (400 μL). The supernatants were collected after 4, 6, and 24 h to monitor continuous antibiotic release. After 24 h, the crosslinked **TAG** was treated with a second dose of 5 nmol TCO-Vanco or TCO-Dapto in 400 μL of PBS. The supernatant solutions were collected in analogous fashion 2, 4, 6 and 24 h post the second pro-drug treatment. Subsequently, a third dose of TCO-Vanco and TCO-Dapto was administered and the supernatant fractions were collected after 2, 4 and 24 h. The supernatant fractions from TCO-Vanco treatments were analyzed by HPLC, while the supernatant fractions from TCO-Dapto treatments were analyzed LC-MS. All kinetic experiments were carried out in duplicate.

Activity of the prodrugs against MRSA biofilm

MRSA biofilm was grown on the porous glass beads with the starting concentration of 10^5 CFU/mL. Each bead was incubated with MRSA in 1mL of Müller-Hinton cation adjusted medium (MHCA), (50 mg Ca_2^+/L) for 24 h at 37°C. Subsequently, the beads were washed with 0.9 % NaCl three times to remove the planktonic bacteria. The washed beads were immersed in **TAG** or **UG** and the gelation of the gel was done with 500 mM $CaCl_2$. After removal of $CaCl_2$ the beads were treated with 1mL MHCA containing TCO-Dapto. The incubation was done for 24 h at 37°C. The beads were separated from the gels and MHCA and washed 3 times with PBS. To quantify the amount of the biofilm bacteria that remained, the beads were sonicated for 30 seconds in 500 μL PBS. The sonication fluid (PBS with the detached bacteria) were plated on the agar plates and the colony forming units were counted the day after.

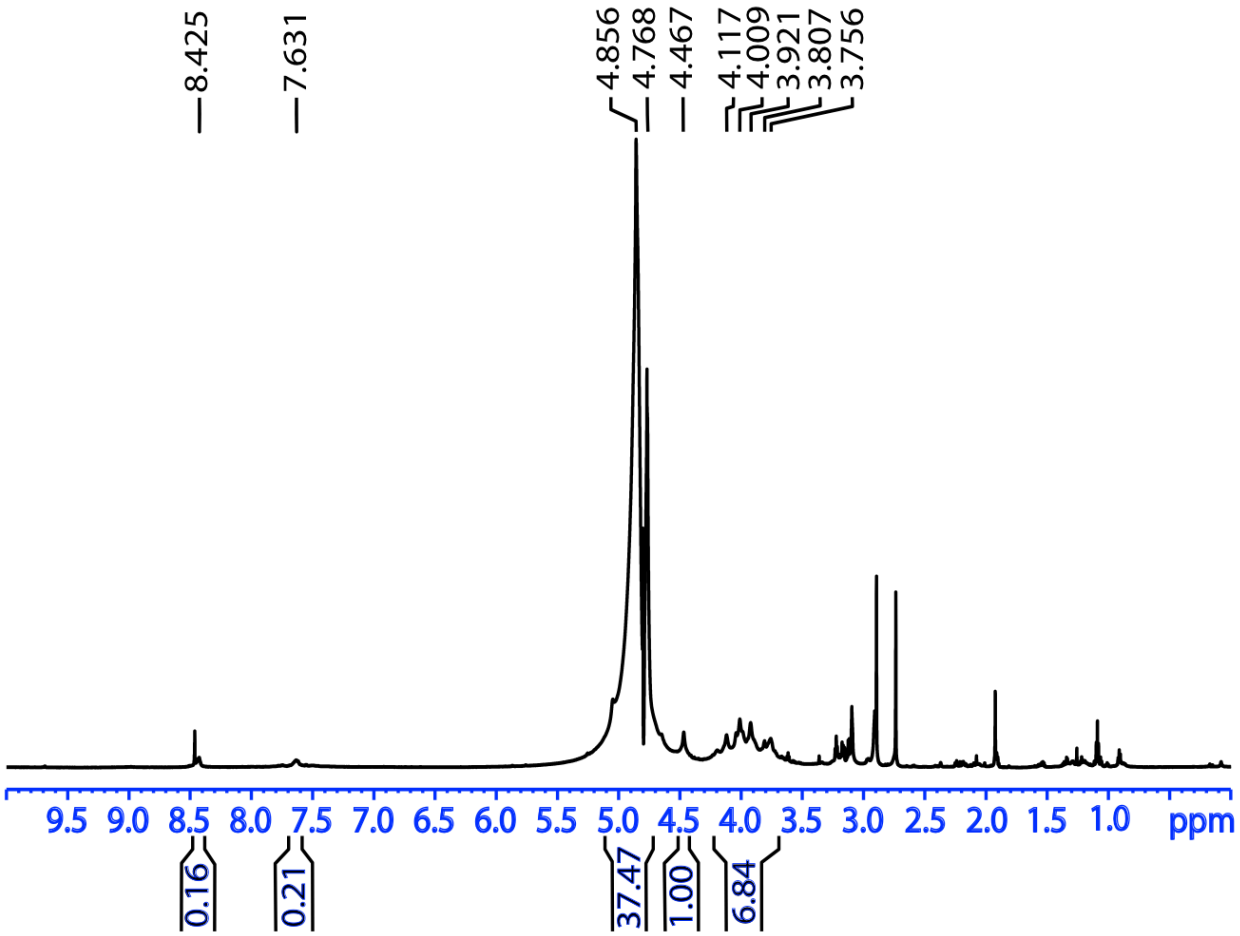


Figure S1. ^{1}H NMR Spectrum of **TAG**.

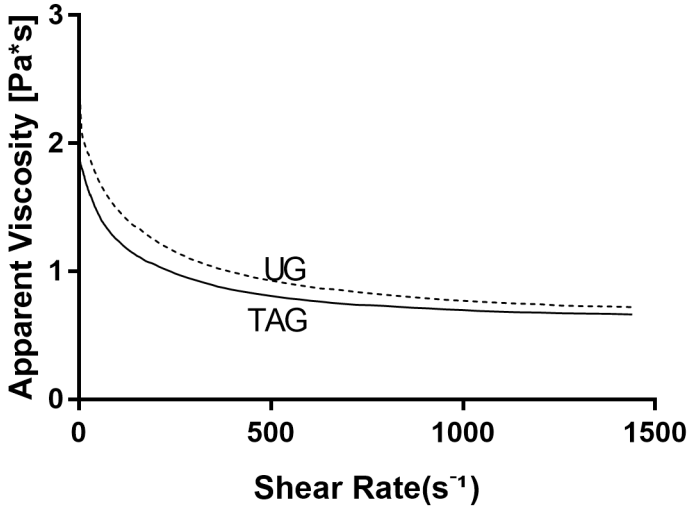


Figure S2. Zeta-potential of **TAG** and **UG** in DI water, adjusted to pH 7.0 at 25°C.

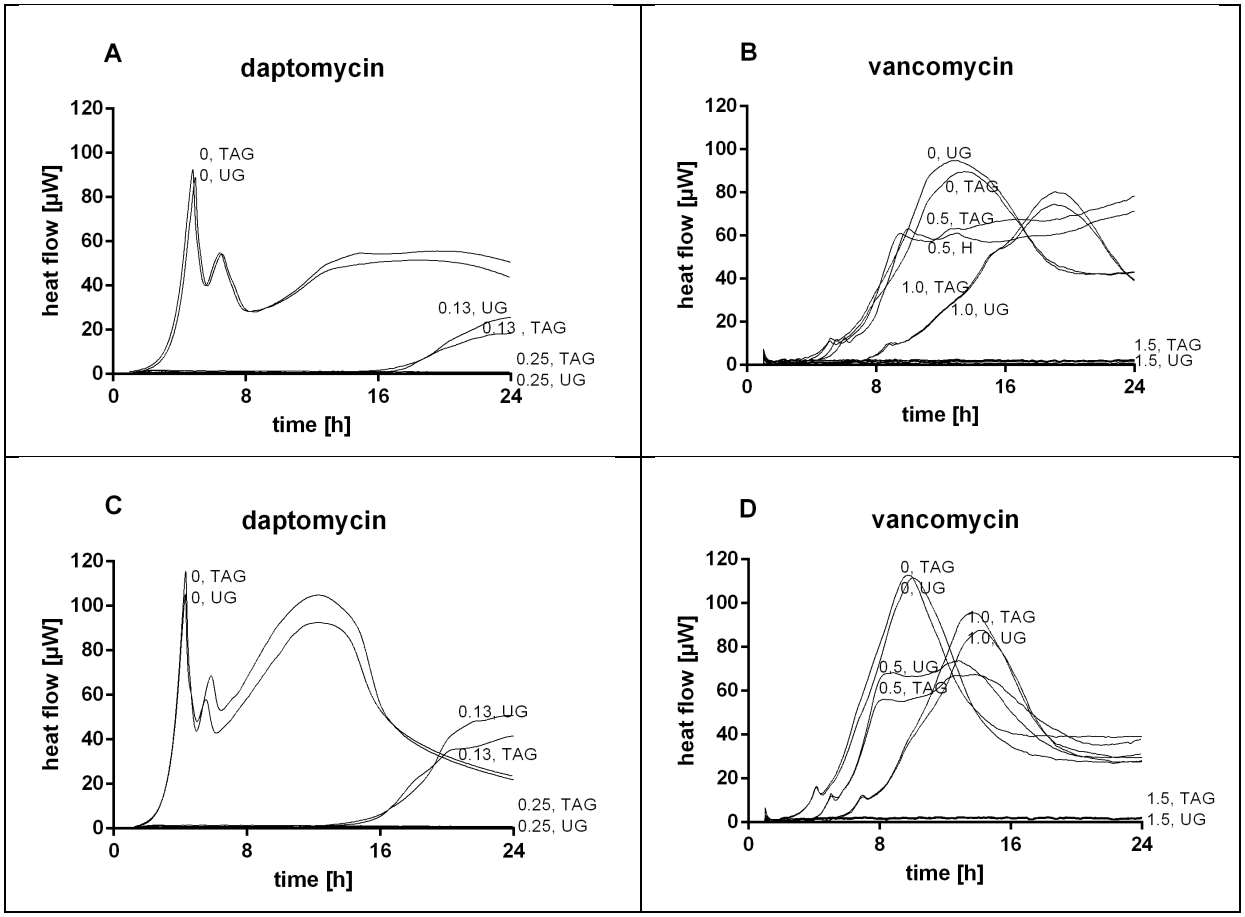


Figure S3. Heat flow measured for 24 h at 37 °C generated by planktonic bacteria. The observed MHIC values when planktonic bacteria was treated with vancomycin or daptomycin were the same in the presence of **TAG** or **UG**. Antibiotic concentrations are expressed in µg/mL. (A) MRSA treated with daptomycin with MHIC 0.25 µg/mL; (B) MRSA treated with vancomycin with MHIC 1.5 µg/mL; (C) MSSA treated with daptomycin with MHIC 0.25 µg/mL; (D) MSSA treated with vancomycin with MHIC 1.5 µg/mL for **TAG** and **UG**.

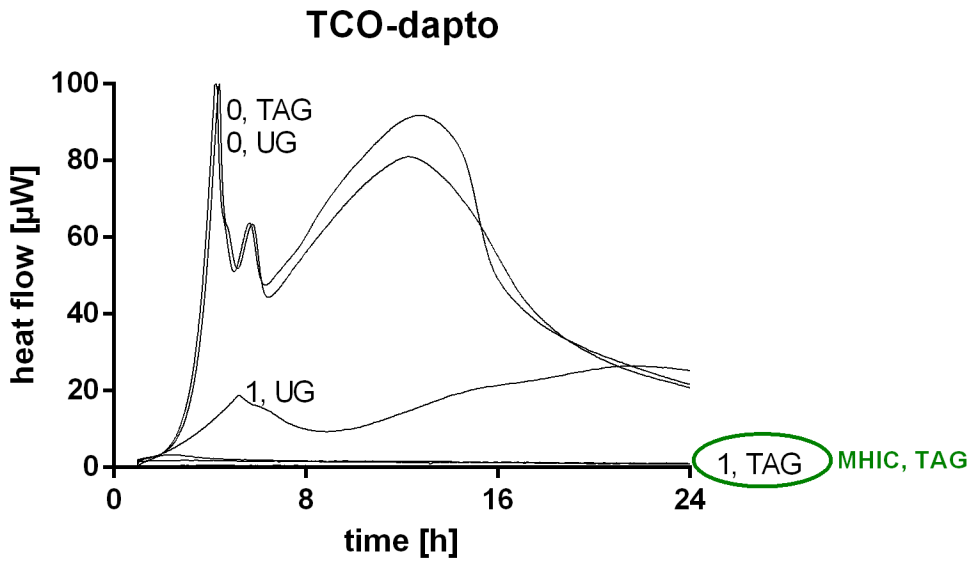


Figure S4. Heat flow measured for 24 h at 37 °C generated by MSSA treated with TCO-Dapto in the presence of either **TAG** or **UG**. The pro-drug's concentrations are expressed in µg/mL. The heat flow graphs illustrate a clear difference of heat generated by bacteria grown in the presence of 1 µg/mL of TCO-Dapto and **TAG** versus **UG**.

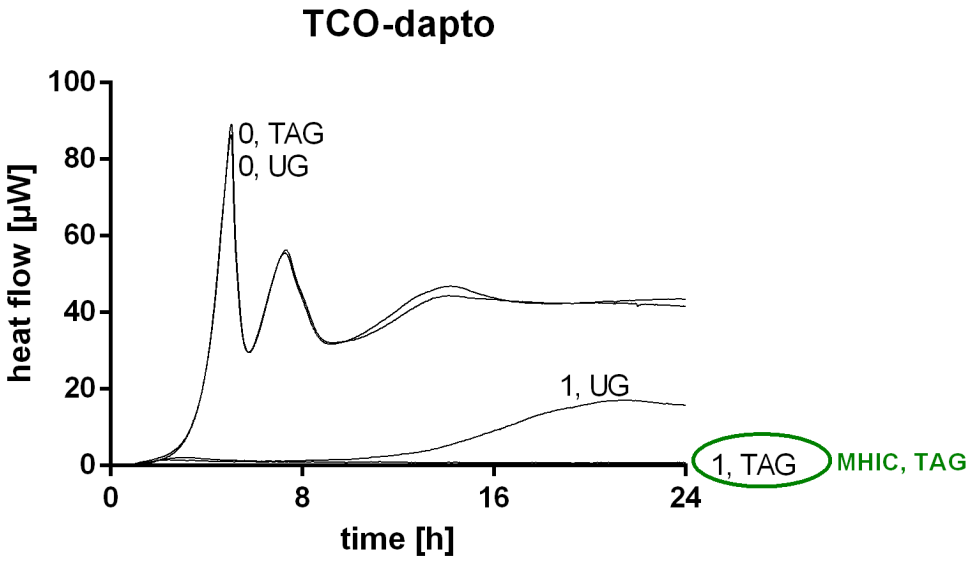


Figure S5. Heat flow measured for 24 h at 37 °C generated by MRSA treated with TCO-Dapto in the presence of either **TAG** or **UG**. The pro-drug's concentrations are expressed in µg/mL. The heat flow graphs illustrate a clear difference of heat generated by bacteria grown in the presence of 1 µg/mL of TCO-Dapto and **TAG** versus **UG**.

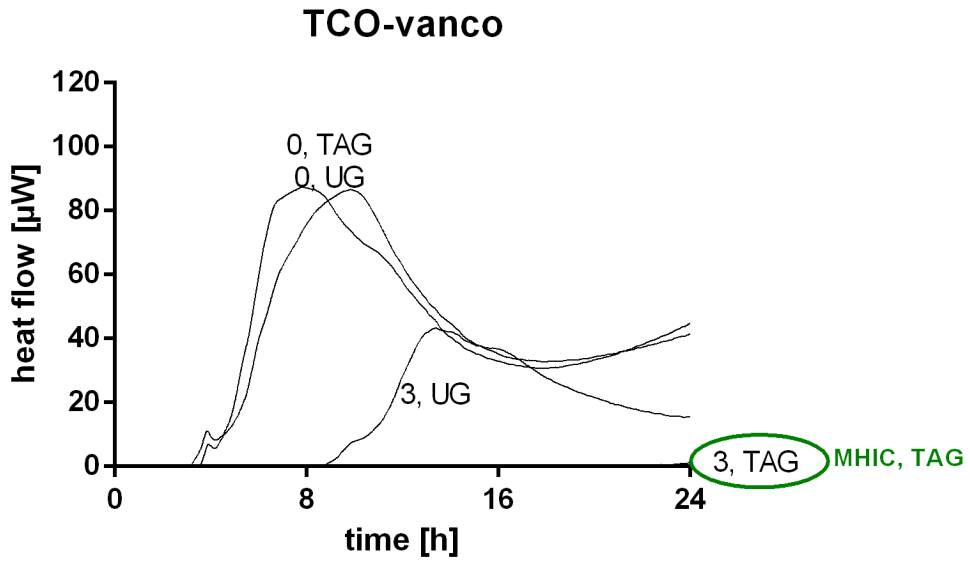


Figure S6. Heat flow measured for 24 h at 37 °C generated by MSSA treated with TCO-Vanco in the presence of either **TAG** or **UG**. The pro-drug's concentrations are expressed in µg/mL. The heat flow graphs illustrate a clear difference of heat generated by bacteria grown in the presence of 3 µg/mL of TCO-Vanco and **TAG** versus **UG**.

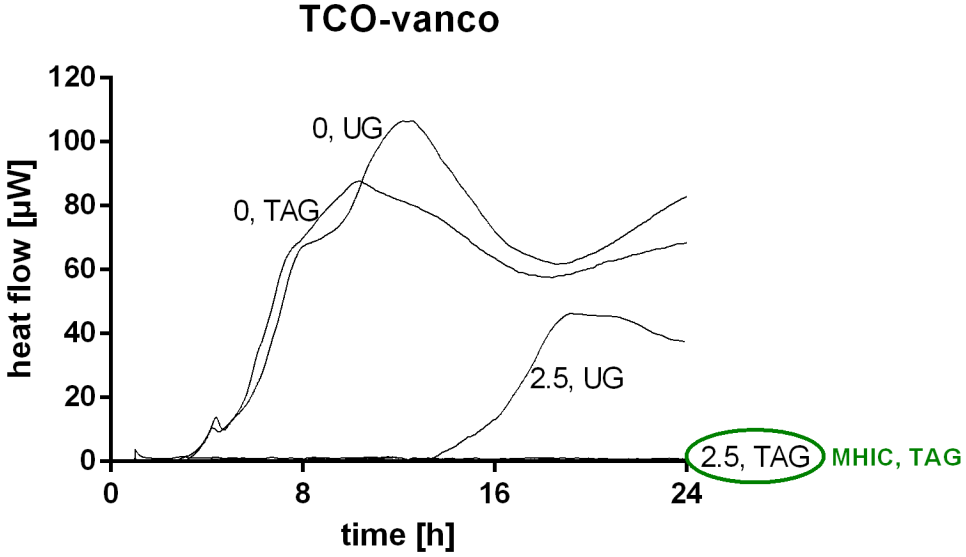


Figure S7. Heat flow measured for 24 h at 37 °C. generated by MRSA treated with TCO-Vanco in the presence of either **TAG** or **UG**. The pro-drug's concentrations are expressed in µg/mL. The heat flow graphs illustrate a clear difference of heat generated by bacteria grown in the presence of 2.5 µg/mL of TCO-Vanco and **TAG** versus **UG**.

Table S1. Thermogenic parameters of planktonic bacteria with and without TCO-Dapto.

		0 µg/ml		0.5 µg/ml		1.0 µg/ml		1.5 µg/ml		2.0 µg/ml		2.5 µg/ml		3.0 µg/ml	
		TAG	UG	TAG	UG	TAG	UG	TAG	UG	TAG	UG	TAG	UG	TAG	UG
MRSA	H [µW]	89.8± 2.8	89.1± 2.5	13.4 ± 15.3	56.2 ± 9.9	0	22.4 ± 17.2	0	10.6± 3,0	0	4.5 ± 3,6	0	0	0	0
	t_{max} [h]	5.0± 0.1	4.9± 0.1	23.2 ± 1.9	9.6± 7.2	0	23.0 ± 1.3	0	22.9 ± 1,3	0	9.6 ± 12,8	0	0	0	0
MSSA	H [µW]	101,7 ± 1,8	104,2 ± 1,2	16,1± 12,5	79,0 ± 1,9	0	26,1± 1,4	0	18,6± 5,7	0	10,6± 4,2	0	5,4 ± 2,4	0	0
	t_{max} [h]	4,3 ± 0,1	7,2 ± 4,9	23,7± 0,6	12,8± 7,1	0	21,1± 0,8	0	20,4± 3,0	0	20,2± 2,6	0	16,8 ± 12,6	0	0

Table S2. Thermogenic parameters of planktonic bacteria with and without TCO-Vanco

		0 µg/ml		2 µg/ml		2.5 µg/ml		3.0 µg/ml		3.5 µg/ml		4.0 µg/ml	
		TAG	UG	TAG	UG	TAG	UG	TAG	UG	TAG	UG	TAG	UG
MRSA	H [µW]	91.2 ± 6.4	103.5 ± 11.6	54.2 ± 65.6	62.3 ±18.9	0	45.2 ± 39.9	0	0	0	0	0	0
	t_{max} [h]	11.8 ± 2.2	11.5 ± 0.6	24 ± 0	16.8 ± 4.0	0	22.4 ± 2.8	0	0	0	0	0	0
MSSA	H (µW)	79,9 ± 11,5	90,9 ± 3,7	-	-	49,4 ± 3,0	53,9 ± 3,1	0	44,7 ± 3,1	0	28,6 ± 16,1	0	0
	t_{max} [h]	8,9 ± 1,5	9,9 ± 0,2	-	-	13,3 ± 0,9	11,3 ±1,1	0	14,2 ± 0,8	0	22,2 ± 2,1	0	0

The real-time microcalorimetric studies allowed to perform an in depth analysis of the antimicrobial activity of activated (**TAG**) vs. non-activated (**UG**) pro-drugs. This analysis is presented in Tables S1 and S2. The untreated bacteria (0 µg/ml column) produced the peak of the maximum heat flow (H 79.9-103.5). As expected the t_{max} values for these samples were the smallest (t_{max} 4.3-11.8 h). There no significant difference between the untreated **TAG** and **UG** samples, showing that the material alone does not eradicate the bacteria.

Increase of pro-drug dosage consistently resulted in a reduction of heat production and delay of t_{max} (lower H values and higher t_{max} values). The **TAG** samples consistently produced lower H values and higher t_{max} values than the **UG** samples. To highlight some key differences, MRSA treated with 0.5 µg/mL TCO-Dapto and **TAG** resulted in H value that is 4.2-times lower and t_{max} that is 13.6 h longer than the analogous treatment with **UG** (Table S1). Meanwhile, MRSA treated with 2 µg/mL TCO-Vanco and **TAG** showed t_{max} value that is 7.2 h longer than the analogous treatment with **UG** (Table S2). These data confirmed efficient *in vitro* activation of the antibiotic pro-drugs using **TAG**.

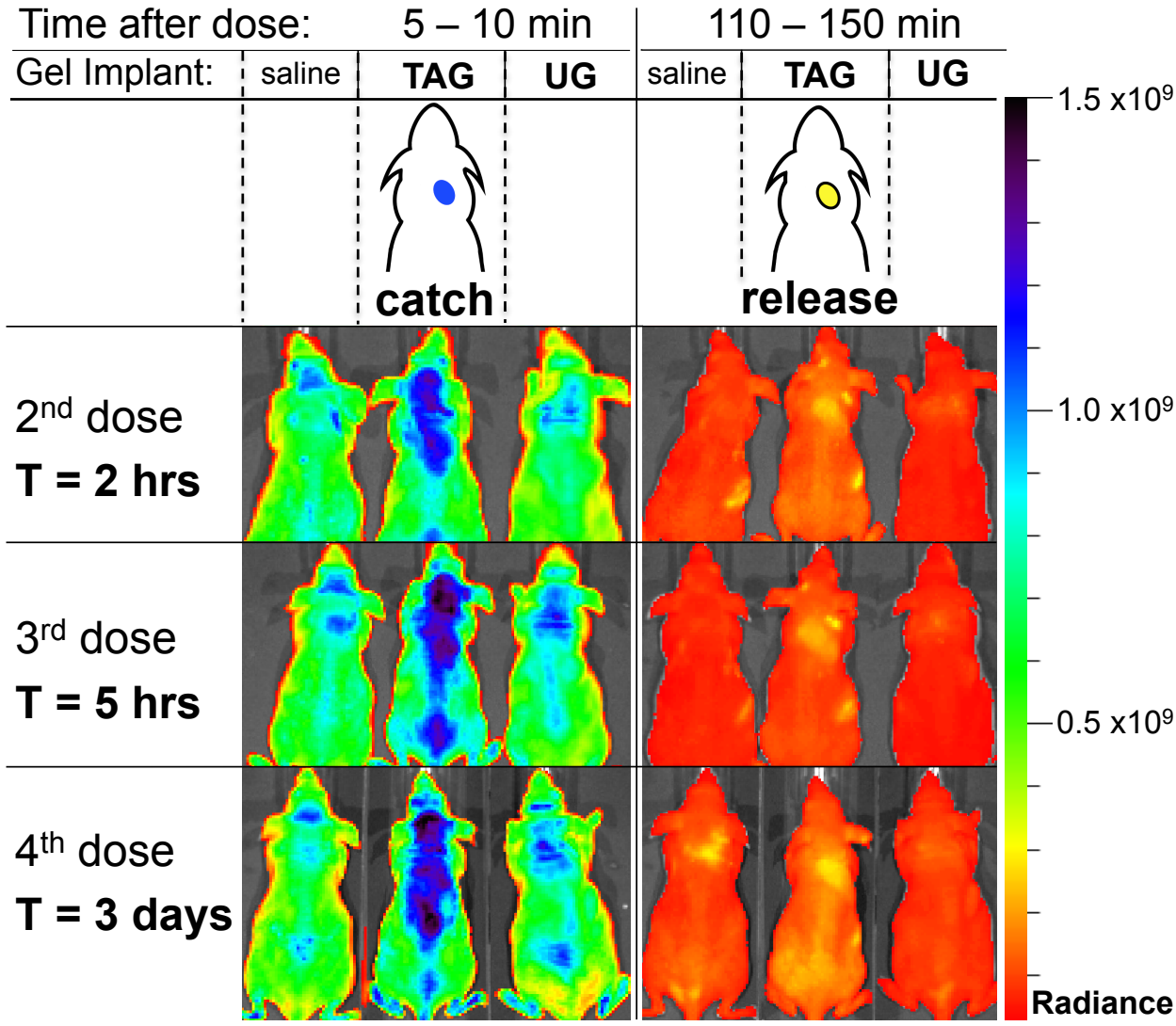


Figure S8. *In vivo* 'catch and release' of TCO-TAMRA. Three mice received 100 µL of a local injection in the dorsum of: saline (column 1), **TAG** (column 2), **UG** (column 3). Then, all mice received four doses of TCO-TAMRA, (100 nmoles) at times 0, 2 h, 5 h and 4 d. After each dose, the mice were imaged after 5-10 min and 110-150 min. These sample images confirm that the largest amount of fluorescence is observed at the dorsum of mouse injected with **TAG** and that the effect is transient, after release and diffusion of the fluorophore.

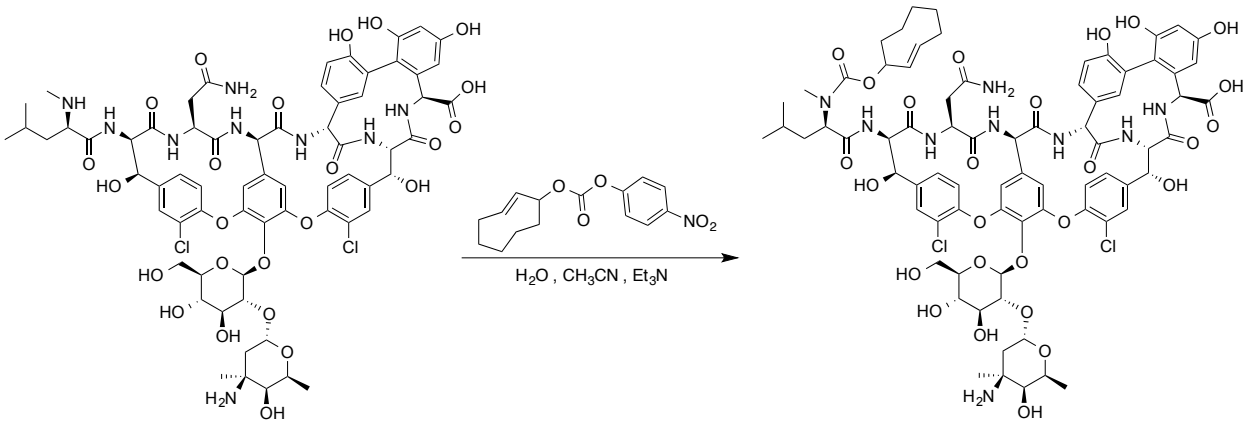


Figure S9. Synthesis of TCO-Vanco.

A solution of TCO-nitrophenylcarbonate (15 mg, 0.053 mmol) in CH_3CN (5 mL) was added dropwise over 2 h to the solution of vancomycin (100 mg, 0.067 mmol) in H_2O (5 mL), containing Et_3N (90 μL). The resulting reaction mixture was stirred at 40 °C for 18 h. After filtration, the title product was isolated by preparative HPLC using a gradient of CH_3CN (0.1% formic acid) in H_2O (0.1% formic acid).

HPLC conditions:

Solvent A: H_2O (0.1% formic acid); Solvent B: CH_3CN (0.1% formic acid); solvent flow: 4 mL/min

Time (min)	Solvent A (%)	Solvent B (%)
0	100	0
2	100	0
17	40	60
19	40	60
20	100	0
22	100	0

^{1}H NMR (500 MHz, DMSO) δ 8.64 (d, *J* = 16.8 Hz, 1H), 8.33 (s, 1H), 7.84 (d, *J* = 10.7 Hz, 1H), 7.65 – 7.44 (m, 3H), 7.33 (t, *J* = 8.3 Hz, 2H), 7.25 (t, *J* = 7.8 Hz, 1H), 7.13 (d, *J* = 12.1 Hz, 1H), 7.03 – 6.86 (m, 1H), 6.86 – 6.53 (m, 3H), 6.43 – 6.31 (m, 1H), 5.90 – 5.70 (m, 1H), 5.70 – 5.37 (m, 2H), 5.37 – 5.20 (m, 2H), 5.15 (dd, *J* = 25.7, 11.8 Hz, 2H), 4.86 (d, *J* = 39.2 Hz, 1H), 4.66 (d, *J* = 33.3 Hz, 1H), 4.55 – 4.35 (m, 1H), 4.13 (d, *J* = 40.1 Hz, 1H), 3.29 (s, 3H), 3.11 (s, 1H), 2.33 (d, *J* = 2.9 Hz, 2H), 2.01 – 1.84 (m, 2H), 1.81 – 1.58 (m, 3H), 1.59 – 1.34 (m, 2H), 1.27 (d, *J* = 29.7 Hz, 3H), 1.14 – 0.94 (m, 3H), 0.94 – 0.68 (m, 6H).

^{13}C NMR (126 MHz, DMSO) δ 174.81, 172.76, 171.29, 170.40, 169.68, 168.88, 167.92, 167.43, 165.72, 158.25, 156.61, 155.25, 152.68, 151.87, 149.26, 149.10, 148.75, 143.02, 140.34, 139.42, 137.93, 136.01, 135.42, 134.90, 132.45, 131.95, 131.07, 130.58, 129.21, 128.49, 127.71, 126.72, 124.75, 123.86, 121.59, 116.39, 114.53, 107.71, 105.19, 105.04, 102.19, 101.74, 97.31, 78.28, 78.00, 77.19, 71.57, 71.23, 70.70, 67.49, 63.66, 62.82, 62.33, 61.74, 58.68, 58.07, 55.42, 54.17, 53.81, 51.46, 49.07, 45.86, 41.53, 36.27, 35.79, 34.62, 34.21, 34.04, 31.26, 28.70, 24.04, 22.91, 22.70, 21.79, 17.33.

HRMS (ESI) *m/z*: calcd. for $C_{75}H_{88}Cl_2N_9O_{26}$ $[M+1]^+$ 1602.4660; found 1602.5219.

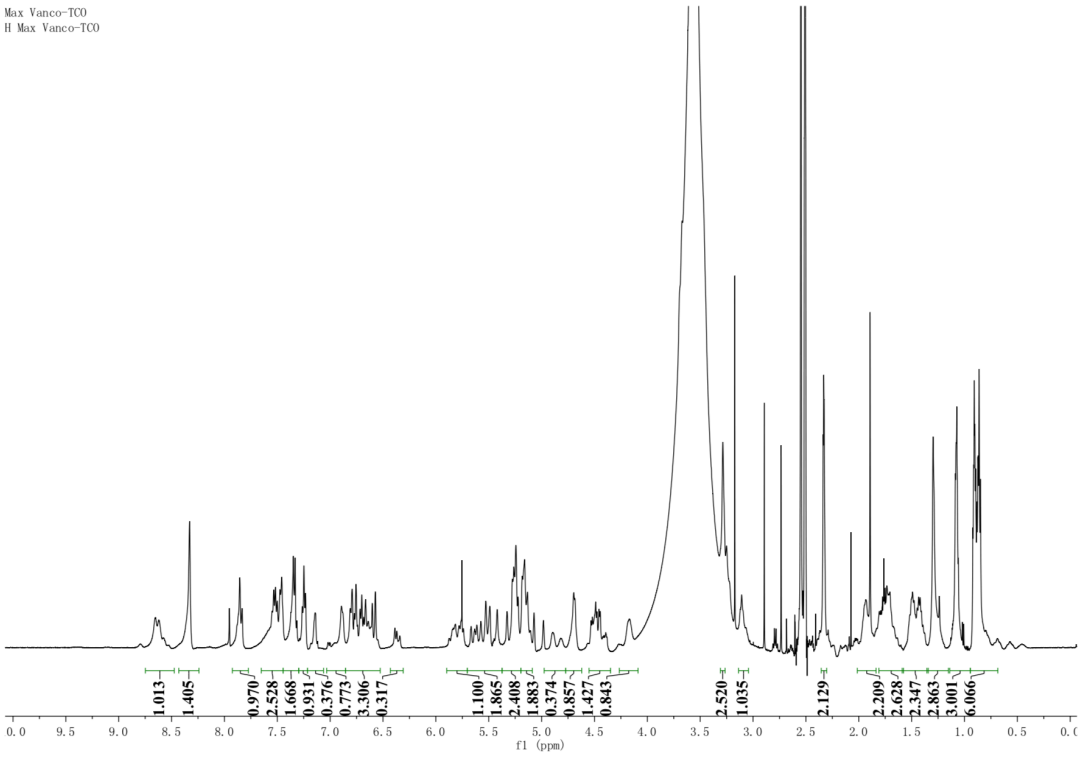


Figure S10. ^{1}H NMR spectrum of TCO-Vanco (500 MHz, DMSO) at 298 K.

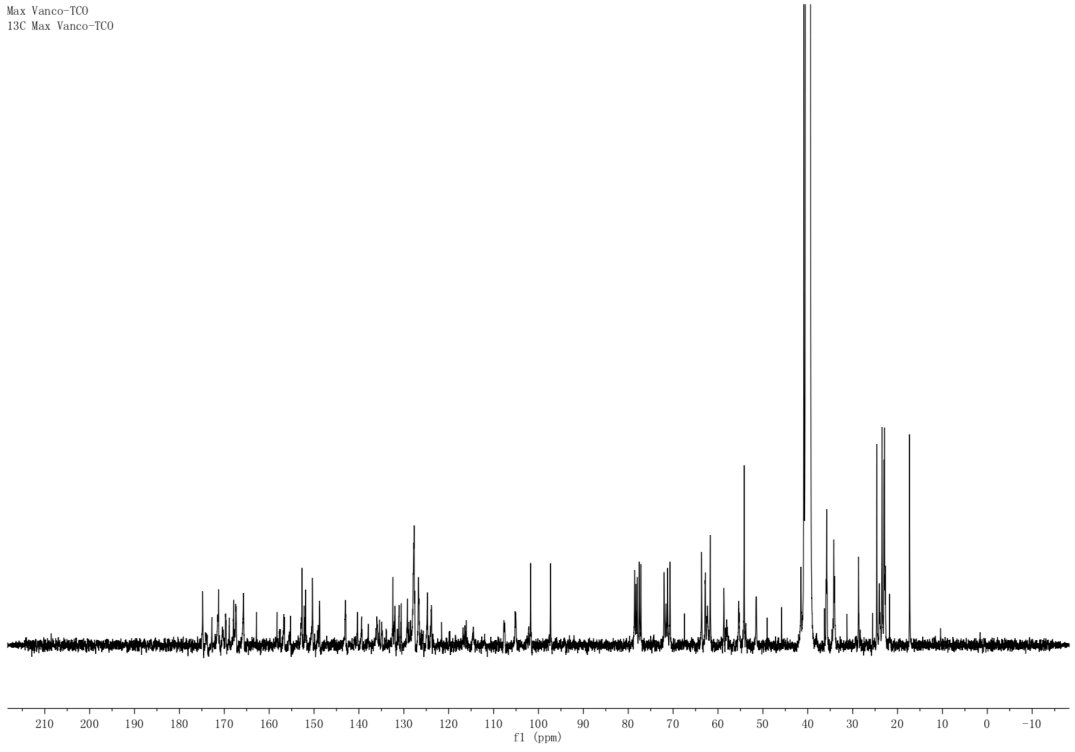


Figure S11. ^{13}C NMR spectrum of TCO-Vanco (500 MHz, DMSO) at 298 K.

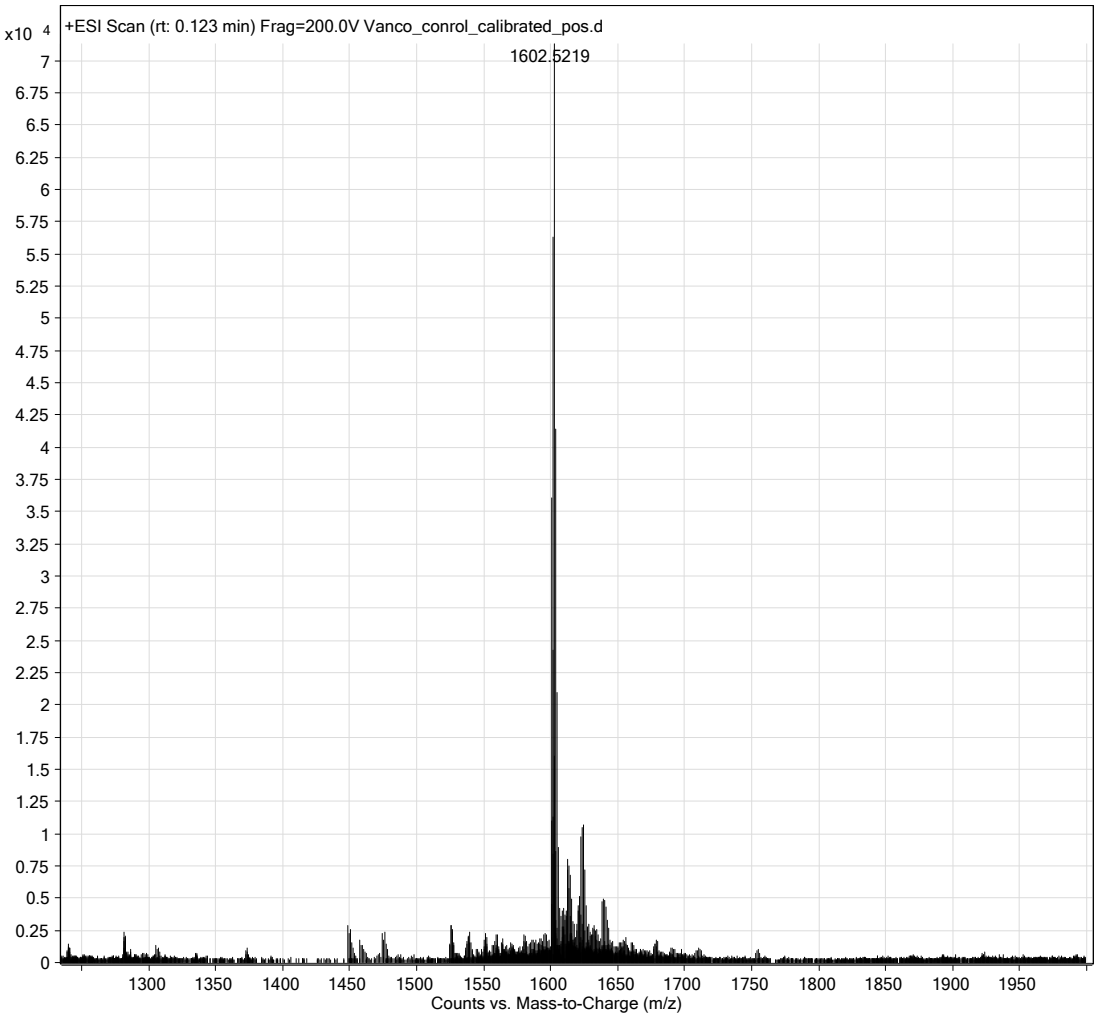


Figure S12. High resolution ESI-MS spectrum of TCO-Vanco.

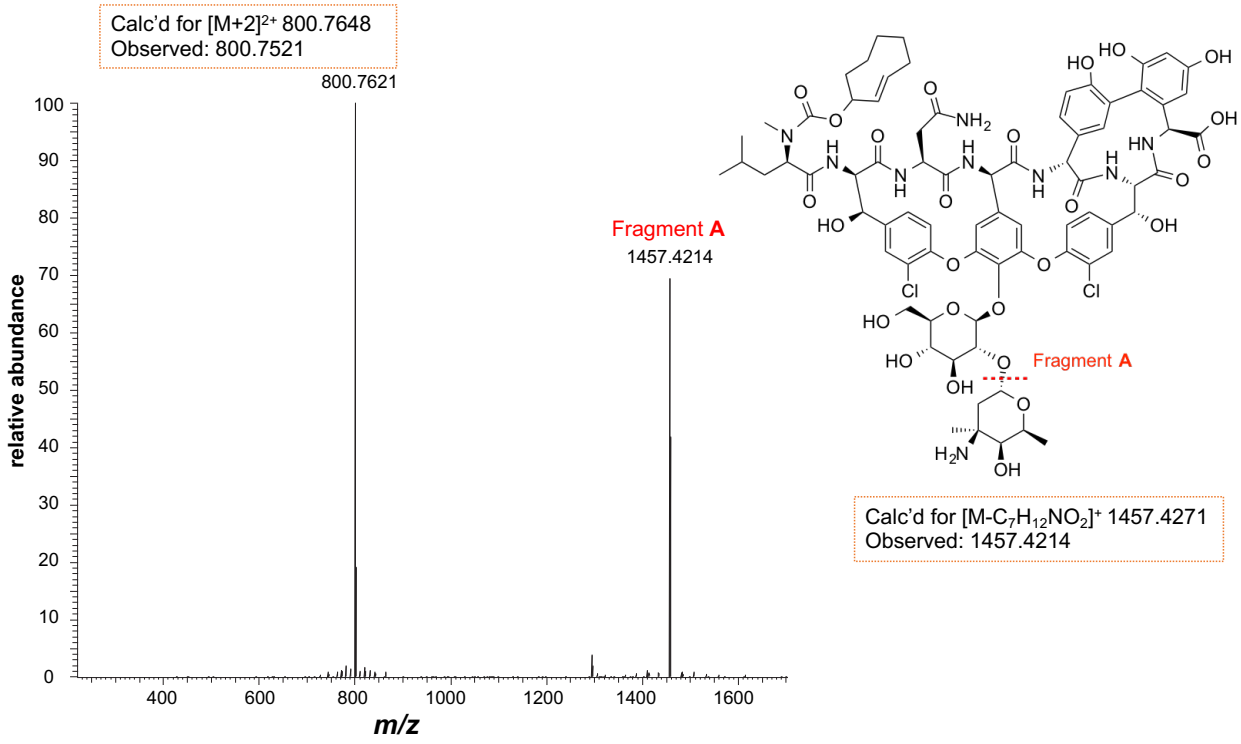


Figure S13. High resolution ESI-MS/MS spectrum of TCO-Vanco. Vancomycin contains two reactive amine groups: at the N-terminal amino acid and at the vancosamine. The Fragment **A** observed using ESI-MS/MS analysis suggests that TCO was coupled to the N-terminal amino acid of vancomycin.

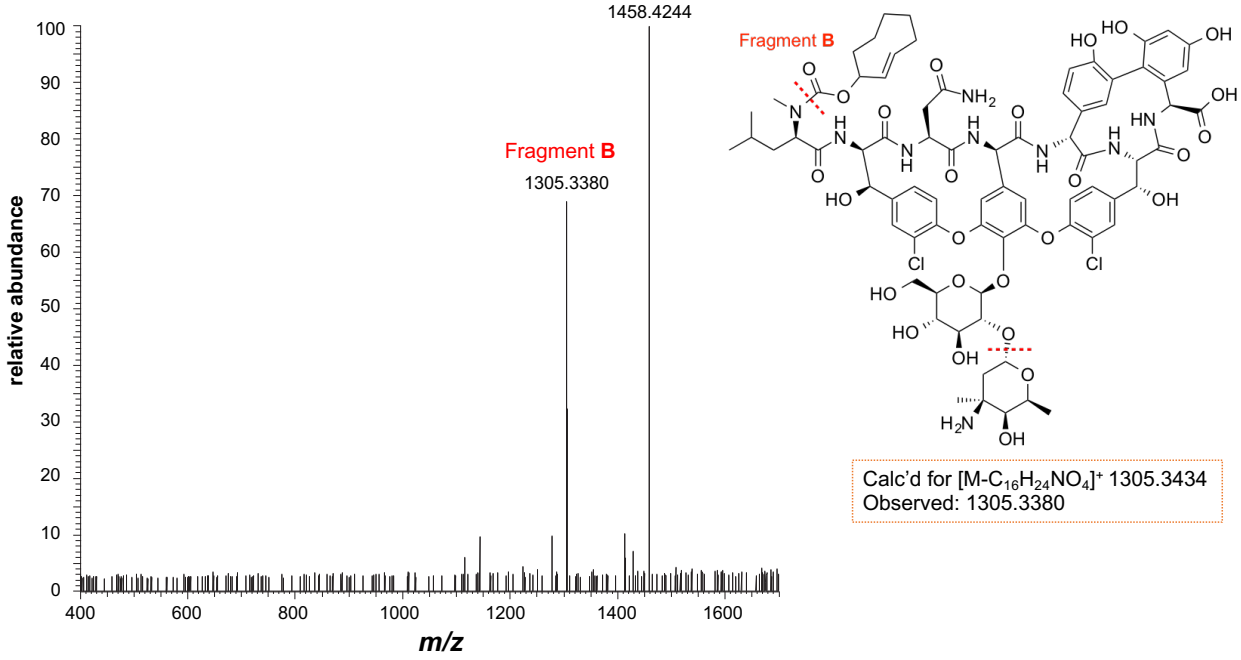


Figure S14. High resolution ESI-MS/MS/MS spectrum of TCO-Vanco, showing Fragment **B** upon further fragmentation of the Fragment **A** shown above.

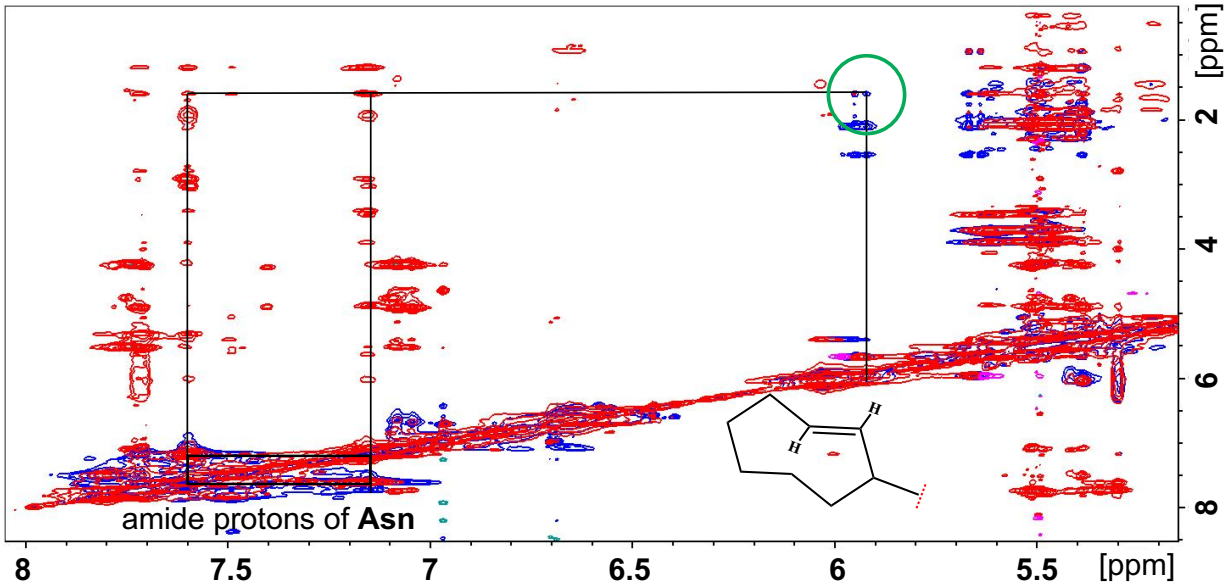
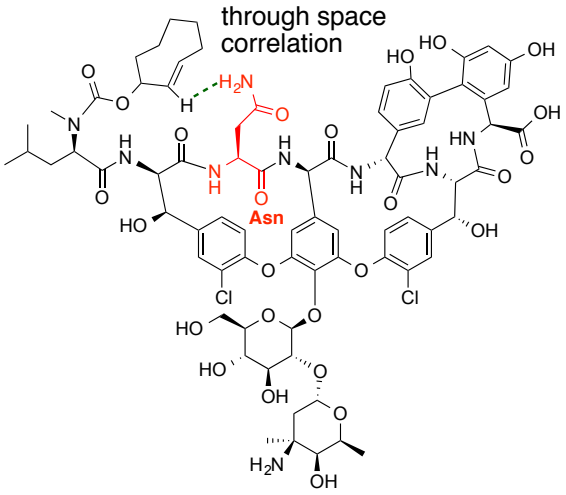


Figure S15. Overlay of TOCSY (red) and NOESY (blue) spectra of TCO-Vanco. The spectra was collected in DMSO-D_6 at 298 K.



Based on the previously reported spectra of vancomycin (*J. Chem. Soc. Perkin Trans. 2* **1995**, 153-157; *Biochemistry*, **1990**, *29*, 2271-2277), the diastereomeric amide peaks corresponding to Asn (red) have been assigned on the TOCSY spectrum that shows through bond correlation of protons 3 bonds apart. NOESY spectrum (through space correlation of protons within 5 Å) shows through space correlation (green circle) between the amide hydrogens of Asn and the alkene proton of the TCO.

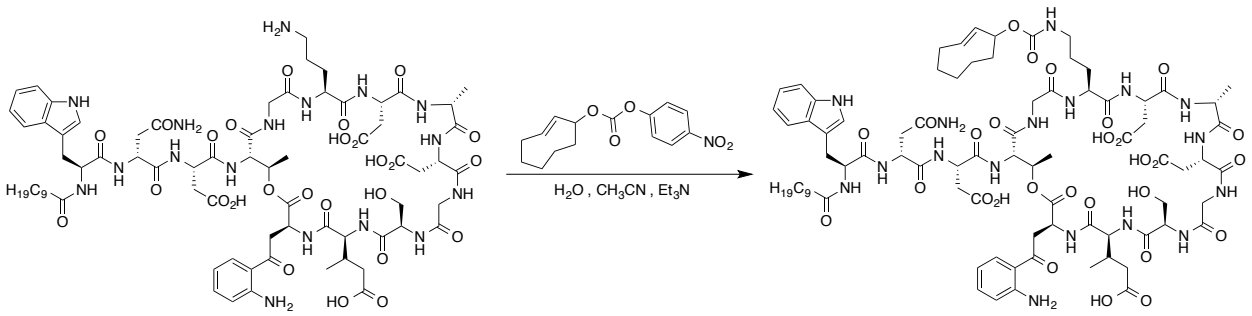


Figure S16. Synthesis of TCO-Dapto.

A solution of TCO-nitrophenylcarbonate (45 mg, 0.15 mmol) in CH_3CN (5 mL) was added dropwise over 2 h to the solution of daptomycin (100 mg, 0.062 mmol) in H_2O (10 mL), containing Et_3N (0.2 mL). The resulting reaction mixture was stirred at rt for 18 h. After filtration, the title product was isolated by preparative HPLC using a gradient of CH_3CN (0.1% formic acid) in H_2O (0.1% formic acid).

HPLC conditions:

Solvent A: H_2O (0.1% formic acid); Solvent B: CH_3CN (0.1% formic acid); solvent flow: 4 mL/min

Time (min)	Solvent A (%)	Solvent B (%)
0	90	10
2	90	10
17	10	90
19	10	90
20	90	10
22	90	10

^{1}H NMR (600 MHz, H_2O+D_2O) δ 10.10 (s, 1H), 8.55 (d, *J* = 6.9 Hz, 1H), 8.52 – 8.47 (m, 1H), 8.34 (ddd, *J* = 25.1, 13.3, 7.5 Hz, 3H), 8.22 (d, *J* = 6.8 Hz, 3H), 8.10 – 7.98 (m, 2H), 7.86 (s, 1H), 7.79 (d, *J* = 6.6 Hz, 1H), 7.69 (t, *J* = 9.3 Hz, 2H), 7.53 (d, *J* = 8.5 Hz, 1H), 7.41 (dd, *J* = 17.5, 9.2 Hz, 2H), 7.30 (s, 1H), 7.26 (t, *J* = 7.8 Hz, 1H), 7.16 (t, *J* = 7.7 Hz, 1H), 6.86 (d, *J* = 8.8 Hz, 1H), 6.80 (s, 1H), 6.76 – 6.67 (m, 2H), 5.91 – 5.81 (m, 1H), 5.67 (d, *J* = 17.3 Hz, 1H), 5.43 (d, *J* = 6.7 Hz, 1H), 5.26 (d, *J* = 31.4 Hz, 1H), 4.36 (s, 1H), 4.22 (s, 1H), 4.13 – 4.05 (m, 1H), 4.05 – 3.97 (m, 1H), 3.97 – 3.86 (m, 2H), 3.84 – 3.73 (m, 1H), 3.58 – 3.50 (m, 1H), 3.35 (dd, *J* = 14.8, 6.4 Hz, 1H), 3.22 (dd, *J* = 14.7, 8.2 Hz, 3H), 2.96 – 2.72 (m, 4H), 2.72 – 2.36 (m, 7H), 2.29 – 2.18 (m, 2H), 2.18 – 2.11 (m, 3H), 2.12 – 1.98 (m, 3H), 1.85 (dt, *J* = 31.4, 16.2 Hz, 4H), 1.74 – 1.51 (m, 3H), 1.48 – 1.40 (m, 5H), 1.28 (dd, *J* = 16.8, 12.2 Hz, 7H), 1.19 (s, 5H), 1.09 (dd, *J* = 15.1, 7.3 Hz, 3H), 0.99 (d, *J* = 7.0 Hz, 3H), 0.95 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (126 MHz, CH_3CN+D_2O) δ 200.13, 193.98, 193.69, 179.15, 176.03, 175.68, 175.05, 174.16, 173.80, 173.57, 173.04, 172.70, 172.12, 171.92, 171.64, 171.46, 171.31, 170.64, 157.71, 151.24, 136.71, 135.97, 132.21, 131.99, 131.76, 127.60, 124.48, 122.04, 116.95, 116.37, 112.07, 109.65, 74.62, 70.92, 62.03, 56.37, 55.50, 54.63, 50.87, 50.67, 50.42, 49.57, 48.61, 43.21, 42.80, 41.76, 40.53, 40.14, 38.41, 36.20, 36.05, 35.83, 35.58, 35.38, 33.56, 31.90, 29.37, 29.28, 28.97, 28.34, 27.29, 26.12, 25.57, 24.15, 22.73, 16.50, 15.19, 14.65, 13.93.

HRMS (ESI) *m/z*: calcd. for $C_{81}H_{112}N_{17}O_{28}$ $[M-1]^-$ 1771.7941; found 1771.7878.

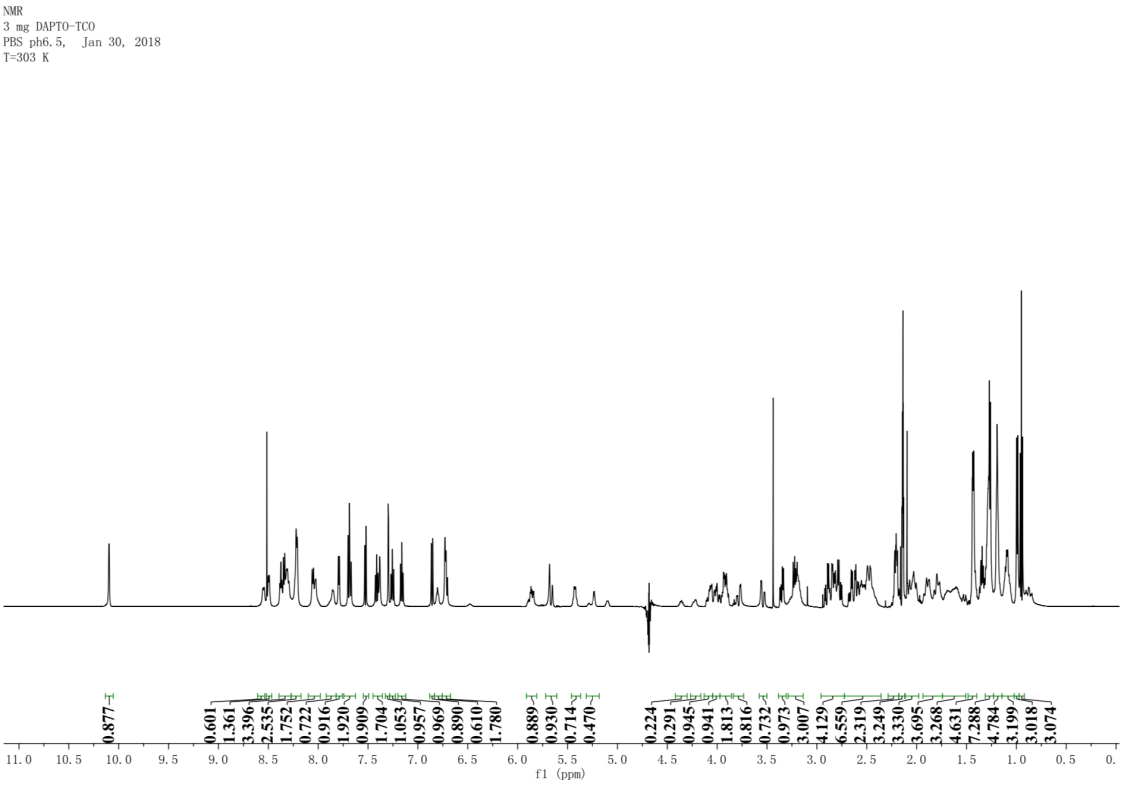


Figure S17. ^{1}H NMR spectrum of TCO-Dapto in phosphate buffer pH 6.5, 10% D_2O at 303 K (600 MHz).

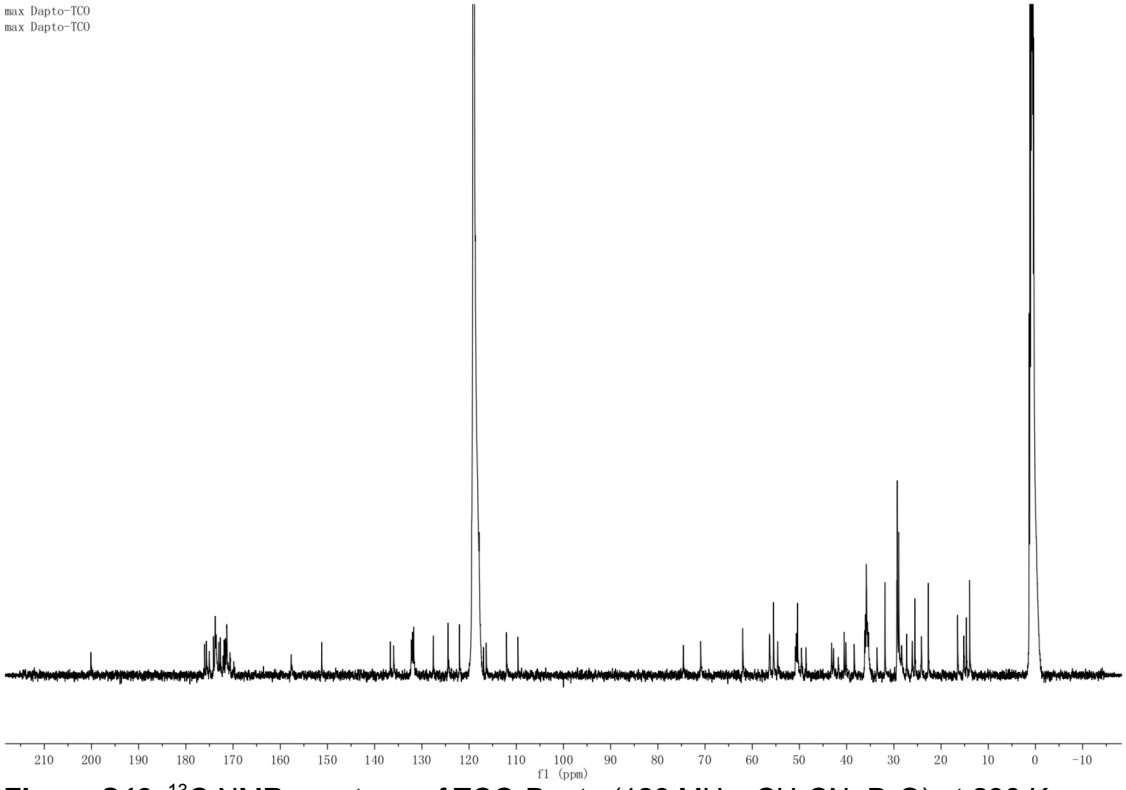


Figure S18. ^{13}C NMR spectrum of TCO-Dapto (126 MHz, CH_3CN+D_2O) at 298 K.

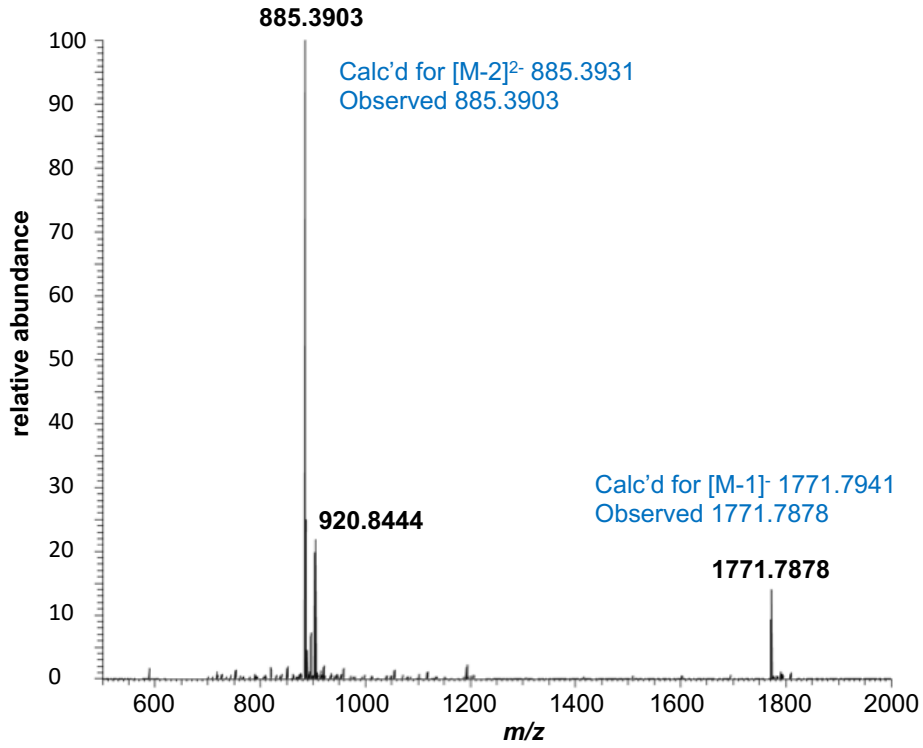


Figure S19. High resolution ESI-MS spectrum of TCO-Dapto.

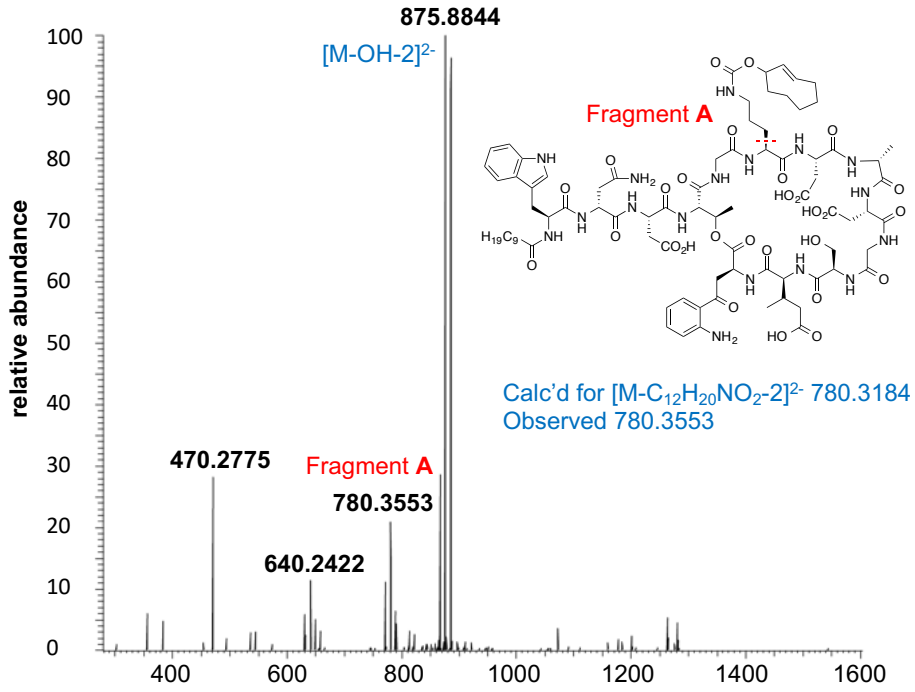


Figure S20. High resolution ESI-MS/MS spectrum of TCO-Dapto. The observed fragment proves that TCO was coupled to the Orn-residue of the cyclic peptide.

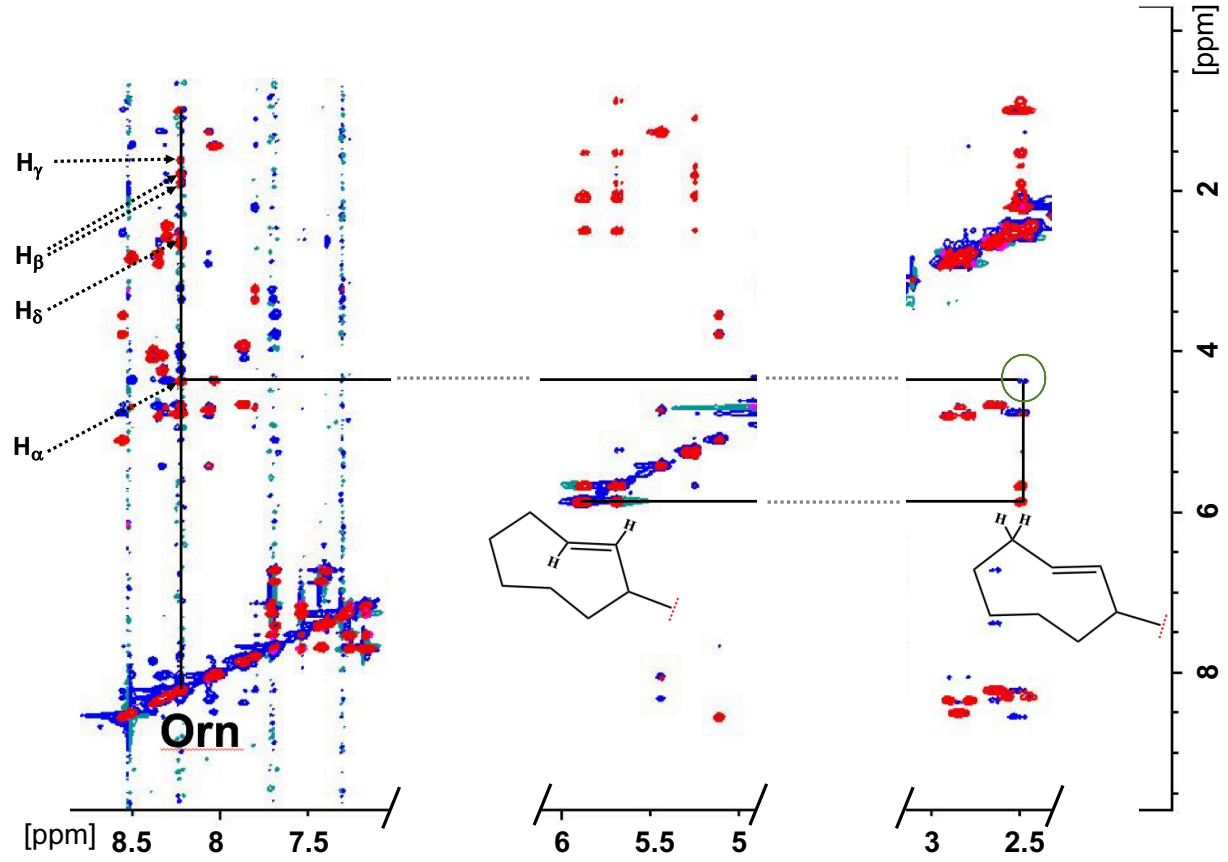
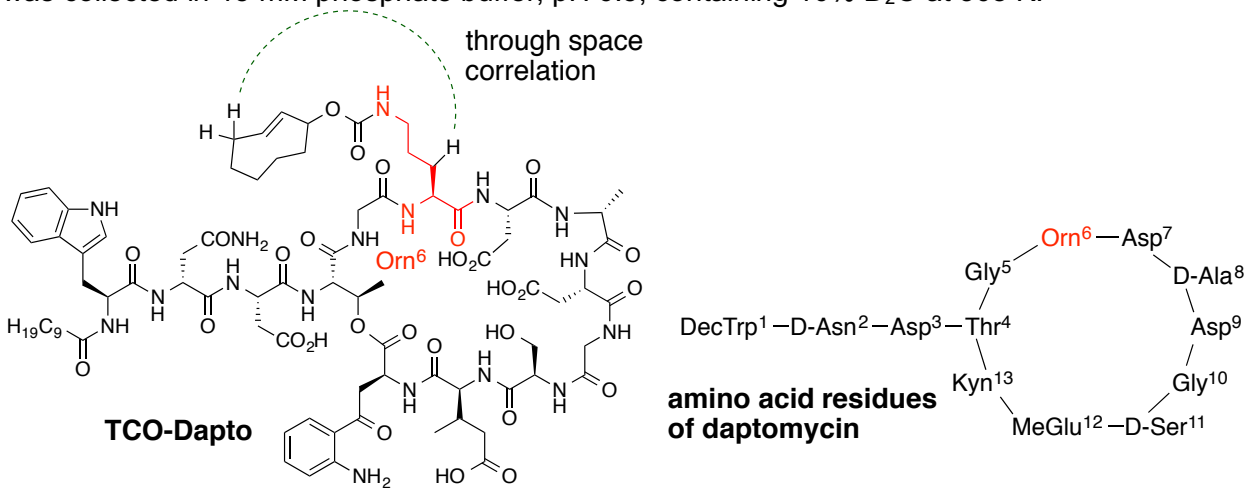


Figure S21. Overlay of TOCSY (red) and NOESY (blue) spectra of TCO-Dapto. The spectra was collected in 15 mM phosphate buffer, pH 6.5, containing 10% D_2O at 308 K.



Analysis of the 2-D NMR data: Previously reported NMR assignments for daptomycin (*Org. Biomol. Chem.* **2004**, *2*, 1872-1878) were utilized to assign the cross peaks corresponding to the **Orn$_6$** amide, as well as the **Orn$_6$** side-chain on the TOCSY spectrum, that shows through bond correlation of protons 3 bonds apart. The TOCSY spectrum also showed a through bond correlation between the alkene peak and the shown allylic proton. Meanwhile, NOESY spectrum (through space correlation of protons within 5 Å) showed a through space correlation (green circle) between the allylic proton and the **H$_\alpha$** of **Orn** sidechain.

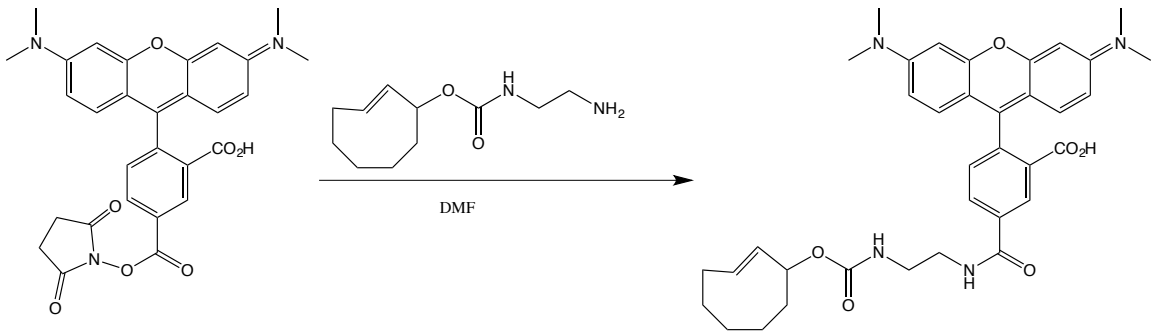


Figure S22. Synthesis of TCO-TAMRA.

The Rhodamine –NHS ester was synthesized as described by:
Brunet, A.; Aslam, T.; Bradley, M. *Bioorg. Med. Chem. Lett.* **2014**, *24*, 3186-3188.

Dissolved rhodamine-NHS ester (50 mg, 0.095 mmol) and (*E*)-cyclooct-2-enyl-2-aminoethylcarbamate (40.0 mg, 0.190 mmol) in CH_2Cl_2 (5 mL). Added triethylamine (129 μL, 0.95 mmol) and stirred at rt for 18 h. Evaporated the solvent under high vacuum and redissolved the reaction mixture in methanol. Purified by preparatory thin layer chromatography using 7.5:2.5:90 MeOH:Et_3N:CH_2Cl_2 mixture as mobile phase.
Yield = 28 mg (47 %)

^{1}H NMR (CD_3OD, 400 MHz) δ 8.54 (s, 1H), 8.04 (d, *J* = 8.2 Hz, 1H), 7.34 (d, *J* = 8.2 Hz, 1H), 7.23 (d, *J* = 9.6 Hz, 1H), 6.99 (dd, J_1 = 2.7 Hz, J_2 = 9.5 Hz, 1H), 6.89 (d, *J* = 2.8 Hz, 1H), 5.85 (t, *J* = 13.7 Hz, 1H), 5.55 (d, *J* = 16.4 Hz, 1H), 5.26 (s, 1H), 3.67-3.36 (m, 4H), 3.32-3.21 (m, 9H), 2.93-2.77 (m, 6H), 2.49-2.36 (m, 1H), 2.10-1.79 (m, 5H), 1.78-1.41 (m, 4H), 1.39-1.25 (m, 1H), 1.22-1.06 (m, 10H), 0.93-0.79 (m, 1H).
^{13}C NMR (CD_3OD, 100 MHz) δ 172.47, 169.41, 161.68, 159.08, 158.76, 142.06, 137.19, 132.96, 132.70, 130.90, 129.85, 129.63, 115.09, 114.86, 97.53, 76.85, 75.38, 41.96, 41.78, 41.01, 37.18, 36.92, 30.21, 25.35.
HRMS (ESI) *m/z*: calcd. for $C_{36}H_{41}N_4O_6$ $[M+1]^+$ 625.3026; found 625.2976.

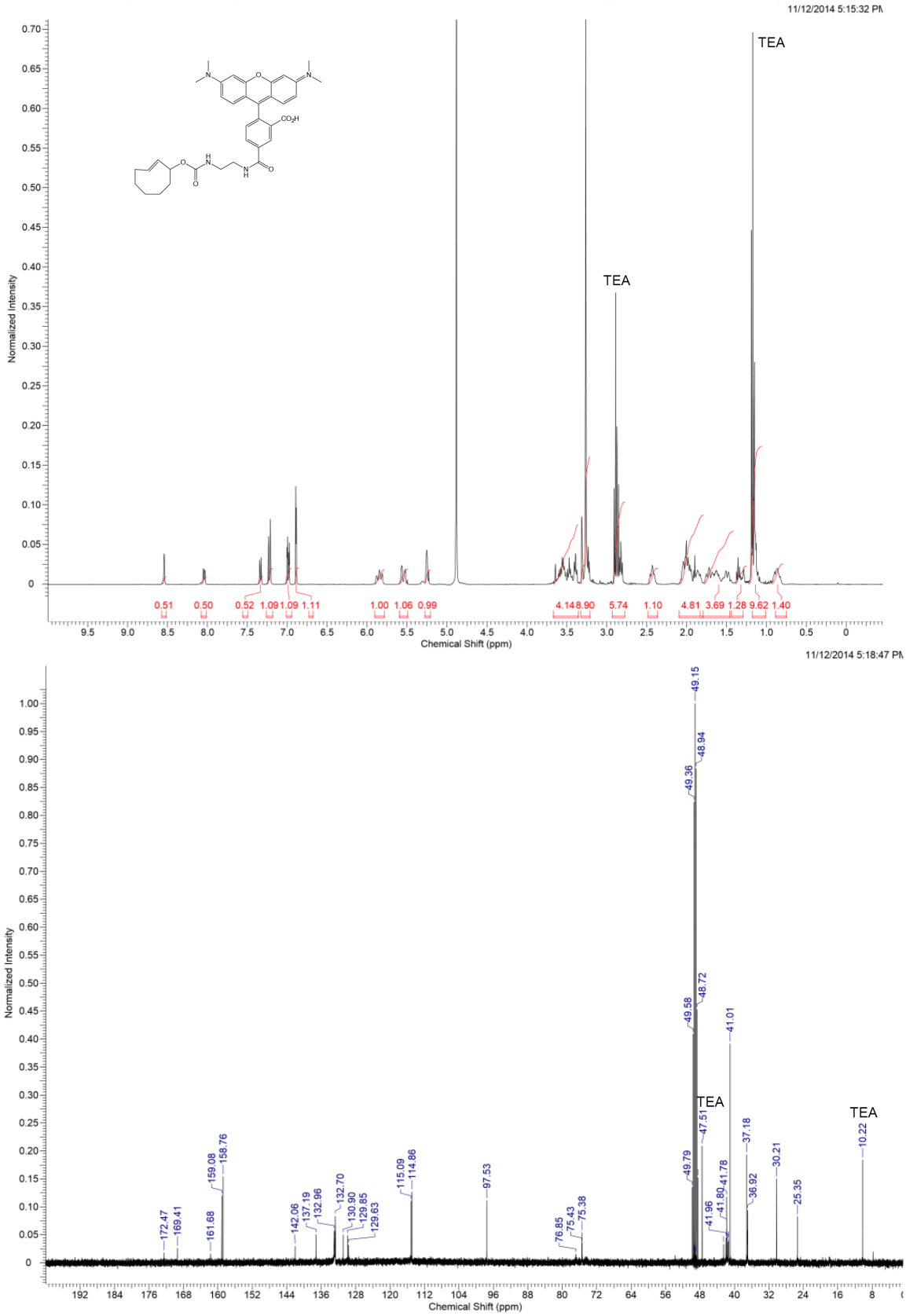


Figure S23. ^{1}H and ^{13}C NMR spectra of TCO-TAMRA.

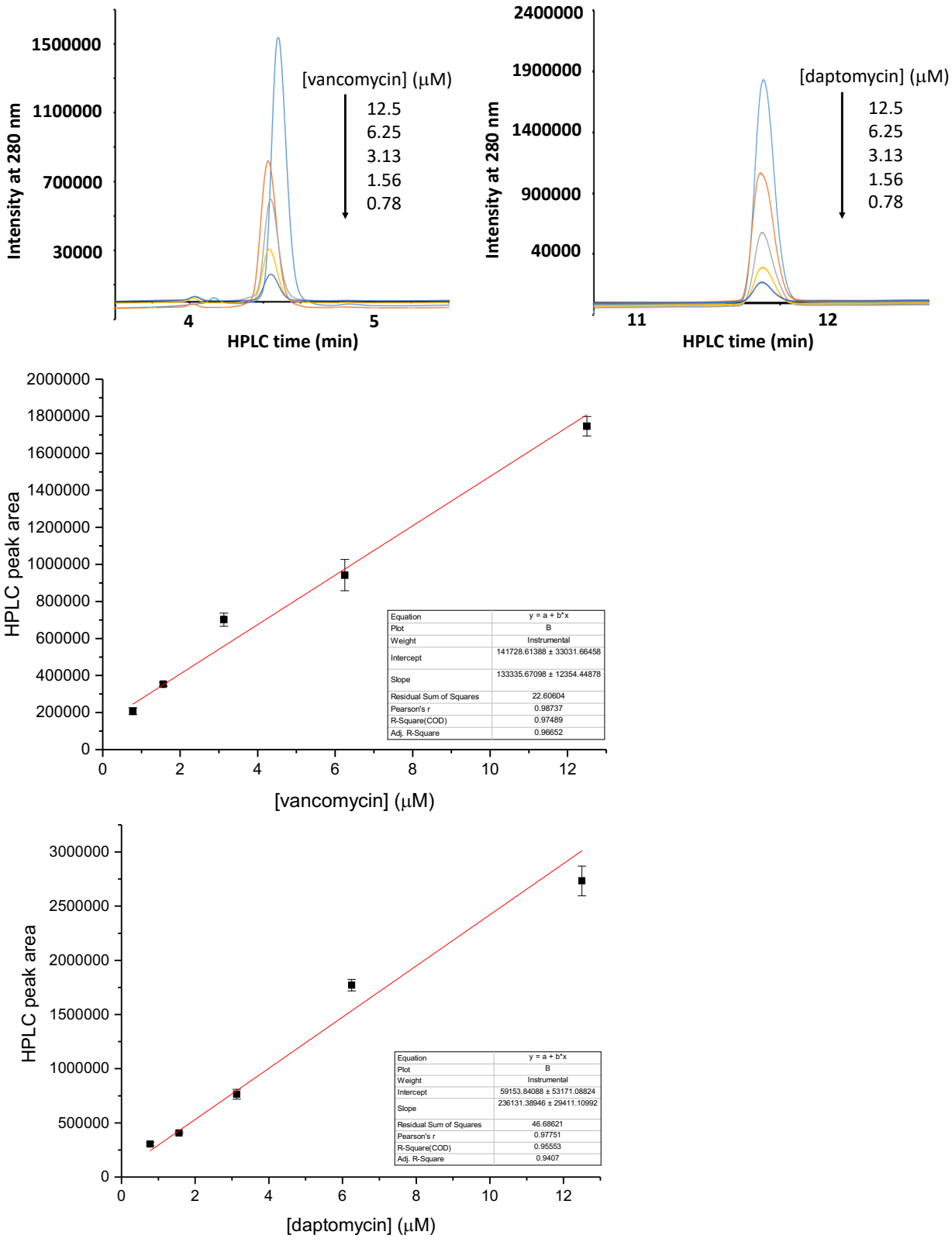


Figure S24. Calibration of the HPLC data for the kinetic studies shown in Figure 2.