Supporting Information

A facile and reproducible synthesis of NIR-fluorescent conjugates with small targeting molecules for microbial infection imaging

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General information

Starting materials, reagents and solvents were purchased from Li-Cor Biosciences, Invitrogen (Fisher Scientific), Sigma–Aldrich, Merck, and Combi-Blocks and were used as received.

High-resolution mass spectrometric measurements were performed using a Thermo scientific LTQ OrbitrapXL spectrometer with ESI ionization.

UPLC-MS measurements were performed on a ThermoFischer Scientific Vanquish UPLC System with a reversed phase C18 column (Acquity UPLC HSS T3 1.8 µm, 2.1 × 150 mm) in combination with an LCQ Fleet mass spectrometer and UV-Vis detector at λ = 300 nm (UV Vis 1), 360 nm (UV Vis 2), 500 nm (UV Vis 3), 680 nm (UV Vis 4). The eluents were acetonitrile and water with triethylammonium acetate (10 mM) and the elution gradient was established from 10% to 90% organic phase.

HPLC analyses were performed on Shimadzu equipment. The eluents were acetonitrile and water with triethylammonium acetate (10 mM). For reaction monitoring a XTerra MS C18 column (3.5 µm, 125 Å, 3.0 x 150 mm) in combination with a photo diode array detector (190 nm – 800 nm) was used with an elution gradient from 5% to 80% organic phase. For semi-preparative chromatography, a Kinetex EVO C18 column (5 µm, 100 Å, 250 x 10.0 mm) with UV detection at 365 nm was used and the elution gradients were established as described below.

UV/Vis absorption spectra were recorded on a JascoV-750 UV/Vis spectrophotometer with photomultiplier tube detection.

Fluorescence microscopy was performed with a Leica AF6000 microscope and the images processed using ImageJ software (National Institutes of Health) and LAS X Life Science.

Synthetic procedures

Vanco-FL-800CW

Vancomycin-BODIPY-FL (10 mg mL^{-1} in DMSO, 100 µL, 0.06 µmol), HBTU (8.8 mg mL^{-1} in DMSO, 1 µL, 0.02 µmol), DIPEA (7.5 mg mL^{-1} in DMSO, 1 µL, 0.06 µmol) and IRdye800CW-NHS ester (5 mg mL^{-1} in DMSO, 2.7 µL, 0.01 µmol) were mixed and left at room temperature overnight. Samples were taken and diluted with H_2O (ca. 1:100) to monitor the conversion by HPLC analysis with PDA detection and UPLC MS.

Ampho-800CW

Amphotericin B (5.2 mg, 5.6 µmol), HBTU (12.7 mg mL^{-1} in DMSO, 64 µL, 2.1 µmol), DIPEA (11.3 mg mL^{-1} in DMSO, 64 µL, 5.6 µmol) and IRdye800CW-NHS ester (5 mg mL^{-1} in DMSO, 100 µL, 0.4 µmol) were mixed and left at room temperature overnight. The mixture was diluted with H_2O and acetonitrile and the product purified by semi-preparative HPLC (elution gradient from 10% to 90% organic phase).

Vanco-700DX

Vancomycin hydrochloride (100 mg mL^{-1} in DMSO, 28.9 µL, 2.0 µmol), HBTU (25.5 mg mL^{-1} in DMSO, 11.4 µL, 0.8 µmol), DIPEA (22.5 mg mL^{-1} in DMSO, 11.4 µL, 2.0 µmol) and IRdye700DX-NHS ester (20 mg mL^{-1} in DMSO, 15 µL, 0.15 µmol) were mixed and left at room temperature overnight. Next, H_2O (0.36 mL) was added to the reaction mixture and the suspension was centrifuged for 10 min at rcf = 16.9x1000 g. The supernatant was centrifuged again, and the pellets re-dissolved in a mixture of DMSO, acetonitrile and H_2O for purification by semi-preparative HPLC (elution gradient from 10% to 70% organic phase).

IRdye800CW-carboxylic acid

H_2O (9 µL) and aq. NaOH (0.05 mM, 2 µL) were added to a solution of IRdye800CW-NHS ester in DMSO (5.0 mg mL^{-1}, 9 µL, 0.04 µmol). The mixture was left at room temperature for 2 h, followed by

addition of aq. HCl (0.05 mM, 2 µL). The hydrolysis of the NHS ester was followed by HPLC analysis with PDA detection.

Safety information

HBTU and analogous coupling reagents should be handled with appropriate personal protection equipment to prevent reactions of hypersensitivity towards these compounds as reported in literature.[1]

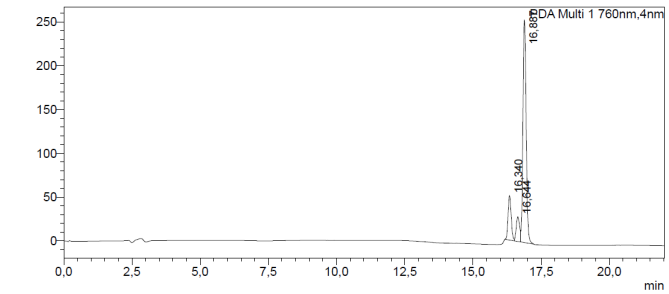
HPLC monitoring of the coupling reaction

Representative HPLC traces recorded at λ = 760 nm (Vanco-700DX: λ = 680 nm) of the corresponding reaction mixture containing the two substrates, DIPEA and HBTU in DMSO are shown below.

Vanco-800CW

Representative HPLC traces recorded at λ = 760 nm of the reaction mixture containing vancomycin hydrochloride, IRdye800CW NHS ester, DIPEA and HBTU in DMSO after indicated reaction times are shown below.

<Chromatogram>



<Peak Table>

PDA Ch1 760nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	16,340	402980	50638	0,000	mg/L		IRdye800CW
2	16,644	245611	28302	0,000	mg/L	V	Vancomycin800CW
3	16,887	2084065	254599	0,000	mg/L	V	IRdye800CW NHS ester
Total		2732656	333539				

Figure S 1. Chromatogram after 1 h reaction time.

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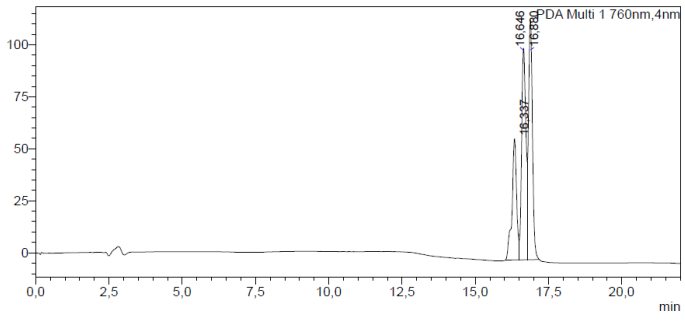


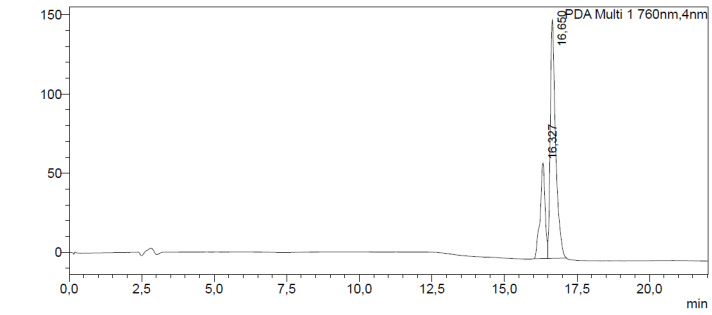
<Peak Table>

PDA Ch1 760nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	16,337	640295	58064	0,000	mg/L		IRdye800CW
2	16,646	1047709	101415	0,000	mg/L	V	Vancomycin800CW
3	16,880	1055888	114867	0,000	mg/L	V	IRdye800CW NHS ester
Total		2743892	274346				

Figure S 2. Chromatogram after 5 h reaction time.

<Chromatogram>



<Peak Table>

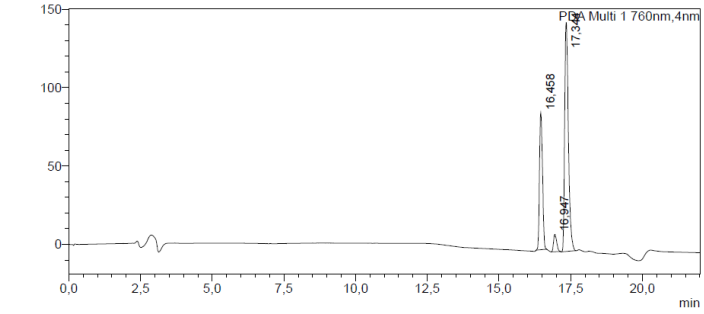
PDA Ch1 760nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	16,327	720154	60361	0,000	mg/L		IRdye800CW
2	16,650	1940354	150287	0,000	mg/L	V	Vancomycin800CW
Total		2660508	210648				

Figure S 3.Chromatogram after 24 h reaction time.

Vanco-FL-800CW

<Chromatogram>



<Peak Table>

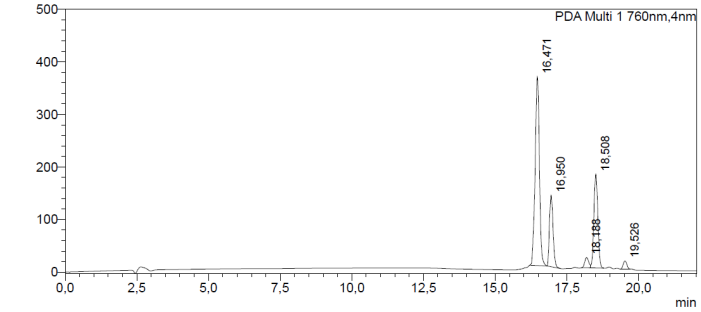
PDA Ch1 760nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	16,458	641332	87450	0,000	mg/L		IRdye800CW
2	16,947	84971	11094	0,000	mg/L		IRdye800CW NHS ester
3	17,344	1323083	146325	0,000	mg/L		Vanco-FL-800CW
Total		2049386	244868				

Figure S 4. Chromatogram of the reaction mixture containing vancomycin-BODIPY-FL, IRdye800CW NHS ester, DIPEA and HBTU in DMSO after 5 days reaction time.

Ampho-800CW

<Chromatogram>



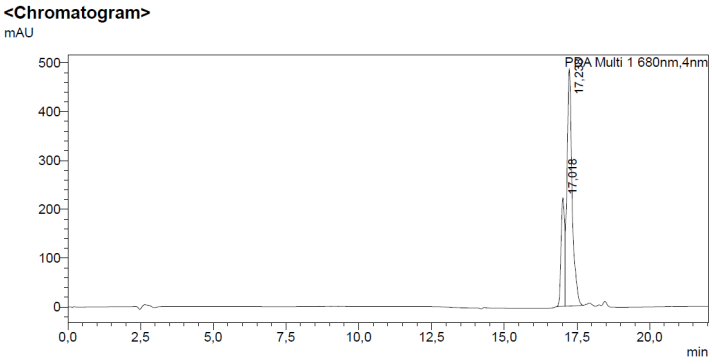
<Peak Table>

PDA Ch1 760nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	16,471	3709437	358909	0,000	mg/L		IRdye800CW
2	16,950	1198489	136162	0,000	mg/L		IRdye800CW NHS ester
3	18,188	188621	19526	0,000			
4	18,508	1723776	178390	0,000	mg/L	V	AmphotericinB800CW
5	19,526	134861	15326	0,000			
Total		6955185	708312				

Figure S 5. Chromatogram (recorded at 760 nm) of the reaction mixture containing IRdye800CW NHS ester, amphotericin B, HBTU and DIPEA in DMSO after 24 h reaction time.

Vanco-700DX

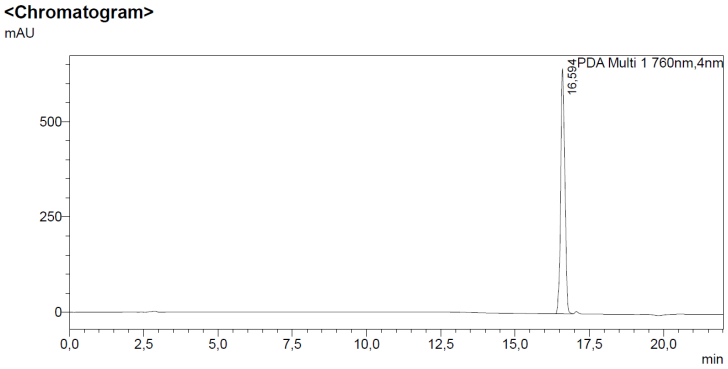


<Peak Table>
PDA Ch1 680nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	17,018	1938082	222249	24,692			
2	17,238	5911051	486812	75,308		V	
Total		7849133	709061				

Figure S 6. Chromatogram (recorded at 680 nm) of the reaction mixture containing IRdye700DX NHS ester, vancomycin hydrochloride, HBTU and DIPEA in DMSO after 24 h reaction time.

HPLC monitoring of hydrolysis of IRdye800CW-NHS ester



<Peak Table>
PDA Ch1 760nm

Peak#	Ret. Time	Area	Height	Conc.	Unit	Mark	Name
1	16,594	6279618	642129	0,000	mg/L		IRdye800CW
Total		6279618	642129				

Figure S 7. Chromatogram after 2h shows full hydrolysis of IRdye800CW-NHS ester to IRdye800CW-carboxylic acid. The chromatogram was recorded at 760 nm after neutralization with aq. dil. HCl.

UPLC-MS analysis of the conjugates

Vanco-800CW

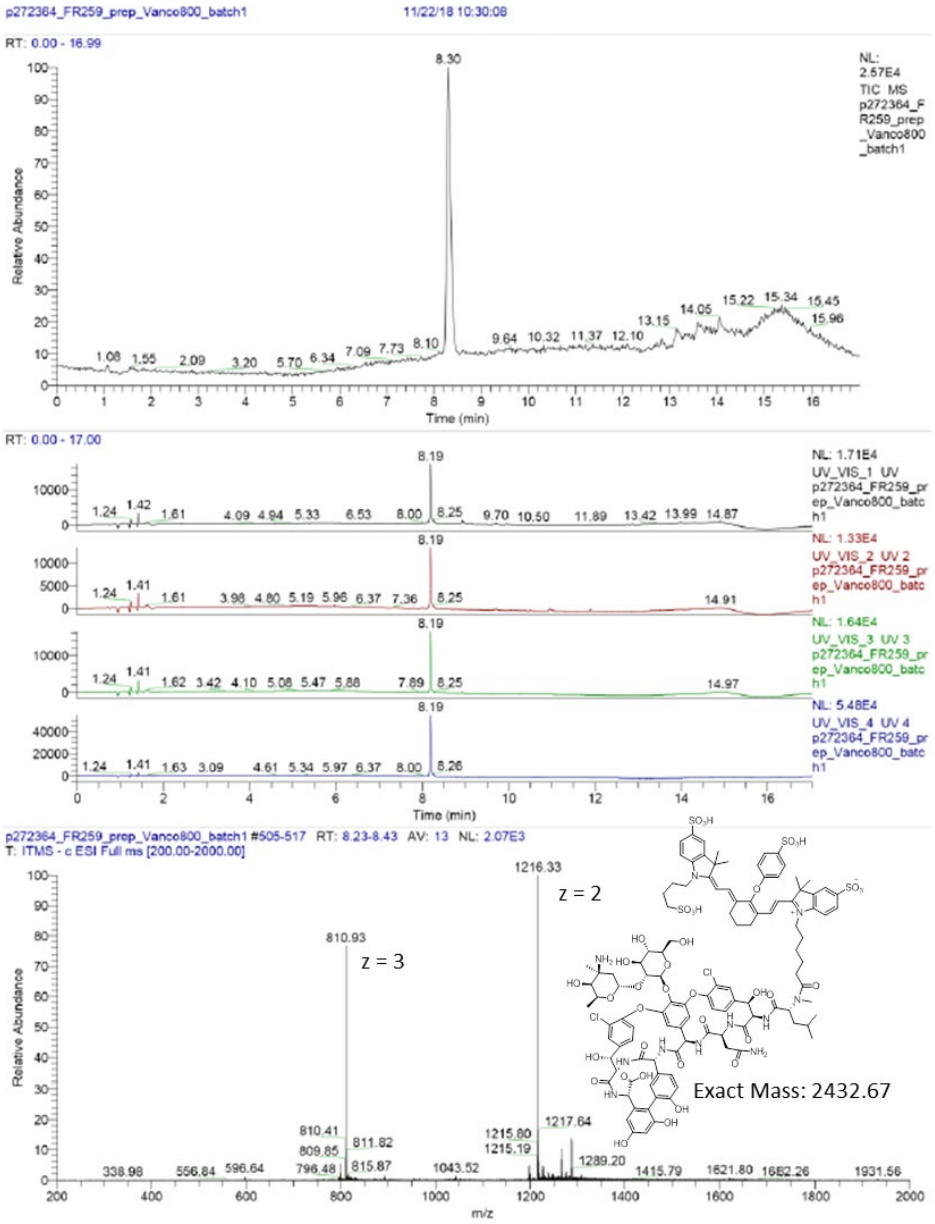


Figure S 8. Chromatogram of purified Vanco-800CW with MS detection and UV-Vis traces at different wavelength (specified above).

Vanco-FL-800CW

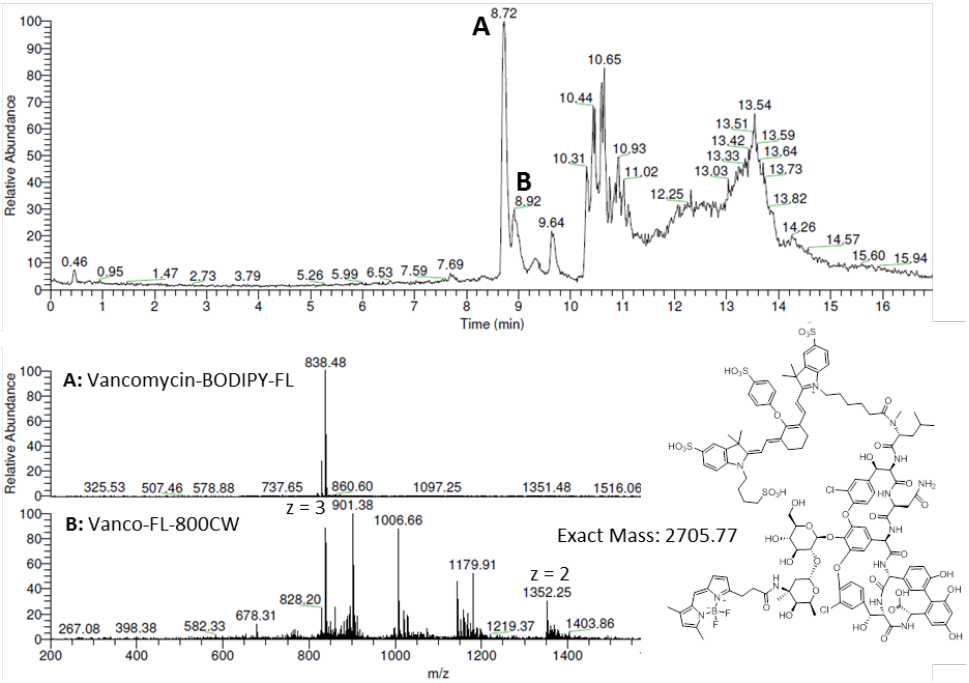


Figure S 9. Chromatogram of the reaction mixture containing vancomycin-BODIPY-FL, IRdye800CW NHS ester, DIPEA and HBTU in DMSO after 3 days reaction time. Peak A in the chromatogram, and the associated mass spectrum, correspond to the starting material (Vancomycin-BODIPY-FL). Peak B in the chromatogram, and the associated mass spectrum, correspond to the product (Vanco-FL-800CW).

Ampho-800CW

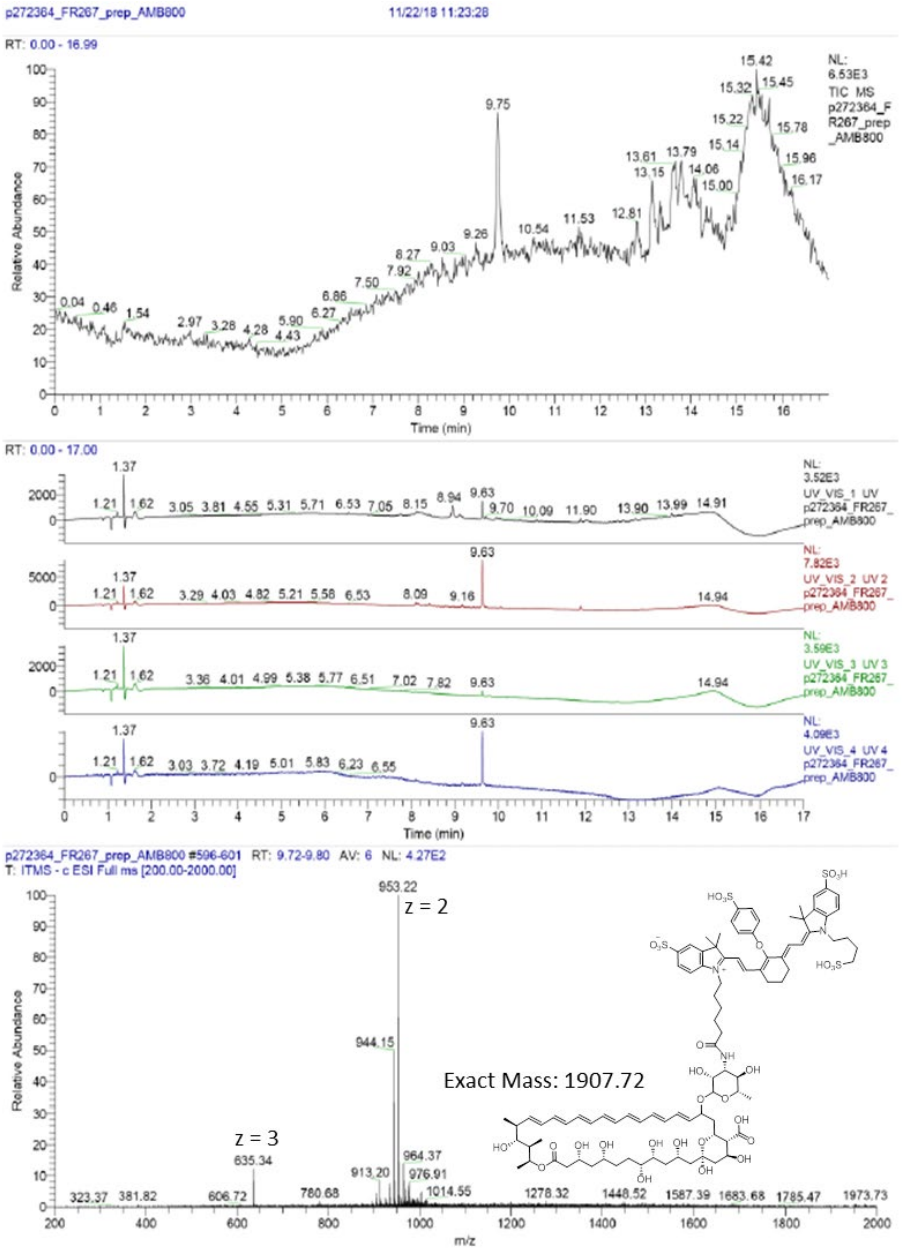


Figure S 10. Chromatogram of purified Ampho-800CW with MS detection and UV-Vis traces at different wavelength (specified above).

Vanco-700DX

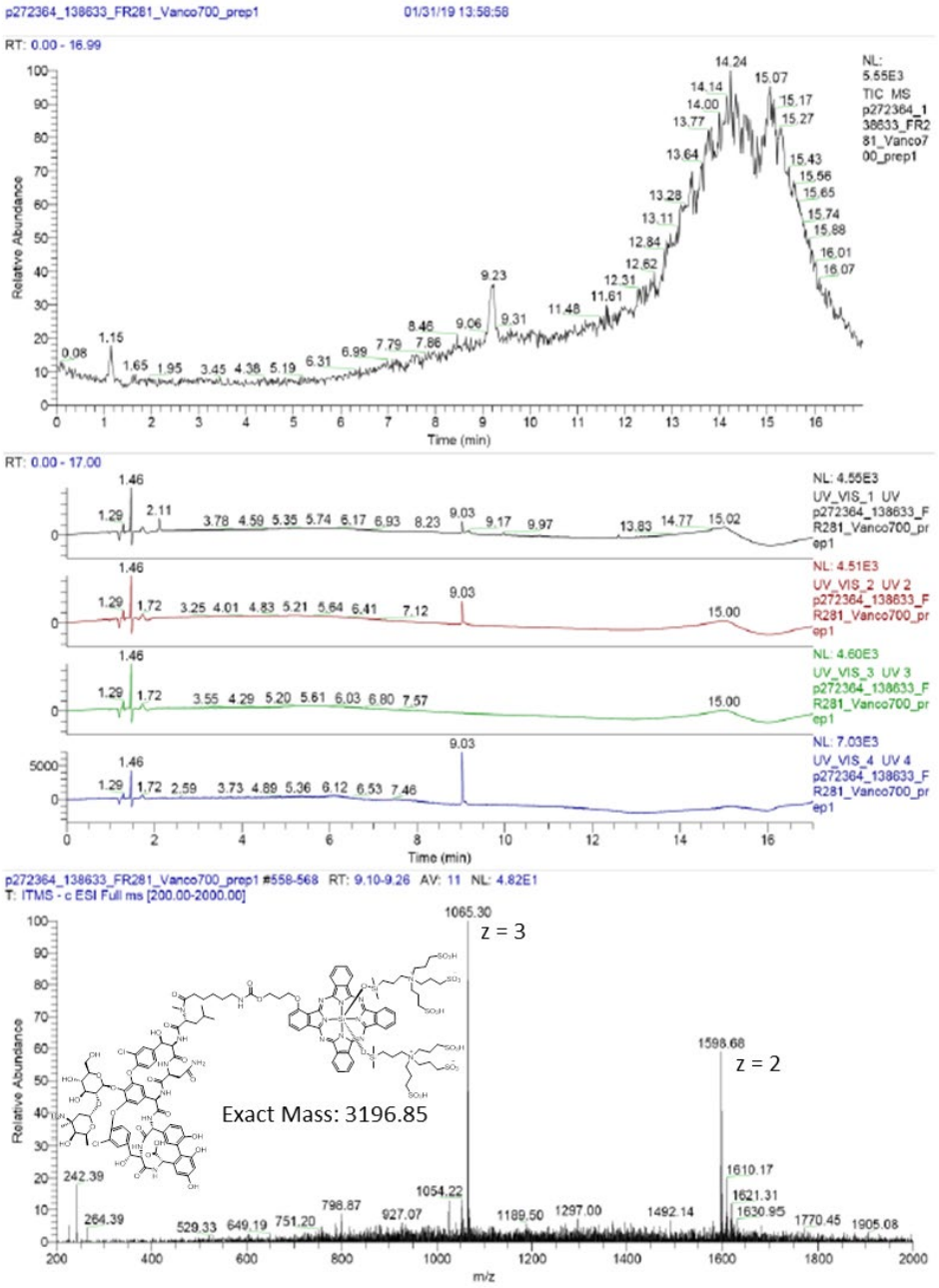


Figure S 11. Chromatogram of purified Vanco-700DX with MS detection and UV-Vis traces at different wavelength (specified above).

High-resolution mass spectrometry of the conjugates

Vanco-800CW

HRMS measurements were performed using a Thermo scientific LTQ OrbitrapXL spectrometer with ESI ionization. The measurement was performed in negative mode, using MeOH with ammonia (0.1 %) as eluents. The detected parent ion m/z: 1216 was fragmented (normalized collision energy = 35) and the detected fragment ions are shown below in m/z-units.

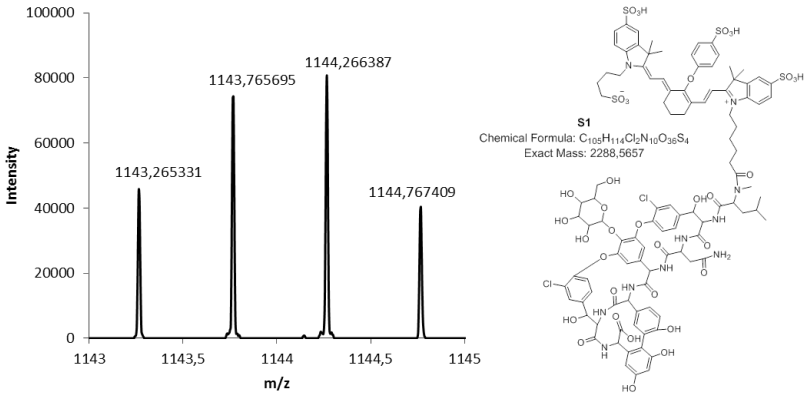


*Figure S 12. Molecular structure of fragment **S1** (Vanco-800CW after loss of one sugar moiety) and the detected isotopic pattern.*

Calculated m/z using https://www.envipat.eawag.ch:

Monoisotopic Mass:	1143.275578
Chemical Formula:	C105H112Cl2N10O36S4
Adduct:	M-2H
Charge:	-2

		m/z	rel. abundance
0	profile:	1143.2751525,	87.72
1	profile:	1143.7772324,	107.46
2	profile:	1144.2758735,	127.84
3	profile:	1144.7768071,	112.28

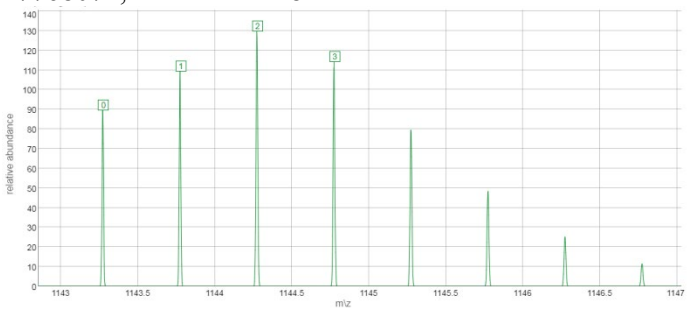


Figure S 13. Predicted isotopic pattern for fragment S1.

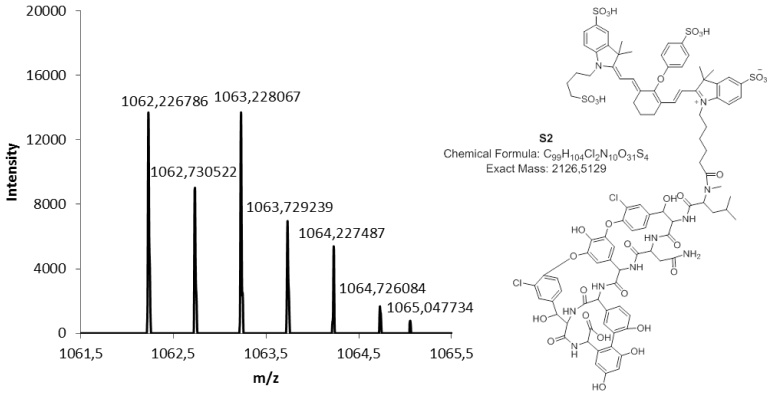


*Figure S 14. Molecular structure of fragment **S2** (Vanco-800CW after loss of two sugar moieties) and the detected isotopic pattern.*

Calculated m/z using https://www.envipat.eawag.ch:
Monoisotopic Mass: 1062.249166
Chemical Formula: C99H102Cl2N10O31S4
Adduct: M-2H
Charge: -2

		m/z	rel. abundance
0	profile:	1062.2483454,	91.86
1	profile:	1062.7500656,	106.37
2	profile:	1063.2496553,	126.16
3	profile:	1063.7492450,	106.93
4	profile:	1064.2498999,	74.20
5	profile:	1064.7494896,	44.07
6	profile:	1065.2501445,	22.49

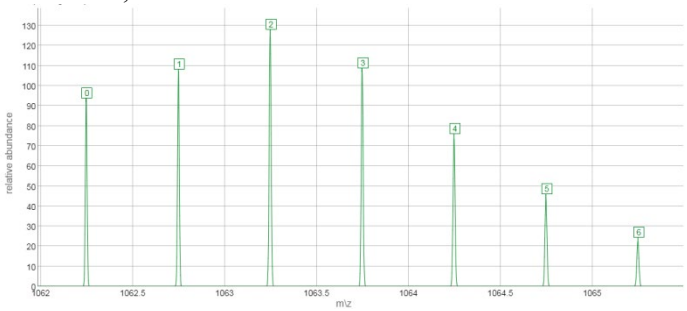


Figure S 15. Predicted isotopic pattern for fragment S2.

Vanco-700DX

The LC-MS measurements were performed at the Interfaculty Mass Spectrometry Center, Groningen on an Orbitrap Velos (Thermo Scientific) coupled to a Shimadzu Prominence UFLC system equipped with a Waters Acquity UPLC BEH-C18 (2.1 × 50 mm, 1.7 µm) column. The eluents were H_2O and acetonitrile both with 0.1% (v/v) formic acid. The elution gradient was established from 1 % to 90% organic phase over 20 min. The ions were detected in positive mode at a resolution of 60000 @ m/z 400. The detected fragments correspond to Vanco-700DX with loss of one or two substituents on the silyl center of the porphyrin. The loss of m/z = 307 observed in the mass spectrum of peak 2 (from m/z = 2701.753 to m/z = 2394.606) suggests the loss of the disaccharide moiety and indicates that the dye is coupled to the secondary amine on the N-terminus of vancomycin.

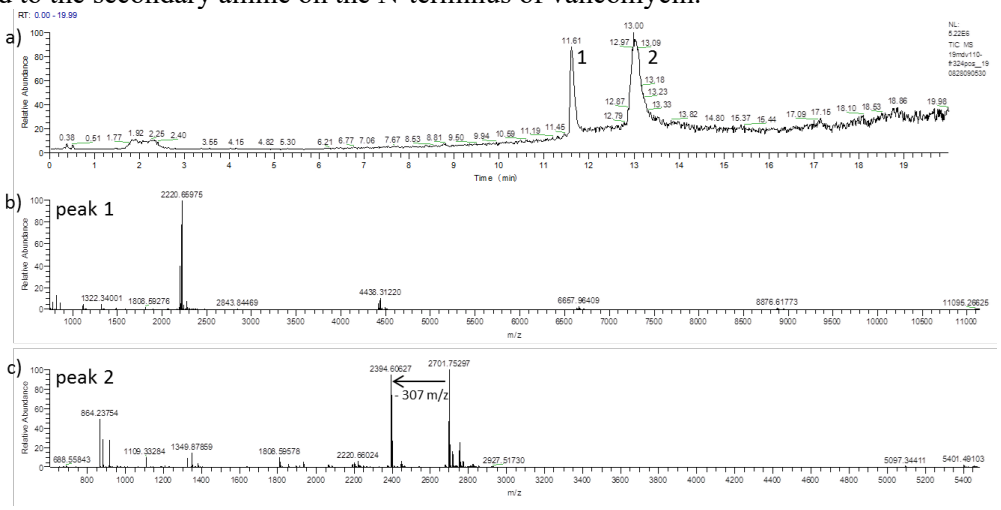


Figure S 16. a) UPLC MS trace (TIC) showing two fragments of Vanco-700DX; b) deconvoluted high-resolution mass spectrum of peak 1; c) deconvoluted high-resolution mass spectrum of peak 2.

Formation of *in vitro* biofilms for bacterial detection with Vanco-800CW

S. aureus clinical strain, *S. epidermidis* ATCC® 38984 and *E. coli* ATCC® 25922 were grown overnight in Tryptic Soy Broth (TSB) using a shaking incubator at 37°C. Bacterial biofilms were grown on chemically resistant borosilicate 18 mm glass coverslips (Marienfeld) in a 12-well plate containing TSB, supplemented with 5% glucose and 4% NaCl, and inoculated with the overnight culture to an optical density at 600 nm (OD_{600}) of 0.1, for 48 h. Coverslips with the respective biofilms were incubated with 0.2 µM of Vanco-800CW, IRdye800CW-carboxylic acid or Vanco-FL-800CW for 15 min in PBS (1x). To remove unbound fluorescent probe, the biofilms were washed 2x with PBS (1x) and, thereafter, fixed in 4% paraformaldehyde. Finally, the coverslips were mounted on microscopy slides and analyzed by fluorescence microscopy.

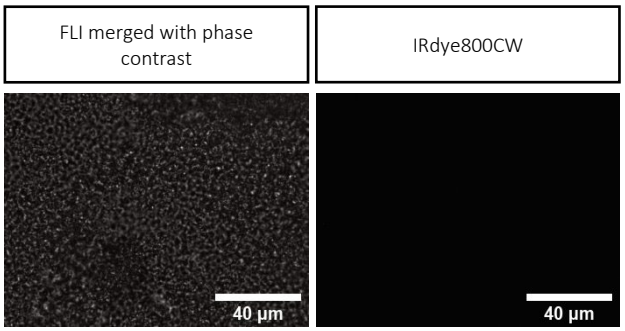


Figure S 17. In vitro detection of bacterial biofilms with IRdye800CW-carboxylic acid. Images recorded by fluorescence microscopy reveal no aspecific binding of IRdye800CW (red) to S. epidermidis. Scale bars: 40 µm.

Production of singlet oxygen by Vanco-700DX

The production of singlet oxygen was assessed by measuring the absorbance decay of 1,3-diphenylisobenzofuran (DPBF, Sigma-Aldrich) due to $^1O_2$ scavenging. Solutions containing DPBF (100 µM) with or without IRDye700DX or Vanco-700DX (0.1 µM) were prepared in DMSO and then irradiated at room temperature for different periods of time with red light at an irradiance of 10 mW cm^{-2}. DPBF absorbance was recorded at 415 nm.

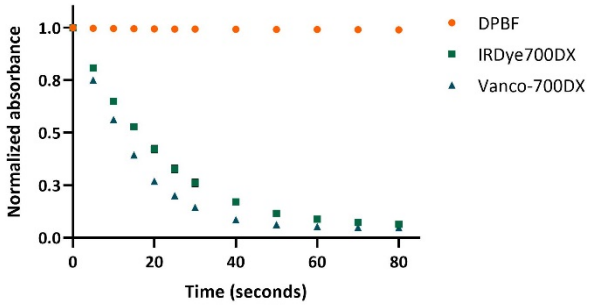
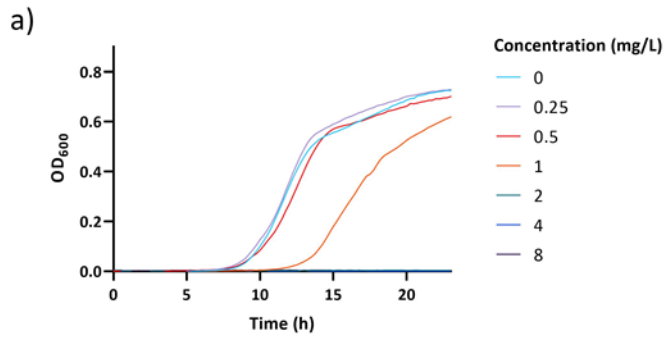
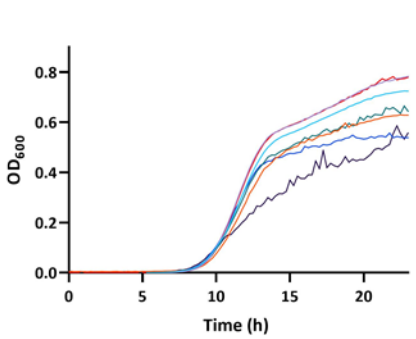


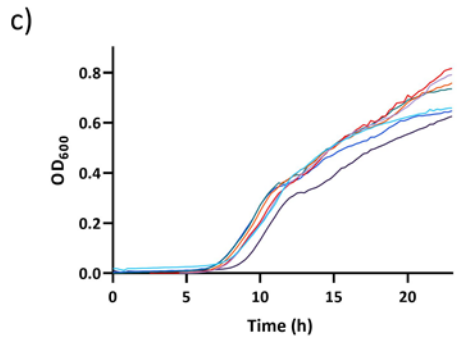
Figure S 18. Photo-oxidation of DPBF (100 µM) in DMSO with or without IRDye700DX or Vanco-700DX (0.1 µM) upon irradiation for different periods of time with a high-output LED (690 nm, 10 mW cm^{-2}). DPBF absorbance was recorded at 415 nm and normalized to the absorbance at the start of the irradiation. Data are the mean value ± SEM of three independent experiments. A one-way ANOVA test with a subsequent Tukey's multiple comparisons test were used for statistical analysis of the DBPF photo-oxidation.

Minimum inhibitory concentrations of Vancomycin, Vanco-800CW and Vanco-700DX

S. epidermidis ATCC® 38984 was grown overnight in TSB using a shaking incubator at 37°C. The overnight culture was diluted to an OD_{600} of 0.05 and grown for 3 h until exponential phase was reached (OD_{600}=0.5). The bacteria were afterwards diluted to an OD_{600} of 0.01 and incubated in a 96-well plate with different concentrations of vancomycin, Vanco-800CW or Vanco-700DX (0 – 8 mg/L) prepared in TSB. The bacteria were grown for 23 h in a microplate spectrophotometer (Synergy™ HT, Biotek instruments), at 37ºC, with 2 min of vigorous shaking every 15 min, prior each absorbance measurement at 600 nm. To complement the study, diffusion disks (Whatman paper) containing 5 µg of vancomycin, Vanco-800CW or Vanco-700DX were placed on a Mueller-Hinton agar plate containing *S. epidermidis* ATCC® 38984 (plated with an OD_{600}=0.1) and incubated overnight, at 37ºC.







d)

S. epidermidis	MIC (mg/L)
Vancomycin	2
Vanco-800CW	> 8
Vanco-700DX	> 8

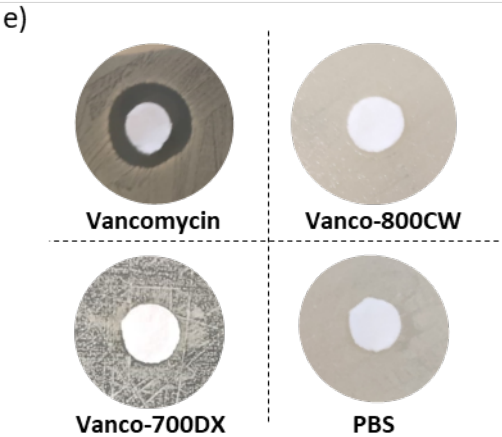


*Figure S 19. Growth curves of S. epidermidis ATCC in the presence of different concentrations (0-8 mg/L) of vancomycin (**a**), Vanco-800CW (**b**) or Vanco-700DX (**c**). The measured MIC values are summarized in **d**. Representative agar plates with S. epidermidis ATCC and disks containing vancomycin, Vanco-800CW or Vanco-700DX (5 µg, **e**). A zone of growth inhibition was only*

observed around the disk with vancomycin. Data are the mean value of two independent experiments.

References

(1) McKnelly, K. J.; Sokol, W.; Nowick, J. S. Anaphylaxis Induced by Peptide Coupling Agents: Lessons Learned from Repeated Exposure to HATU, HBTU, and HCTU. *J. Org. Chem.* **2020**, *85,* 1764–1768.