Supplementary Material (ESI) for Organic & Biomolecular Chemistry

Using Chemical Probes to Investigate the Sub-Inhibitory Effects of Azithromycin

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SUPPORTING INFORMATION

General Experimental. All experiments were performed under a nitrogen atmosphere, using anhydrous solvents, except where stated. Dichloromethane, methanol, toluene, acetonitrile and hexane were distilled from calcium hydride. Diethyl ether was distilled from calcium hydride and $LiAlH_4$. Tetrahydrofuran was distilled from calcium hydride and $LiAlH_4$ with triphenylmethane as indicator. All other reagents were purified in accordance with the instructions in "Purification of Laboratory Chemicals"[1] or used as obtained from commercial sources. Yields refer to chromatographically and spectroscopically pure compounds. Ambient temperature refers to 20 - 25 ºC. Analytical thin layer chromatography (TLC) was carried out on Merck Kieselgel 60 F254 plates with visualization by ultraviolet light or staining with ceric ammonium molybdate or potassium permanganate. Retention factors (R_f) are quoted to 0.01. Flash column chromatography was carried out using slurry-packed Merck 9385 Kieselgel 60 under a positive pressure of nitrogen. Full analytical data is given for new compounds. Optical rotations were recorded on a Perkin Elmer 343 polarimeter. $[\alpha]_D^{25}$ values are reported in 10^{-1} deg cm^2 g^{-1} at 589 nm, concentration (*c*) is given in g(100 mL)$^{-1}$. Infrared spectra were recorded on a Perkin Elmer Spectrum One infrared spectrophotometer with internal referencing as neat films. Wavelengths of maximum absorbance (ν_{max}) are quoted in wavenumbers (cm_{-1}); the abbreviations s and b indicate sharp and broad peaks respectively. ^{1}H Nuclear magnetic resonance (NMR) spectra were recorded using an internal deuterium lock at ambient probe temperatures on the following instruments: Bruker DPX-400 (400MHz), Bruker Avance DRX-400 (400 MHz), Bruker Avance 500 BB-ATM (500 MHz) and Bruker Avance 500 Cryo Ultrashield (500MHz). An internal reference of δ_H 7.26 was used for the residual $CHCl_3$ in $CDCl_3$. Data are represented as follows: chemical shift (in ppm to the nearest 0.01 ppm), integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant (*J* in Hz to the nearest 1 Hz) and assignment. Assignments were determined either on the basis of unambiguous chemical shift or coupling pattern, by patterns observed in 2D experiments (^{1}H-^{1}H COSY and HMQC) or by analogy to fully interpreted spectra for related compounds. ^{13}C NMR spectra were recorded by broadband proton spin decoupling at ambient probe temperature using an internal deuterium lock. All chemical shift values are reported in ppm to the nearest 0.01 ppm. An internal reference of δ_C 77.0 was used for $CDCl_3$. Assignments were supported by DEPT editing and determined either on the basis of unambiguous chemical shift, by patterns observed in 2D experiments (HMQC) or by analogy to fully interpreted spectra for related compounds. Low resolution mass spectra (ESI and APCI) were recorded using an LCMS system (Agilent 1200 series LC with and ESCi Multi-Mode Ionization Waters ZQ spectrometer using MassLynx 4.1 software). Only molecular ions are quoted. High resolution mass spectra were obtained with a Finnigan MAT 900 XLT or a Finnigan MAT 95XP spectrometer at the EPSRC National Mass Spectrometry Service Centre in Swansea, or with a Micromass Q-TOF or a Micromass LCT Premier spectrometer at the Department of Chemistry, University of Cambridge, using chemical ionization or electron impact. The parent ion [M]+ or [M+H]+ is quoted and the reported mass values are within the error limits of ±5 ppm mass units. Proteomics data were obtained with an ESI-TRAP instrument using MS/MS Ion Search with a peptide mass tolerance of ±1 Da.

Experimental section

The numbered assignments in the NMR spectra correspond to the numbering system used in the figures and schemes in the paper.

Erythromycin A 9-oxime 5. Erythromycin **(4)** (3.65 g, 5.0 mmol) was dissolved in pyridine (25 mL, 323 mmol). Hydroxylamine hydrochloride (3.48 g, 50 mmol) was added to this solution. The reaction mixture was stirred for 24h. Pyridine was removed *in vacuo* and the residue was dissolved in CH_2Cl_2. The organic layer was washed

[1] W. L.F. Armarego and C. L. L. Chai. *Purification of Laboratory Chemicals*, 5th edn., Butterworth-Heinmann, **2003**.

with brine, dried (Na_2SO_4) and concentrated *in vacuo* to give the crude product, which was purified by flash chromatography (CH_2Cl_2:MeOH:aq. NH_3 90:8:2) to give **5** as a white solid (2.16 g, 2.88 mmol, 79 %): R_f 0.32 (CH_2Cl_2:MeOH:aq. NH_3 90:8:2); $[\alpha]_D^{25}$ -62.3 (*c* = 0.97, $CHCl_3$); m.p. 158 ºC (CH_2Cl_2:MeOH) [lit. 157-164 ºC (CH_2Cl_2:hexane)[19]]; ν_{max} (thin film /cm^{-1}) 3429 (b, OH), 2970 (s, CH), 1737 (s, lactone carbonyl C=O), 1706 (s, oxime C=N); δ_H (500 MHz, $CDCl_3$) 5.01 (1H, dd, *J* 11, 3 Hz, C_{13}-H), 4.86 (1H, d, *J* 5 Hz, C_1''-H), 4.44 (1H, s, C_{11}-OH), 4.42 (1H, s, C_{11}-H), 4.35 (1H, d, *J* 7 Hz, C_1'-H), 4.00-3.93 (1H, m, C_5''-H), 3.97 (1H, d, *J* 10 Hz, C_3-H), 3.74-3.69 (1H, m, C_8-H), 3.62 (1H, s, OH), 3.51 (1H, d, *J* 7 Hz, C_5-H), 3.45-3.38 (1H, m, C_5'-H), 3.37 (1H, bs, C_{12}-OH), 3.25 (3H, s, C_3''-OCH_3), 3.17 (1H, dd, *J* 10, 7 Hz, C_2'-H), 3.11 (1H, s, C_6-OH), 3.06 (1H, s, OH), 2.94 (1H, app. t, *J* 10 Hz, C_4''-H), 2.84 (1H, dq, *J* 7, 3 Hz, C_2-H), 2.62 (1H, q, *J* 7 Hz, C_{10}-H), 2.34 (1H, ddd, *J* 12, 10, 4 Hz, C_3'-H), 2.29 (1H, d, *J* 15 Hz, C_2''-H^{eq}), 2.22 (6H, s, C_3'-$N(CH_3)_2$), 2.20 (1H, d, *J* 10 Hz, C_4''-OH), 1.93 (1H, ddq, *J* 7, 7, 1 Hz, C_4-H), 1.88-1.80 (1H, m, C_{13}-CH_2-CH_3), 1.69 (1H, s, OH), 1.60 (1H, ddd, *J* 13, 4, 2 Hz, C_4'-H^{eq}), 1.55-1.47 (2H, m, C_7-H_2), 1.50 (1H, d, *J* 15 Hz, C_2''-H^{ax}), 1.44 (3H, s, C_6-CH_3), 1.44-1.37 (1H, m, C_{13}-CH_2-CH_3), 1.23 (3H, d, *J* 6 Hz, C_5''-CH_3), 1.19-1.15 (1H, m, C_4'-H^{ax}), 1.17 (3H, s, C_3''-CH_3), 1.16 (3H, d, *J* 7 Hz, C_5'-CH_3), 1.12 (6H, 2 × d, *J* 7 Hz, C_2-CH_3 and C_{10}-CH_3), 1.06 (3H, s, C_{12}-CH_3), 1.04 (3H, d, *J* 8 Hz, C_4-CH_3), 0.98 (3H, d, *J* 7 Hz, C_8-CH_3), 0.77 (3H, t, *J* 7 Hz, C_{13}-CH_2-CH_3); δ_C (125 MHz, $CDCl_3$) 175.5 (C_1), 171.8 (C_9), 103.2 (C_1'), 96.4 (C_1''), 83.4 (C_5), 80.2 (C_3), 78.1 (C_4''), 77.2 (C_{13}), 75.4 (C_6), 74.0 (C_{12}), 72.2 (C_3''), 71.1 (C_2'), 70.8 (C_{11}), 68.8 (C_5'), 65.5 (C_3' & C_5''), 49.5 (C_3''-OCH_3), 44.7 (C_2), 40.3 (C_3'-$N(CH_3)_2$), 39.0 (C_4), 37.8 (C_7), 35.1 (C_2''), 32.8 (C_{10}), 29.0 (C_4'), 27.0 (C_6-CH_3), 25.5 (C_8), 21.3 (C_5'-CH_3 & C_3''-CH_3), 21.0 (C_{13}-CH_2), 18.6 (C_8-CH_3 & C_5''-CH_3), 16.3 (C_{12}-CH_3), 16.2 (C_2-CH_3), 14.3 (C_{10}-CH_3), 10.7 (C_{13}-CH_2-CH_3), 9.2 (C_4-CH_3); HRMS (ES^+) calculated for $C_{37}H_{68}N_2O_{13}$ $[M+H]^+$ 749.4794, found 749.4792.

9-Deoxo-6-deoxy-6,9-epoxy-9,9a-didehydro-9a-aza-homoerythromycin A 7. Tosyl chloride (2.55 g, 13.35 mmol) in acetone (30 mL) and sodium hydrogen carbonate (2.24 g, 26.7 mmol mmol) in H_2O (100 mL) were added simultaneously to a solution of **5** (5.0 g, 6.68 mmol) in acetone (80 mL) over 2h at 0-5 ºC. The reaction mixture was stirred at this temperature for an additional 3h. Acetone was removed *in vacuo*. CH_2Cl_2 was added to the obtained suspension. The reaction mixture was acidified with 2N HCl to pH 5. The layers were separated and the acidic aqueous layer was extracted with CH_2Cl_2. Extraction with CH_2Cl_2 was repeated at pH 6 and pH 8. The combined extracts at pH 8 were dried (K_2CO_3) and concentrated *in vacuo* to give **7** as an off-white solid (3.60 g, 4.93 mmol, 74 %): $[\alpha]_D^{25}$ -57.8 (*c* = 0.83, $CHCl_3$) [lit. -54.6 (*c* = 1, CH_2Cl_2)[21]]; m.p. 131 ºC (CH_2Cl_2) [lit. 128-131 ºC (CH_2Cl_2)[21]]; ν_{max} (thin film /cm^{-1}) 3426 (b, OH), 2970 (s, CH), 2936 (s, CH), 1725 (s, lactone carbonyl C=O), 1699 (s, imine C=N); δ_H (500 MHz, $CDCl_3$) 5.09 (1H, d, *J* 5 Hz, C_1''-H), 4.83 (1H, dd, *J* 10, 2 Hz, C_{13}-H), 4.40 (1H, d, *J* 7 Hz, C_1'-H), 4.04-3.97 (1H, m, C_5''-H), 3.91 (1H, app. t, *J* 3 Hz, C_3-H), 3.87 (1H, d, *J* 7 Hz, C_5-H), 3.69 (1H, dq, *J* 7, 7 Hz, C_{10}-H), 3.64-3.61 (1H, m, C_{11}-H), 3.51-3.44 (1H, m, C_5'-H), 3.38 (1H, s, OH), 3.29 (3H, s, C_3''-OCH_3), 3.28 (1H, s, OH), 3.13 (1H, dd, *J* 10, 7 Hz, C_2'-H), 3.00 (1H, app. t, *J* 10 Hz, C_4''-H), 2.85-2.77 (1H, m, C_8-H), 2.67 (1H, dq, *J* 7, 3 Hz, C_2-H), 2.60 (1H, s, OH), 2.45-2.36 (1H, m, C_3'-H), 2.36 (1H, d, *J* 15 Hz, C_2''-H^{eq}), 2.24 (6H, s, C_3'-$N(CH_3)_2$), 2.06 (1H, d, *J* 10 Hz, C_4''-OH), 1.96 (1H, dd, *J* 13, 8 Hz, C_7-H), 1.87-1.76 (2H, m, C_4-H and C_{13}-CH_2-CH_3), 1.65-1.60 (1H,m, C_4'-H^{eq}), 1.60-1.56 (1H, m, C_7-H), 1.56 (1H, dd, *J* 15, 5 Hz, C_2''-H^{ax}), 1.45-1.41 (1H, m, C_{13}-CH_2-CH_3), 1.39 (3H, s, C_6-CH_3), 1.25 (3H, d, *J* 6 Hz, C_5''-CH_3), 1.22 (1H, d, *J* 7 Hz, C_{10}-CH_3), 1.20 (3H, s, C_3''-CH_3), 1.19-1.17 (1H, m, C_4'-H^{ax}), 1.16 (6H, 2 × d, *J* 7 Hz, C_8-CH_3 & C_5'-CH_3), 1.13 (3H, d, *J* 7 Hz, C_2-CH_3), 1.06 (3H, d, *J* 8 Hz, C_4-CH_3), 1.03 (3H, s, C_{12}-CH_3), 0.82 (3H, t, *J* 8 Hz, C_{13}-CH_2-CH_3); δ_C (125 MHz, $CDCl_3$) 178.5 (C_1), 165.2 (C_9), 102.8 (C_1'), 94.8 (C_1''), 88.3 (C_6), 79.2 (C_5), 78.1 (C_4''), 78.1 (C_{13}), 76.4 (C_3), 75.0 (C_{12}), 73.0 (C_3''), 72.3 (C_{11}), 70.7 (C_2'), 69.0 (C_5'), 65.9 (C_3'), 65.7 (C_5''), 52.6 (C_{10}), 49.5 (C_3''-OCH_3), 44.6 (C_2), 42.7 (C_4), 40.4 (C_3'-$N(CH_3)_2$), 37.2 (C_7), 35.5 (C_8), 34.6 (C_2''), 28.6 (C_4'), 25.7 (C_6-CH_3), 21.6 (C_3''-CH_3), 21.5 (C_{13}-CH_2), 21.4 (C_5'-CH_3), 18.3 (C_5''-CH_3), 18.1 (C_8-CH_3), 17.4 (C_{12}-CH_3), 15.9 (C_{10}-CH_3), 13.4 (C_2-CH_3), 11.1 (C_{13}-CH_2-CH_3), 9.2 (C_4-CH_3); HRMS (ES^+) calculated for $C_{37}H_{66}N_2O_{12}$ $[M+H]^+$ 731.4689, found 731.4718.

9-Deoxo-9a-aza-9a-homoerythromycin A 9. Compound **7** (3.80 g, 5.20 mmol) was dissolved in MeOH (35 mL) and cooled to 0 ºC. Sodium borohydride (3.93 g, 104 mmol) was added in small portions. The reaction mixture was stirred at 0 ºC for 4h, then allowed to warm to ambient temperature and stirred for 36h. The reaction mixture was concentrated *in vacuo*. Chloroform and H_2O were added to the residue. The organic layer was dried (K_2CO_3) and concentrated *in vacuo* to give the crude product, which was purified by flash chromatography (CH_2Cl_2:MeOH:aq. NH_3 90:6:1.5) to give **9** as a white solid (2.94 g, 4.00 mmol, 77 %): R_f 0.15 (CH_2Cl_2:MeOH:aq. NH_3 90:6:1.5); $[\alpha]_D^{25}$ -32.0 (*c* = 0.90, $CHCl_3$) [lit. -33.9 (*c* = 1, CH_2Cl_2)[21]]; m.p. 115 ºC (CH_2Cl_2:MeOH) [lit. 113-116 ºC (CH_2Cl_2)[21]]; ν_{max} (thin film /cm^{-1}) 3600-3550 (m, NH), 3449 (b, OH), 2970 (s, CH), 2933 (s, CH), 1731 (s, lactone carbonyl C=O); δ_H (500 MHz, $CDCl_3$) 5.05 (1H, d, *J* 5 Hz, C_1''-H), 4.71 (1H, dd, *J* 10, 2 Hz, C_{13}-H), 4.42 (1H, d, *J* 7 Hz, C_1'-H), 4.33 (1H, dd, *J* 5, 2 Hz, C_3-H), 4.07 (1H, dq, *J* 10, 6 Hz, C_5''-H), 3.65 (1H, d, *J* 7 Hz, C_5-H), 3.53-3.47 (1H, m, C_5'-H), 3.45 (1H, d, *J* 2 Hz, C_{11}-H), 3.37 (1H, s, OH), 3.32 (3H, s, C_3''-OCH_3), 3.27 (1H, s, OH), 3.22 (1H, dd, *J* 10, 7 Hz, C_2'-H), 3.03 (1H, d, *J* 10 Hz, C_9-H), 3.02 (1H, app. t, *J* 10 Hz, C_4''-H), 2.81-2.74 (1H, m, C_2-H), 2.59-2.54 (1H, m, C_{10}-H), 2.46-2.40 (1H, m, C_3'-H), 2.33 (1H, d, *J* 15 Hz, C_2''-H^{eq}), 2.27 (6H, s, C_3'-$N(CH_3)_2$), 2.25 (1H, s, OH), 2.06 (1H, d, *J* 11 Hz, C_4''-OH), 1.96-1.91 (1H, m, C_4-H), 1.90-1.84 (1H, m, C_{13}-CH_2-CH_3), 1.81 (1H, d, *J* 11 Hz, C_9-H), 1.73 (1H, d, *J* 14 Hz, C_7-H), 1.74-1.70 (1H, m, C_8-H), 1.67-1.62 (1H, m, C_4'-H^{eq}), 1.57 (1H, dd, *J* 15, 5 Hz, C_2''-H^{ax}), 1.50-1.46 (1H, m, C_{13}-CH_2-CH_3), 1.39-1.34 (1H, m, C_7-H), 1.31 (3H, d, *J* 6 Hz, C_5''-CH_3), 1.28 (3H, s, C_6-CH_3), 1.25-1.20 (1H, m, C_4'-H^{ax}), 1.23 (3H, s, C_3''-CH_3), 1.21 (6H, app. t, *J* 7 Hz, C_2-CH_3 & C_5'-CH_3), 1.13 (1H, d, *J* 7 Hz, C_{10}-CH_3), 1.06 (3H, s, C_{12}-CH_3), 1.04 (3H, d, *J* 7 Hz, C_4-CH_3), 0.92 (3H, d, *J* 7 Hz, C_8-CH_3), 0.88 (3H, t, *J* 7 Hz, C_{13}-CH_2-CH_3); δ_C (125 MHz, $CDCl_3$) 178.5 (C_1), 103.1 (C_1'), 95.3 (C_1''), 83.8 (C_5), 78.6 (C_3), 78.2 (C_4''), 77.9 (C_{13}), 74.1 (C_{12}), 73.7 (C_6), 73.5 (C_{11}), 73.0 (C_3''), 71.0 (C_2'), 68.8 (C_5'), 65.7 (C_3'), 65.5(C_5''), 57.1 (C_9), 56.4 (C_{10}), 49.4 (C_3''-OCH_3), 45.2 (C_2), 42.3 (C_7), 41.7 (C_4), 40.4 (C_3'-$N(CH_3)_2$), 35.0 (C_2''), 30.0 (C_8), 28.8 (C_4'), 27.4 (C_6-CH_3), 21.9 (C_8-CH_3), 21.6 (C_3''-CH_3), 21.4 (C_5'-CH_3), 21.0 (C_{13}-CH_2), 18.4 (C_5''-CH_3), 16.2 (C_{12}-CH_3), 15.3 (C_2-CH_3), 14.2 (C_{10}-CH_3), 11.2 (C_{13}-CH_2-CH_3), 9.2 (C_4-CH_3); HRMS (ES^+) calculated for $C_{37}H_{70}N_2O_{12}$ $[M+H]^+$ 735.5002, found 735.5017.

9-Deoxo-9a-aza-9a-methyl-9a-homoerythromycin A (Azithromycin) 1. A mixture of **9** (100 mg, 0.14 mmol), 37 % aqueous formaldehyde (0.12 mL, 0.34 mmol) and formic acid (0.013 mL, 0.34 mol) in chloroform (3 mL) was stirred at 70 ºC for 2h. The mixture was then allowed to cool to ambient temperature and partitioned between CH_2Cl_2 and sat. aqueous potassium carbonate. The aqueous layer was re-extracted with CH_2Cl_2. The combined extracts were dried ($MgSO_4$) and concentrated *in vacuo* to give the crude product, which was purified by flash chromatography (CH_2Cl_2:MeOH:aq. NH_3 90:8:2) to give **1** as a white solid (70.4 mg, 0.10 mmol, 71 %): R_f 0.57 (CH_2Cl_2:MeOH:aq. NH_3 90:8:2); $[\alpha]_D^{25}$ -44.6 (*c* = 0.75, $CHCl_3$); m.p. 120 ºC (CH_2Cl_2:MeOH) [lit. 187-188 ºC (EtOH)[24]]; ν_{max} (thin film /cm^{-1}) 3468 (b, OH), 2972 (s, CH), 2936 (s, CH), 1726 (s, lactone carbonyl C=O); δ_H (500 MHz, $CDCl_3$) 9.44 (1H, bs, $C_{2'}$-OH), 5.13 (1H, d, *J* 5 Hz, C_1''-H), 4.74 (1H, d, *J* 6 Hz, C_{11}-OH), 4.68 (1H, dd, *J* 10, 2 Hz, C_{13}-H), 4.43 (1H, d, *J* 7 Hz, C_1'-H), 4.29 (1H, dd, *J* 5, 2 Hz, C_3-H), 4.08 (1H, dq, *J* 10, 6 Hz, C_5''-H), 3.68 (1H, d, *J* 5 Hz, C_{11}-H), 3.65 (1H, d, *J* 7 Hz, C_5-H), 3.55-3.47 (1H, m, C_5'-H), 3.35 (1H, s, C_{12}-OH), 3.34 (3H, s, C_3''-OCH_3), 3.23 (1H, dd, *J* 10, 7 Hz, C_2'-H), 3.03 (1H, app. t, *J* 10 Hz, C_4''-H), 2.89 (1H, s, C_6-OH), 2.78-2.72 (1H, m, C_2-H), 2.69 (1H, q, *J* 7 Hz, C_{10}-H), 2.52 (1H, d, *J* 10 Hz, C_9-H), 2.46-2.41 (1H, m, C_3'-H), 2.35 (1H, d, *J* 15 Hz, C_2''-H^{eq}), 2.31 (3H, s, C_1*-H_3), 2.28 (6H, s, C_3'-$N(CH_3)_2$), 2.15 (1H, d, *J* 11 Hz, C_4''-OH), 2.07-1.95 (2H, m, C_4-H &

C_8-H), 2.02 (1H, d, *J* 10 Hz, C_9-H), 1.93-1.85 (1H, m, C_{13}-CH_2-CH_3), 1.79 (1H, d, *J* 15 Hz, C_7-H), 1.67-1.63 (1H,m, C_4'-H^{eq}), 1.58 (1H, dd, *J* 15, 5 Hz, C_2''-H^{ax}), 1.51-1.43 (1H, m, C_{13}-CH_2-CH_3), 1.32 (3H, d, *J* 5 Hz, C_5''-CH_3), 1.31 (3H, s, C_6-CH_3), 1.24 (3H, s, C_3''-CH_3), 1.26-1.21 (2H, m, C_7-H & C_4'-H^{ax}), 1.22 (3H, d, *J* 6 Hz, C_5'-CH_3), 1.19 (3H, d, *J* 7 Hz, C_2-CH_3), 1.08 (1H, d, *J* 7 Hz, C_{10}-CH_3), 1.08 (3H, s, C_{12}-CH_3), 1.04 (3H, d, *J* 7 Hz, C_4-CH_3), 0.90 (3H, d, *J* 7 Hz, C_8-CH_3), 0.89 (3H, t, *J* 7 Hz, C_{13}-CH_2-CH_3); δ_C (125 MHz, $CDCl_3$) 178.9 (C_1), 102.9 (C_1'), 94.5 (C_1''), 83.3 (C_5), 78.1 (C_4''), 77.6 (C_3), 77.4 (C_{13}), 74.2 (C_{12}), 73.6 (C_6), 73.6 (C_{11}), 72.9 (C_3''), 70.8 (C_2'), 70.1 (C_9), 68.7 (C_5'), 65.9 (C_3'), 65.5 (C_5''), 62.4 (C_{10}), 49.4 (C_3''-OCH_3), 45.3 (C_2), 42.3 (2C, C_4 & C_7), 40.3 (C_3'-N(CH_3)$_2$), 36.2 (C_1*), 34.7 (C_2''), 28.7 (C_4'), 27.6 (C_6-CH_3), 26.7 (C_8), 22.0 (C_8-CH_3), 21.6 (C_3''-CH_3), 21.3 (C_5'-CH_3), 21.3 (C_{13}-CH_2), 18.2 (C_5''-CH_3), 16.2 (C_{12}-CH_3), 14.6 (C_2-CH_3), 11.2 (C_{13}-CH_2-CH_3), 9.0 (C_4-CH_3), 7.3 (C_{10}-CH_3); HRMS (ES^+) calculated for $C_{38}H_{72}N_2O_{12}$ $[M+H]^+$ 749.5158, found 749.5185. The data was consistent with that reported previously.[24]

9-Deoxo-9a-aza-9a-(β-cyanoethyl)-9a-homoerythromycin A 10. A solution of **1** (49 mg, 0.07 mmol) in acrylonitrile (1 mL) was stirred at 60 ºC for 16h. The mixture was then concentrated *in vacuo* to give the crude product, which was purified by flash chromatography (CH_2Cl_2:MeOH:aq. NH_3 90:6:1.5) to give **10**[26a] as a white solid (33.0 mg, 0.042 mmol, 63 %): R_f 0.36 (CH_2Cl_2:MeOH:aq. NH_3 90:6:1.5); $[\alpha]_D^{25}$ -45.2 (*c* = 0.81, $CHCl_3$); m.p. 90 ºC (CH_2Cl_2:MeOH); ν_{max} (thin film /cm^{-1}) 3498 (b, OH), 2972 (s, CH), 2937 (s, CH), 2158 (C≡N), 1721 (s, lactone carbonyl C=O); δ_H (500 MHz, $CDCl_3$) 4.94 (1H, d, *J* 4 Hz, C_1''-H), 4.63 (1H, dd, *J* 10, 2 Hz, C_{13}-H), 4.44 (1H, d, *J* 7 Hz, C_1'-H), 4.13 (1H, dd, *J* 7, 3 Hz, C_3-H), 4.04 (1H, dq, *J* 9, 6 Hz, C_5''-H), 3.75 (1H, d, *J* 5 Hz, C_{11}-OH), 3.64 (1H, d, *J* 5 Hz, C_{11}-H), 3.60 (1H, d, *J* 6 Hz, C_5-H), 3.55-3.49 (1H, m, C_5'-H), 3.29 (3H, s, C_3''-OCH_3), 3.26 (1H, dd, *J* 10, 7 Hz, C_2'-H), 3.02 (1H, app. t, *J* 10 Hz, C_4''-H), 2.86-2.79 (2H, m, C_2-H & C_{10}-H), 2.79-2.69 (1H, m, C_9-H), 2.67-2.59 (1H, m, C_1*-H_2), 2.54-2.42 (4H, m, C_3'-H & C_1*-H_2 (1H) & C_2*-H_2 (2H)), 2.35 (1H, d, *J* 15 Hz, C_2''-H^{eq}), 2.31 (6H, s, C_3'-N(CH_3)$_2$), 2.21-2.16 (2H, m, C_9-H & C_4''-OH), 1.97-1.91 (2H, m, C_4-H & C_8-H), 1.88-1.82 (1H, m, C_{13}-CH_2-CH_3), 1.71-1.67 (1H, m, C_7-H), 1.57 (1H, dd, *J* 15, 5 Hz, C_2''-H^{ax}), 1.53-1.47 (2H, m, C_4'-H^{eq} & C_{13}-CH_2-CH_3), 1.30 (3H, d, *J* 7 Hz, C_5''-CH_3), 1.29 (3H, s, C_6-CH_3), 1.26-1.19 (11H, m, C_3''-CH_3 & C_5'-CH_3 & C_2-CH_3 & C_7-H & C_4'-H^{ax}), 1.10 (1H, d, *J* 7 Hz, C_{10}-CH_3), 1.07 (3H, d, *J* 8 Hz, C_4-CH_3), 1.05 (3H, s, C_{12}-CH_3), 0.96 (3H, d, *J* 7 Hz, C_8-CH_3), 0.88 (3H, t, *J* 7 Hz, C_{13}-CH_2-CH_3); δ_C (125 MHz, $CDCl_3$) 178.0 (C_1), 119.1 (C_3*), 103.2 (C_1'), 96.1 (C_1''), 84.7 (C_5), 79.5 (C_3), 78.2 (C_{13}), 77.9 (C_4''), 75.1 (C_{11}), 74.8 (C_{12}), 74.5 (C_6), 72.7 (C_3''), 70.8 (C_2'), 68.9 (C_5'), 65.8 (C_5''), 65.4 (C_3'), 64.6 (C_9), 60.0 (C_{10}), 49.4 (C_3''-OCH_3), 47.9 (C_1*), 45.1 (C_2), 40.8 (C_7), 40.6 (C_4), 40.4 (C_3'-N(CH_3)$_2$), 35.0 (C_2''), 29.0 (C_4'), 29.0 (C_8), 26.0 (C_6-CH_3), 22.2 (C_8-CH_3), 21.5 (C_3''-CH_3), 21.3 (C_{13}-CH_2), 21.3 (C_5'-CH_3), 18.3 (C_5''-CH_3), 17.3 (C_2*), 16.4 (C_{12}-CH_3), 15.5 (C_2-CH_3), 11.2 (C_{13}-CH_2-CH_3), 9.7 (C_4-CH_3), 8.7 (C_{10}-CH_3); HRMS (ES^+) calculated for $C_{40}H_{73}N_3O_{12}$ $[M+H]^+$ 788.5267, found 788.558.

9-Deoxo-9a-aza-9a-(γ-aminopropyl)-9a-homoerythromycin A 11 & Dimer of 9-Deoxo-9a-aza-9a-(γ-aminopropyl)-9a-homoerythromycin A 12. Compound **10** (51 mg, 0.065 mmol) was dissolved in EtOH (2 mL) and hydrogenated by flow using an H-Cube® (Raney nickel cartridge, flow rate 1 mL/min at 40 bar and 30 ºC). The eluent was collected and concentrated *in vacuo* to give the crude product, which was purified by flash chromatography (CH_2Cl_2:MeOH:aq. NH_3 90:8:2) to give both **11** and the minor product, the dimer **12**, as white solids. **11** (22.7 mg, 0.029 mmol, 45 %): R_f 0.16 (CH_2Cl_2:MeOH:aq. NH_3 90:8:2); $[\alpha]_D^{25}$ -44.3 (*c* = 0.59, $CHCl_3$); m.p. 108 ºC (CH_2Cl_2:MeOH) [lit. 180-183 ºC (Et_2O)[26a]]; ν_{max} (thin film /cm^{-1}) 3481 (b, OH/NH), 2971 (s, CH), 2936 (s, CH), 1727 (s, lactone carbonyl C=O); δ_H (500 MHz, $CDCl_3$) 5.09 (1H, d, *J* 5 Hz, C_1''-H), 4.95 (1H, dd, *J* 11, 2 Hz, C_{13}-H), 4.49 (1H, d, *J* 7 Hz, C_1'-H), 4.21 (1H, dd, *J* 6, 2 Hz, C_3-H), 4.10 (1H, dq, *J* 9, 6 Hz, C_5''-H), 3.64 (1H, s, C_{11}-H), 3.62 (1H, d, *J* 7 Hz, C_5-H), 3.57-3.51 (1H, m, C_5'-H), 3.35 (3H, s, C_3''-OCH_3), 3.26 (1H, dd, *J* 10, 7 Hz, C_2'-H), 3.08-2.96 (3H, m, C_4''-H & C_3*-H_2), 2.86 (1H, app. quintet, *J* 7 Hz, C_2-H), 2.79-2.71 (2H, m, C_{10}-H & C_1*-H_2), 2.65 (1H, dd, *J* 14, 6 Hz, C_9-H), 2.51-2.47 (1H, m, C_3'-H), 2.39 (1H, d, *J* 15 Hz, C_2''-H^{eq}), 2.34 (1H, d, dd, *J* 6 Hz, C_9-H), 2.31 (6H, s, C_3'-N(CH_3)$_2$), 2.26-2.20 (2H, m, C_1*-H_2 & C_4''-OH), 2.07-2.02 (2H, m, C_4-H & C_8-H), 1.95-1.87 (1H, m, C_{13}-CH_2-CH_3), 1.80-1.72 (1H, m, C_2*-H_2), 1.69-1.65 (1H, m, C_4'-H^{eq}), 1.60 (1H, dd, *J* 15, 5 Hz, C_2''-H^{ax}), 1.59-1.53 (2H, m, C_7-H & C_2*-H_2), 1.52-1.43 (1H, m, C_{13}-CH_2-CH_3), 1.32 (3H, d, *J* 6 Hz, C_5''-CH_3), 1.30 (3H, s, C_6-CH_3), 1.27-1.20 (2H, m, C_7-H & C_4'-H^{ax}), 1.26 (3H, s, C_3''-CH_3), 1.24 (3H, d, *J* 6 Hz, C_5'-CH_3), 1.21 (3H, d, *J* 7 Hz, C_2-CH_3), 1.11 (3H, d, *J* 7 Hz, C_4-CH_3), 1.08 (3H, s, C_{12}-CH_3), 1.07-1.04 (6H, m, C_{10}-CH_3 & C_8-CH_3), 0.87 (3H, t, *J* 7 Hz, C_{13}-CH_2-CH_3); δ_C (125 MHz, $CDCl_3$) 177.2 (C_1), 102.8 (C_1'), 95.5 (C_1''), 84.1 (C_5), 79.0 (C_3), 78.0 (C_4''), 77.6 (C_{13}), 74.7 (C_{12}), 74.4 (C_{11}), 74.3 (C_6), 72.8 (C_3''), 70.9 (C_2'), 68.9 (C_5'), 65.6 (C_5''), 65.5 (C_3'), 64.0 (C_9), 58.2 (C_{10}), 49.4 (C_3''-OCH_3), 48.4 (C_1*), 45.0 (C_2), 41.1 (C_4), 40.5 (C_7), 40.4 (C_3'-N(CH_3)$_2$), 39.8 (C_3*), 35.0 (C_2''), 29.2 (C_8), 28.9 (C_4'), 28.9 (C_2*), 25.9 (C_6-CH_3), 23.5 (C_8-CH_3), 21.6 (C_3''-CH_3), 21.4 (C_5'-CH_3), 21.1 (C_{13}-CH_2), 18.2 (C_5''-CH_3), 16.4 (C_{12}-CH_3), 15.3 (C_2-CH_3), 11.0 (C_{13}-CH_2-CH_3), 9.6 (C_4-CH_3), 6.6 (C_{10}-CH_3); HRMS (ES^+) calculated for $C_{40}H_{77}N_3O_{12}$ $[M+H]^+$ 792.5580, found 792.5587. **12** (2.0 mg, 0.0013 mmol, 2 %): R_f 0.20 (CH_2Cl_2:MeOH:aq. NH_3 90:8:2); $[\alpha]_D^{25}$ -49.3 (*c* = 0.30, $CHCl_3$); m.p. 132 ºC (CH_2Cl_2:MeOH); ν_{max} (thin film /cm^{-1}) 3447 (b, OH/NH), 2972 (s, CH), 2937 (s, CH), 1727 (s, lactone carbonyl C=O); δ_H (500 MHz, $CDCl_3$) 5.04-5.00 (2H, m, C_1''-H), 4.82 (2H, d, *J* 9 Hz, C_{13}-H), 4.42 (2H, d, *J* 7 Hz, C_1'-H), 4.14 (2H, d, *J* 4 Hz, C_3-H), 4.03 (2H, dq, *J* 9, 3 Hz, C_5''-H), 3.63 (2H, s, C_{11}-H), 3.58 (2H, d, *J* 7 Hz, C_5-H), 3.52-3.44 (2H, m, C_5'-H), 3.29 (6H, s, C_3''-OCH_3), 3.21 (2H, dd, *J* 10, 7 Hz, C_2'-H), 3.16-3.08 (2H, m, C_3*-H_2), 2.99 (2H, app. t, *J* 11 Hz, C_4''-H), 2.80-2.66 (6H, m, C_2-H & C_{10}-H & C_3*-H_2), 2.63 (2H, d, *J* 10 Hz, C_9-H), 2.54-2.46 (2H, m, C_1*-H_2), 2.46-2.38 (2H, m, C_3'-H), 2.32 (2H, d, *J* 15 Hz, C_2''-H^{eq}), 2.25 (12H, s, C_3'-N(CH_3)$_2$), 2.21-2.14 (2H, m, C_9-H), 2.19 (2H, d, *J* 10 Hz, C_4''-OH), 2.28-2.14 (2H, m, C_1*-H_2), 2.04-1.94 (4H, m, C_4-H & C_8-H), 1.88-1.78 (2H, m, C_{13}-CH_2-CH_3), 1.76-1.68 (2H, m, C_2*-H_2), 1.64-1.58 (2H, m, C_4'-H^{eq}), 1.58-1.48 (6H, m, C_2''-H^{ax} & C_7-H & C_2*-H_2), 1.48-1.38 (2H, m, C_{13}-CH_2-CH_3), 1.28-1.23 (12H, m, C_5''-CH_3 & C_6-CH_3), 1.21-1.14 (22H, m, C_3''-CH_3 & C_5'-CH_3 & C_2-CH_3 & C_7-H & C_4'-H^{ax}), 1.06-1.01 (18H, m, C_4-CH_3 & C_{12}-CH_3 & C_{10}-CH_3), 0.95 (6H, d, *J* 7 Hz, C_8-CH_3), 0.82 (6H, t, *J* 7 Hz, C_{13}-CH_2-CH_3); δ_C (125 MHz, $CDCl_3$) 177.3 (C_1), 102.9 (C_1'), 95.3 (C_1''), 84.1 (C_5), 78.7 (C_3), 78.0 (C_4''), 77.6 (C_{13}), 74.7 (C_{12}), 74.5 (C_{11}), 74.3 (C_6), 72.8 (C_3''), 70.9 (C_2'), 68.8 (C_5'), 65.5 (C_5''), 65.5 (C_3'), 64.3 (C_9), 59.4 (C_{10}), 49.4 (C_3''-OCH_3), 47.9 (C_1*), 47.0 (C_3*), 45.0 (C_2), 41.3 (C_4), 41.2 (C_7), 40.3 (C_3'-N(CH_3)$_2$), 35.0 (C_2''), 28.8 (C_4'), 28.8 (C_8), 26.8 (C_2*), 26.1 (C_6-CH_3), 23.1 (C_8-CH_3), 21.5 (C_3''-CH_3), 21.3 (C_5'-CH_3), 21.3 (C_{13}-CH_2), 18.2 (C_5''-CH_3), 16.5 (C_{12}-CH_3), 15.1 (C_2-CH_3), 11.0 (C_{13}-CH_2-CH_3), 9.6 (C_4-CH_3), 7.8 (C_{10}-CH_3); HRMS (ES^+) calculated for $C_{80}H_{151}N_5O_{24}$ $[M+H]^+$ 1567.0827, found 1567.0783.

9-Deoxo-9a-aza-9a-(γ-(D-(+)-biotin-amido-*N*-propyl))-9a-homoerythromycin A 2. A solution of biotin (10.0 mg, 0.041 mmol), EDC hydrochloride (7.9 mg, 0.041 mmol) and DMAP (3.9 mg, 0.032 mmol) was stirred in DMF (0.5 mL). To this solution was added a solution of **11** (25 mg, 0.032 mmol) in DMF (0.5 mL). The solution was stirred at overnight at ambient temperature. The solution was concentrated *in vacuo* to give the crude product, which was purified by flash chromatography (CH_2Cl_2:MeOH: aq. NH_3 90:12:3) to give **2** as a white solid (21.2 mg, 0.021 mmol, 65 %): R_f 0.39 (CH_2Cl_2:MeOH:aq. NH_3 90:12:3); $[\alpha]_D^{25}$ -21.3 (*c* = 0.93, $CHCl_3$); m.p. 125 ºC (CH_2Cl_2:MeOH); ν_{max} (thin film /cm^{-1}) 3298 (b, OH/NH), 2971 (s, CH), 2936 (s, CH), 1694 (s, C=O), 1657 (s, C=O), 1648 (s, C=O); δ_H (500 MHz, $CDCl_3$) 4.87 (1H, d, *J* 4 Hz, C_1''-H), 4.78 (1H, d, *J* 10 Hz, C_{13}-H), 4.58 (1H, app. t, *J* 6 Hz, C_{12}*-H), 4.44 (1H, d, *J* 7 Hz, C_1'-H), 4.32 (1H, app. t, *J* 6 Hz, C_{10}*-H), 4.03 (1H, dq, *J* 9, 6 Hz, C_5''-H), 3.98 (1H, dd, *J* 7, 2 Hz, C_3-H), 3.69 (1H, d, *J* 5 Hz, C_{11}-H), 3.59 (1H, d, *J* 6 Hz, C_5-H), 3.50-3.45 (1H, m, C_5'-H), 3.29 (3H, s, C_3''-OCH_3), 3.21 (1H, dd, *J* 10, 8 Hz, C_2'-H), 3.16-3.08 (3H, m, C_9*-H & C_3*-H_2), 3.01 (1H, app. t, *J* 10Hz, C_4''-H), 2.89-2.83 (3H, m, C_2-H & C_{10}-H & C_{13}*-H^A), 2.73 (1H,

d, *J* 13 Hz, C_{13}*-H^{B}), 2.72-2.67 (1H, m, C_9-H), 2.60 (2H, t, *J* 7 Hz, C_5*-H$_2$), 2.48-2.38 (1H, m, C_3′-H), 2.34 (1H, d, *J* 15 Hz, C_2″-H^{eq}), 2.28 (6H, s, C_3′-N(CH$_3$)$_2$), 2.31-2.24 (2H, m, C_1*-H$_2$), 2.15-2.12 (1H, m, C_9-H), 2.04-2.02 (1H, m, C_4-H), 2.01-1.93 (1H, m, C_8-H), 1.88-1.75 (3H, m, C_{13}-CH$_2$-CH$_3$ (1H) & C_2*-H$_2$ (1H) & C_7-H), 1.73-1.60 (6H, m, C_4′-H^{eq} & C_8*-H$_2$ (2H) & C_6*-H$_2$ (2H) & C_2*-H$_2$ (1H)), 1.56 (1H, dd, *J* 15, 5 Hz, C_2″-H^{ax}), 1.52-1.38 (3H, C_{13}-CH$_2$-CH$_3$ (1H) & C_7*-H$_2$), 1.30 (3H, s, C_6-CH$_3$), 1.26 (3H, d, *J* 6 Hz, C_5″-CH$_3$), 1.22-1.12 (11H, m, C_3″-CH$_3$ & C_5′-CH$_3$ & C_2-CH$_3$ & C_7-H & C_4′-H^{ax}), 1.10-1.05 (9H, m, C_4-CH$_3$ & C_{12}-CH$_3$ & C_{10}-CH$_3$), 0.92 (3H, d, *J* 7 Hz, C_8-CH$_3$), 0.83 (3H, t, *J* 7 Hz, C_{13}-CH$_2$-CH$_3$); δ_C (125 MHz, $CDCl_3$) 176.9 (C_1), 173.7 (C_4*), 164.7 (C_{11}*), 102.7 (C_1′), 95.9 (C_1″), 83.8 (C_5), 79.9 (C_3), 77.8 (C_4″), 77.3 (C_{13}), 74.6 (C_{12}), 74.6 (C_6), 73.7 (C_{11}), 72.7 (C_3″), 71.0 (C_2′), 68.7 (C_5′), 67.0 (C_9), 65.7 (C_5″), 65.5 (C_3′), 62.6 (C_{10}), 62.0 (C_{10}*), 59.9 (C_{12}*), 56.2 (C_9*), 49.8 (C_1*), 49.4 (C_3″-OCH$_3$), 44.7 (C_2), 41.0 (C_{13}*), 40.7 (C_7), 40.3 (C_3′-N(CH$_3$)$_2$), 39.8 (C_4), 37.6 (C_3*), 35.9 (C_5*), 35.0 (C_2″), 29.7 (C_8), 28.9 (C_4′), 28.9 (C_7*), 28.1 (C_2*), 27.7 (C_8*), 27.5 (C_6-CH$_3$), 25.9 (C_6*), 22.5 (C_8-CH$_3$), 21.5 (C_3″-CH$_3$), 21.4 (C_5′-CH$_3$), 21.2 (C_{13}-CH$_2$), 18.7 (C_5″-CH$_3$), 16.6 (C_{12}-CH$_3$), 15.9 (C_2-CH$_3$), 11.0 (C_{13}-CH$_2$-CH$_3$), 9.6 (C_4-CH$_3$), 7.6 (C_{10}-CH$_3$); HRMS (ES$^+$) calculated for $C_{50}H_{91}N_5O_{14}$ [M+H]$^+$ 1018.6263, found 1018.6400.

2-Azidoacetic acid 14. Synthesized in two steps *via* methyl 2-azidoacetate. Methyl bromoacetate (0.63 mL, 6.67 mmol) was added to a solution of sodium azide (0.65 g, 10.0 mmol) in DMSO (17.5 mL). The mixture was stirred for 20h and then poured into H_2O (40 mL). The resulting solution was extracted with Et_2O. The combined organic fractions were washed with H_2O and brine, dried ($MgSO_4$) and concentrated *in vacuo* to give methyl 2-azidoacetate[28] as a colourless oil (0.72 g, 6.25 mmol, 94 %): ν_{max} (thin film /cm^{-1}) 2958 (s, CH), 2917 (s, CH), 2849 (s, CH), 2107 (s, N=N=N), 1748 (s, C=O); δ_H (500 MHz, $CDCl_3$) 3.91 (2H, s, C_5*-H$_2$), 3.83 (3H, s, C_4*-OCH$_3$); δ_C (125 MHz, $CDCl_3$) 168.7 (C_4*), 52.6 (C_4*-OCH$_3$), 50.3 (C_5*); *m/z* (APCI+) 116 (100 %, M+H$^+$). Methyl 2-azidoacetate (0.68 g, 5.9 mmol) was dissolved in a THF/MeOH/H_2O (3:1:1) solution (15 mL) and LiOH was added (1.24 g, 29.5 mmol). The resulting solution was stirred for 15h and then diluted with THF. The solution was acidified to pH 3 by addition of 3N HCl. The resulting solution was extracted with ether, the combined organic fractions were dried ($MgSO_4$) and concentrated *in vacuo* to give **14**[28] as a light yellow oil (0.59 g, 5.83 mmol, 99%): ν_{max} (thin film /cm^{-1}) 3200 (b, OH), 2920 (s, CH), 2850 (s, CH), 2110 (s, azide), 1726 (s, C=O); δ_H (500 MHz, $CDCl_3$) 3.98 (2H, s, C_5*-H$_2$); δ_C (125 MHz, $CDCl_3$) 172.8 (C_4*), 50.0 (C_5*); *m/z* (APCI+) 102 (100%, M+H$^+$).

9-Deoxo-9a-aza-9a-(γ-(2-azidoacetamido)-propyl)-9a-homoerythromycin A 13. EDC hydrochloride (48.5 mg, 0.25 mmol) was added to a solution of **14** (20.4 mg, 0.20 mmol) and **11** (80.0 mg, 0.10 mmol) in CH_2Cl_2 with 30% pyridine (7.0 mL). The resulting solution was stirred for 20h and then concentrated *in vacuo* to give the crude product, which was purified by flash chromatography (CH_2Cl_2:MeOH:aq. NH_3 90:8:2) to give **13** as a white solid (70.3 mg, 0.080 mmol, 80%): R_f 0.47 (CH_2Cl_2:MeOH:aq. NH_3 90:8:2); $[\alpha]_D^{25}$ -40.8 (*c* = 0.61, $CHCl_3$); m.p. 105 ºC (CH_2Cl_2:MeOH); ν_{max} (thin film /cm^{-1}) 3374 (b, OH/NH), 2972 (s, CH), 2935 (s, CH), 2105 (s, N=N=N), 1727 (s, C=O), 1669 (s, C=O); δ_H (500 MHz, $CDCl_3$) 5.00 (1H, d, *J* 5 Hz, C_1″-H), 4.68 (1H, d, *J* 9 Hz, C_{13}-H), 4.47 (1H, d, *J* 7 Hz, C_1′-H), 4.18 (1H, dd, *J* 7, 2 Hz, C_3-H), 4.10 (1H, dq, *J* 9, 6 Hz, C_5″-H), 3.97 (2H, s, C_5*-H$_2$), 3.77 (1H, d, *J* 5 Hz, C_{11}-H), 3.67 (1H, d, *J* 7 Hz, C_5-H), 3.54 (1H, m, C_5′-H), 3.36-3.32 (1H, m, C_3*-H$_2$), 3.34 (3H, s, C_3″-OCH$_3$), 3.30-3.20 (2H, m, C_2′-H & C_3*-H$_2$), 3.06 (1H, app. t, *J* 10 Hz, C_4″-H), 2.90 (1H, app. quintet, *J* 7 Hz, C_2-H), 2.87-2.83 (1H, m, C_{10}-H), 2.75-2.71 (1H, m, C_9-H), 2.63-2.59 (1H, m, C_1*-H$_2$), 2.54-2.43 (1H, m, C_3′-H), 2.38 (1H, d, *J* 15 Hz, C_2″-H^{eq}), 2.34 (6H, s, C_3′-N(CH$_3$)$_2$), 2.26-2.17 (3H, m, C_9-H & C_1*-H$_2$ & C_4″-OH), 2.08-1.99 (2H, m, C_4-H & C_8-H), 1.95-1.87 (1H, m, C_{13}-CH$_2$-CH$_3$), 1.82-1.69 (3H, m, C_2*-H$_2$ & C_4′-H^{eq} & C_7-H), 1.62 (1H, dd, *J* 15, 5 Hz, C_2″-H^{ax}), 1.56-1.49 (1H, m, C_{13}-CH$_2$-CH$_3$), 1.46-1.39 (1H, m, C_2*-H$_2$), 1.36 (3H, s, C_6-CH$_3$), 1.35 (3H, d, *J* 6 Hz, C_5″-CH$_3$), 1.29-1.22 (11H, m, C_3″-CH$_3$ & C_5′-CH$_3$ & C_2-CH$_3$ & C_7-H & C_4′-H^{ax}), 1.13 (3H, d, *J* 8 Hz, C_4-CH$_3$), 1.12 (3H, d, *J* 7 Hz, C_{10}-CH$_3$), 1.09 (3H, s, C_{12}-CH$_3$), 0.98 (3H, d, *J* 7 Hz, C_8-CH$_3$), 0.92 (3H, t, *J* 7 Hz, C_{13}-CH$_2$-CH$_3$); δ_C (125 MHz, $CDCl_3$) 177.8 (C_1), 167.0 (C_4*), 103.0 (C_1′), 95.9 (C_1″), 84.0 (C_5), 79.6 (C_3), 78.0 (C_{13}), 77.9 (C_4″), 74.7 (C_{12}), 74.5 (C_6), 74.3 (C_{11}), 72.8 (C_3″), 70.8 (C_2′), 68.8 (C_5′), 65.9 (C_5″), 65.6 (C_3′), 64.5 (C_9), 60.5 (C_{10}), 52.6 (C_5*), 49.4 (C_3″-OCH$_3$), 49.0 (C_1*), 44.9 (C_2), 40.5 (C_7), 40.4 (C_3′-N(CH$_3$)$_2$), 40.2 (C_4), 37.8 (C_3*), 35.0 (C_2″), 29.2 (C_4′), 28.9 (C_8), 27.6 (C_2*), 26.8 (C_6-CH$_3$), 22.7 (C_8-CH$_3$), 21.5 (C_3″-CH$_3$), 21.3 (C_5′-CH$_3$), 21.2 (C_{13}-CH$_2$), 18.4 (C_5″-CH$_3$), 16.4 (C_{12}-CH$_3$), 15.5 (C_2-CH$_3$), 11.1 (C_{13}-CH$_2$-CH$_3$), 9.6 (C_4-CH$_3$), 8.1 (C_{10}-CH$_3$); HRMS (ES$^+$) calculated for $C_{42}H_{78}N_6O_{13}$ [M+H]$^+$ 875.5705, found 875.5736.

Sepharose-azithromycin construct 26. Beads used: Amersham Biosciences CNBr-activated Sepharose 4 Fast Flow. Buffers: acidification solution (1 mM HCl), coupling buffer (0.2 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3), blocking buffer (1 M ethanolamine in coupling buffer, pH 8.0), wash buffer (0.1 M acetic acid and 0.5 M NaCl, pH 4). The propargylamine solution was prepared at a concentration of 20 mg/mL in coupling buffer. The sepharose beads (170 mg) were swollen in the acidification solution (1 mL) for 15 min, forming approximately 0.6 mL of gel. The gel was transferred to a sinter and washed with 40 mL of acidification solution, follwed by 1 mL of coupling buffer. The gel was then immediately transferred to the propargylamine solution (1.2 mL) and rotated gently for 3.5h. The coupled beads were then washed 3 times with coupling buffer. The coupling buffer was drained from the beads and incubated with blocking buffer (1.2 mL) for 2h. The beads were then washed (4 × 10 mL), alternating between coupling buffer and wash buffer. The beads were air-dried under suction for 30 min and placed under high vacuum for 14h to give sepharose-alkyne beads **15**. The azide **13** (55 mg) was dissolved in ^{t}BuOH (0.7 mL). The sepharose-alkyne beads **15** were suspended in H_2O (0.1 mL) and added to the azide solution. Sodium ascorbate (0.38 mL of a 1M solution in H_2O) was then added, followed by $CuSO_4.5H_2O$ (0.38 mL of a 0.1 M solution in H_2O). The reaction mixture was rotated in an end-over-end mixer for 3 days. The solution was drained under positive nitrogen pressure and washed/drained with wash buffer (20 mL), H_2O (20 mL), CH_2Cl_2:MeOH 1:1 (20 mL), CH_2Cl_2 (20 mL) and CH_2Cl_2:MeOH 1:1 (20 mL). The beads were air-dried under suction for 1h and placed under high vacuum for 6h to give sepharose-azithromycin construct **3**.

General experimental for biological assays

Media used. LB medium (Luria-Bertani broth) contains: Tryptone, 10 g/L, Yeast extract 5 g/L, NaCl 5 g/L. The solution was brought to pH 7.0 using NaOH. AGS medium (alanine glycerol salts) contains: L-alanine 5 g/L, K_2HPO_4 (dibasic) 3 g/L, NaCl 5 g/L.The solution was brought to pH 7.0 using conc. HCl and autoclaved together. To this, salts and glycerol were added to a final concentration of: 0.1 mM $CaCl_2$, 10 mM $MgSO_4$, 5 µM $FeCl_2$, 7.5 µM $ZnCl_2$, 0.5 % v/v glycerol.

Gels used for polyacrylamide gel electrophoresis. (Percentage indicates the amount of acrylomide monomer.) 5 % Stacking gel contains per 6 mL: H_2O 4.1 mL, 30 % Acrylomide 1.0 mL, 1.0 M Tris pH 6.8 0.75 mL, 10 % SDS, 60 µL, 10 % APS 60 µL, TEMED 6 µL. 12 % Resolving gel contains per 20 mL: H_2O 6.6 mL, 30 % Acrylomide 8.0 mL, 1.5 M Tris pH 8.8 5.0 mL, 10 % SDS 0.2 mL, 10 % APS 0.2 mL, TEMED 8 µL. 20 % Gradient resolving gel: gradient mix of 10 % and 20 % gel.

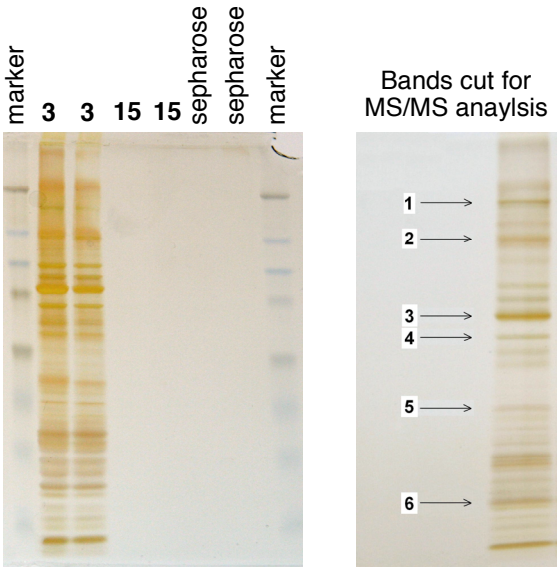
Strains used. PAO 1: wild-type *Pseudomonas aeruginosa*.

MIC assay. Stock solutions in 0.1M K_2HPO_4 (dibasic) of the compounds to be tested were made, all samples were filtered through a sterilizing filter. Overnight cultures of PAO 1 in 10 mL AGS medium were set up and left to grow at 37 ºC. The OD_{600} of an overnight culture grown in AGS on the rolling drum is usually around 2. The next day, dilutions were set up of the compounds in 10 mL AGS medium, starting from 1000 µg/mL and decreasing down by factors of 2 to 2 µg/mL, plus a control without any compound added. 20 µL of overnight culture was added to each dilution, which was then incubated at 37 ºC overnight. After this the dilutions were examined, the MIC could be determined from the cut-off point between dilutions that showed growth and no growth.

Biofilm microtitre plate assay. 1.0 mg/mL solutions in 0.1M K_2HPO_4 (dibasic) of the compounds to be tested were made, all samples were filtered through a sterilizing filter. An overnight culture of PAO 1 in 10 mL AGS medium was set up and left to grow at 37 ºC. The next day, a reading of the optical density at 600 nm (OD_{600}) was taken of the overnight culture, the culture was diluted with AGS medium to give an OD_{600} reading of around 0.2. After this, 200 µL of the diluted cultures were aliquoted into the wells of a 96-well plate. 4 µL of the 1.0 mg/mL compound solutions were added to each 200 µL well, giving a final concentration of 20 µg/mL in the wells. (Varying stock solutions were used for assays with varying concentrations in the wells.) The plate was left in a humid environment at 37 ºC for 5 days. Visualization: the liquid was carefully removed from the wells of the plate by aspiration and the wells were washed with 240 µL deionised water, being as careful as possible not to disturb cells adhering to the sides of the wells. 260 µL of crystal violet (0.1 % in H_2O) was added to each well and left to stain for 1 hour. The crystal violet was carefully removed from the wells of the plate by aspiration and the wells were washed with 3 × 400 µL deionised water, purple-stained cells were now visible adhering to the sides of the wells. 240 µL 50 % EtOH was added to each well and the plate was agitated gently for an hour to allow the crystal violet to dissolve. The more intense the purple colour, the greater the extent of adhesion / biofilm formation. Purple colour was quantified by measurement of the absorbance at 595nm (A_{595}).

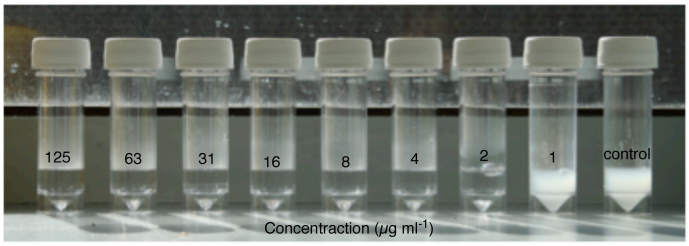
Pull-down assay. An overnight culture of PAO 1 in 10 mL AGS medium was set up and left to grow at 37 ºC. The next day, 100 mL of AGS medium was inoculated to OD_{600} = 0.01 in a 500 mL baffled flask, where the cells were grown for 20h at 37 ºC. Aliquots of 20 mL from this culture were then spun down at 4000 rpm for 10 min at 4 ºC, the supernatant was removed and the cell pellets were frozen for future use. Cell pellets of PAO 1 were resuspended in 2 mL ice-cold PBS, then spun down at 4000 rpm for 10 min at 4 ºC, after which the liquid was removed. The cell pellets were once again suspended in 2 mL ice-cold PBS and sonicated for 3 × 10 seconds at setting 3. The suspensions were then spun down at 13000 rpm for 30 min at 4 ºC, after which 1.5 mL of the liquid was transferred to new Eppendorffs. Batches of 100 µL of bead suspensions (10 mg beads / 1.0 mL PBS) were spun down at 13000 rpm for 5 min at 4 ºC, after which the liquid was removed. The beads were once again suspended in 100 µL ice-cold PBS, and were then added to the 1.5 mL batches of cell solution. These suspensions were then gently rotated on the end-over-end mixer for 30 min. Washes: the suspensions were spun down at 13000 rpm for 5 min at 4 ºC, after which the liquid was removed. The beads were then resuspended in 1.5 mL of ice-cold PBS, spun down at 13000 rpm for 5 min at 4 ºC, after which the liquid was removed. The washes were repeated 3 times. The beads were then suspended in 100 µL 2 % SDS and heated at 100 ºC for 5 min, then spun down at 13000 rpm for 5 min at ambient temperature. The liquid (containing the targets) was transferred to new Eppendorffs. Some of this solution was kept for a Protein Assay. Gel solutions were made using 3 × SDS Sample Buffer (New England BioLabs 3 × SDS Sample Buffer, #B7703S), after which these solutions were frozen.

1D SDS-PAGE. Visualization was conducted using the Shevechenko Silver Stain method, which used: Silver Stain Fixing Solution - MeOH:acetic acid:H_2O 45:10:45, Silver Stain Sensitization Solution - 0.02 % sodium thiosulphate, Silver Stain Solution - 0.1 % silver nitrate, Silver Stain Developer - 20 g/L sodium bicarbonate and 0.4 g/L paraformaldehyde. The 5 % stacking gel was made using either a 10-well comb. The gel solution samples were defrosted, resuspended and heated at 100 ºC for 5 min to denature the proteins. 30 µL of sample was loaded into each well, or 10 µL marker (New England BioLabs Prestained Protein Marker, Broad Range, Premixed, #P7708S). The gel was run under 60 V for about 30 min, which was put up to 110 V once the dye front had reached the resolving gel. Approximately 2 hours later, when the dye front had reached the bottom of the gel, the gel was transferred to the fixing solution, in which the gel was left overnight. The gel was rinsed with distilled H_2O for at least one hour, and then sensitized with sensitization solution for 10 min. The gel was rinsed with 2 changes of distilled H_2O (one minute each) and then incubated with chilled silver stain solution for 30 min at 4 ºC. The gel was rinsed with 2 changes of distilled H_2O (one minute each) and then developed with silver stain developer. The reaction was stopped using 1 % acetic acid.

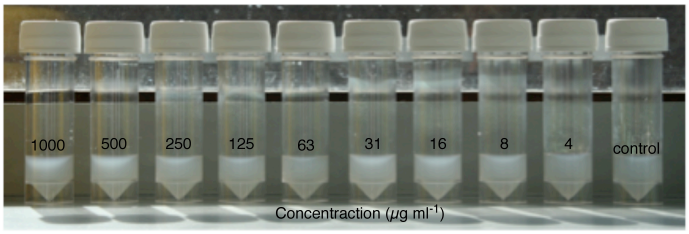


Protein assay. Used Bio-Rad Detergent Compatible Protein Assay. Preparation of BSA standard: the BSA standards were prepared in duplicate, in a total volume of 25 µL per standard, using Lysis buffer and a 10 mg/mL BSA stock. A range from 0 - 1.6 mg/mL in 0.2 mg/mL increments was used. Protein Estimation Procedure: to 25 µL sample and BSA standard, 125 µL of Working Reagent A' was added, and the mixtures were vortexed briefly. (Working Reagent A' = 49 parts Reagent A + 1 part Reagent S). 1 mL of Reagent B was added to each solution and vortexed immediately. The reactions were incubated at ambient temperature for 20 min. The absorbance at 750 nm (A_{750}) was measured for each sample. The average response was calculated and the a BSA standard curve was plotted, hence the protein concentration in the samples could be determined.

Results of the MIC assay with azithromycin and tobramycin. A series of cultures of *P. aeruginosa* PAO1 (wild-type) in the AGS medium were set up overnight in the presence of different concentrations of macrolides. A trial experiment was run with tobramycin, an antibiotic known to have an MIC against *P. aeruginosa* of around 3 μg/mL. The results indicated an MIC of 2.0 μg/mL in AGS medium (supporting information figure 1). An MIC of > 1000 μg/mL was recorded for azithromycin (supporting information figure 2). Also, at azithromycin concentrations < 64 μg/mL, in shake flasks in liquid culture, there is no effect on the growth curves (rate or stationary phase OD reached) compared to untreated samples.

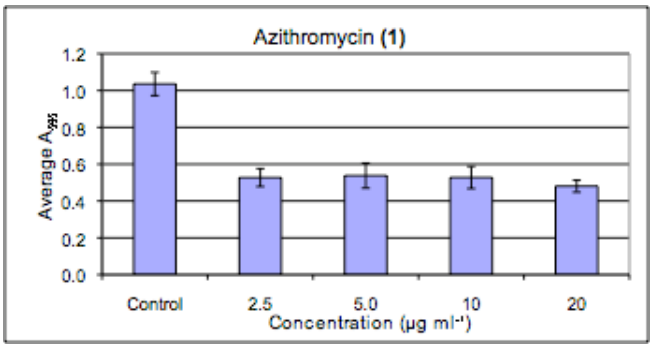


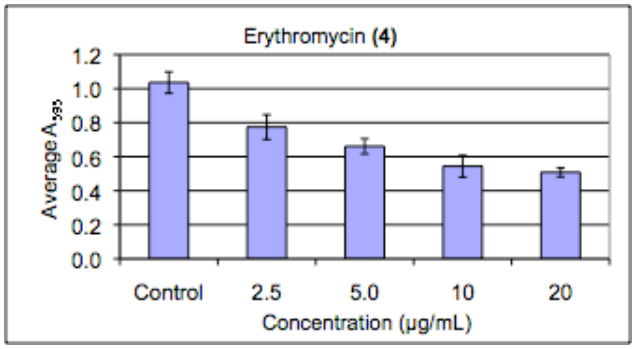
Supporting Information Figure 1. *P. aeruginosa* MIC experiment with tobramycin.

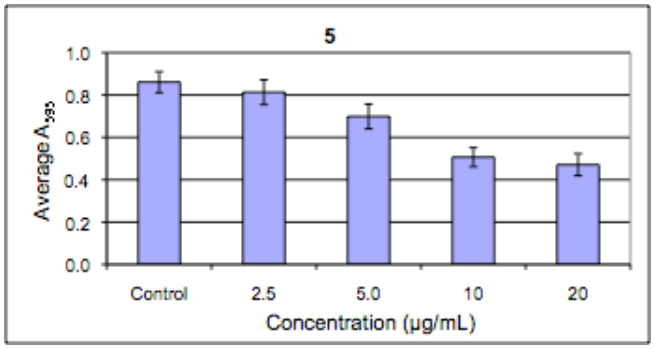


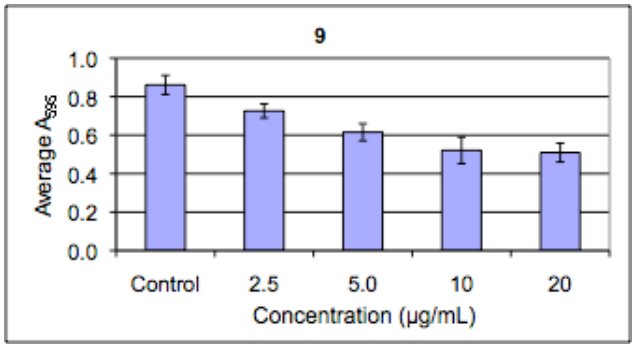
Supporting Information Figure 2. *P. aeruginosa* MIC experiment with azithromycin.

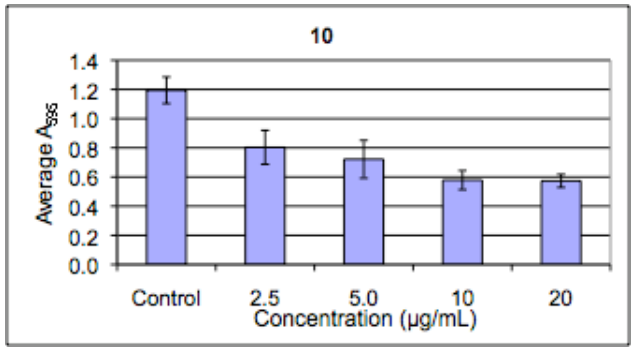
Results of the biofilm microtitre plate assay. In addition to screening azithromycin **(2)**, erythromycin **(4)**, and compounds **5** and **9**-**11** were screened. The absorption at 595 nm (A_{595}) at various concentrations (supporting information figure 3) and a comparison at 20 μg/mL (supporting information figure 4) can be seen below:

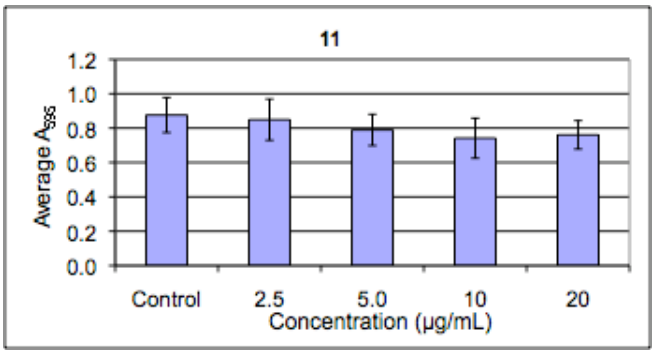


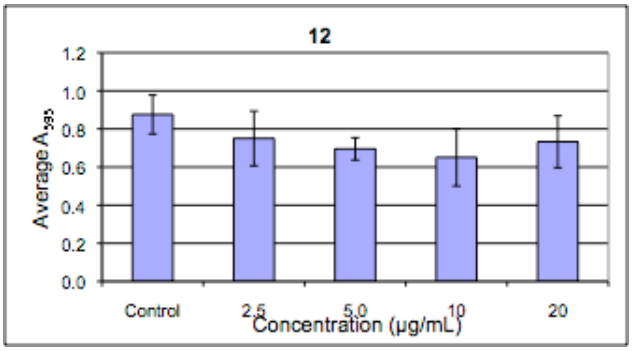




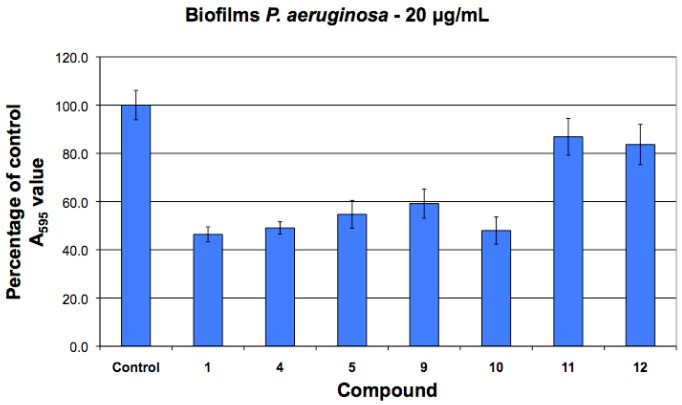




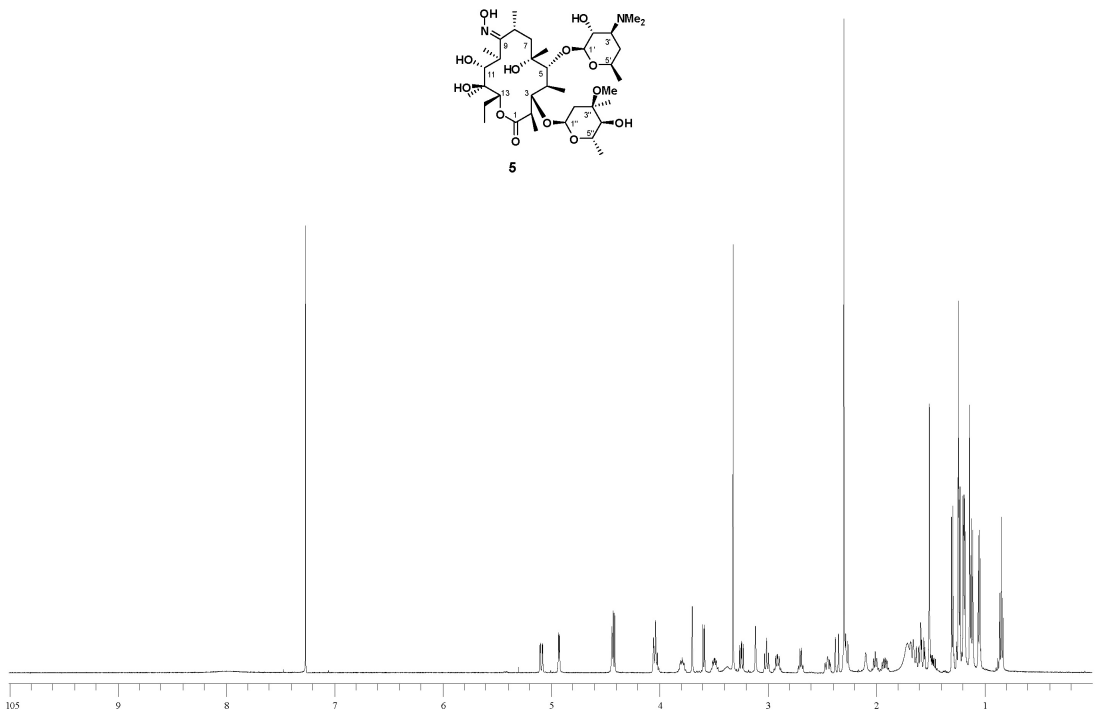




Supporting Information Figure 3. *P. aeruginosa* biofilm assays with various compounds at various concentrations.



Supporting Information Figure 4. A comparison of the *P. aeruginosa* biofilm assays with various compounds at 20 μg/mL.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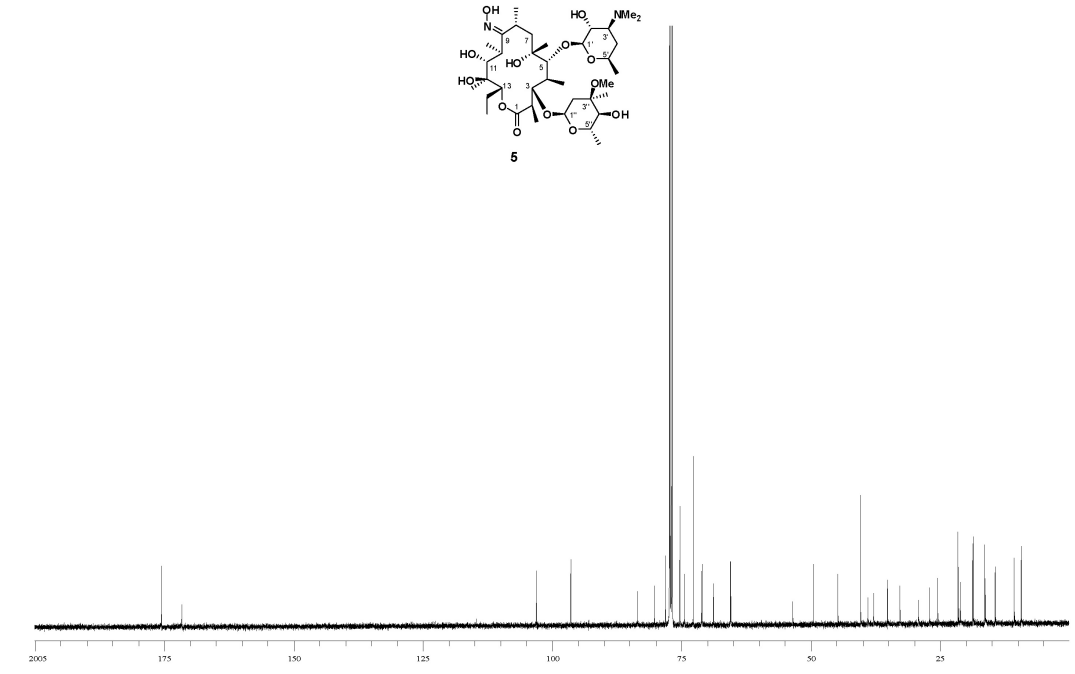
1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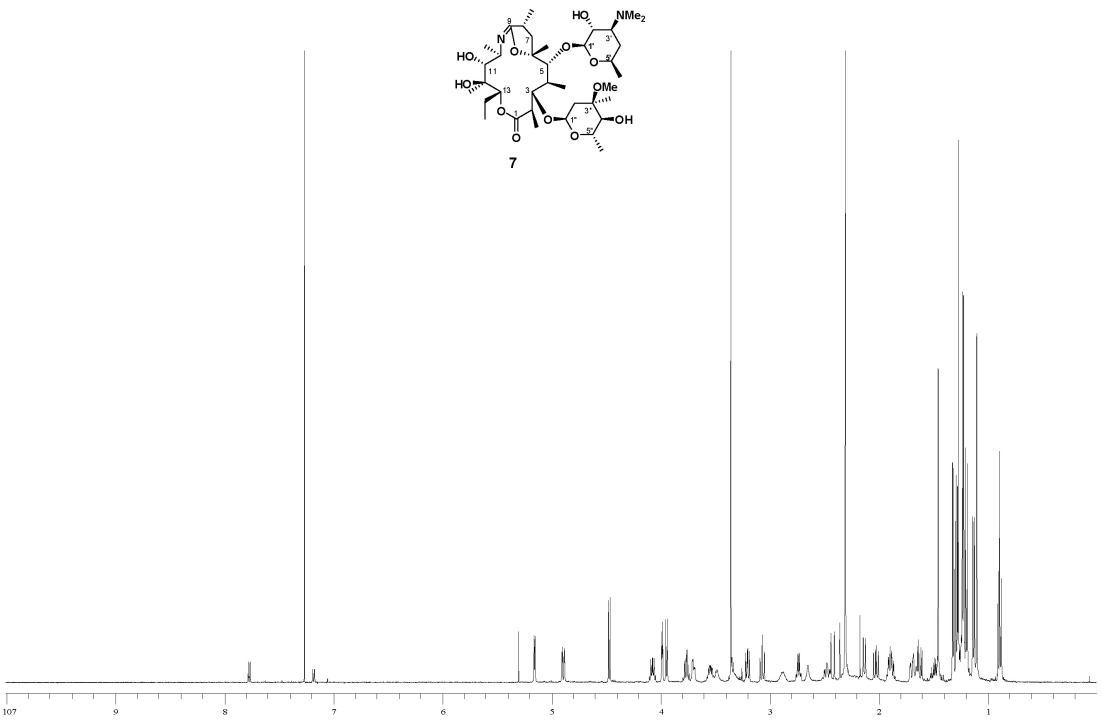
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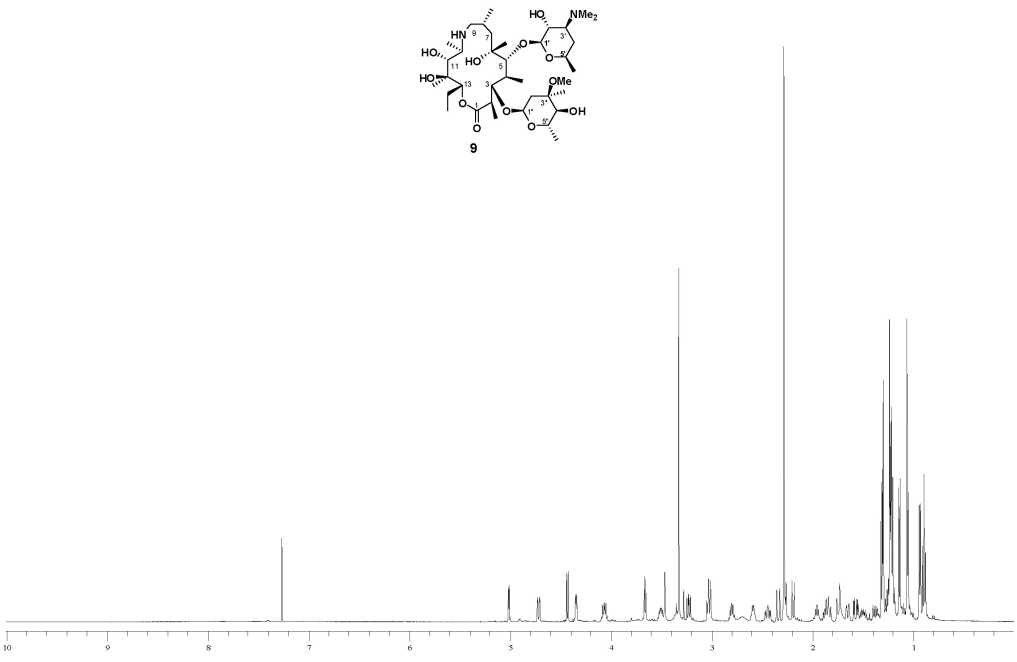
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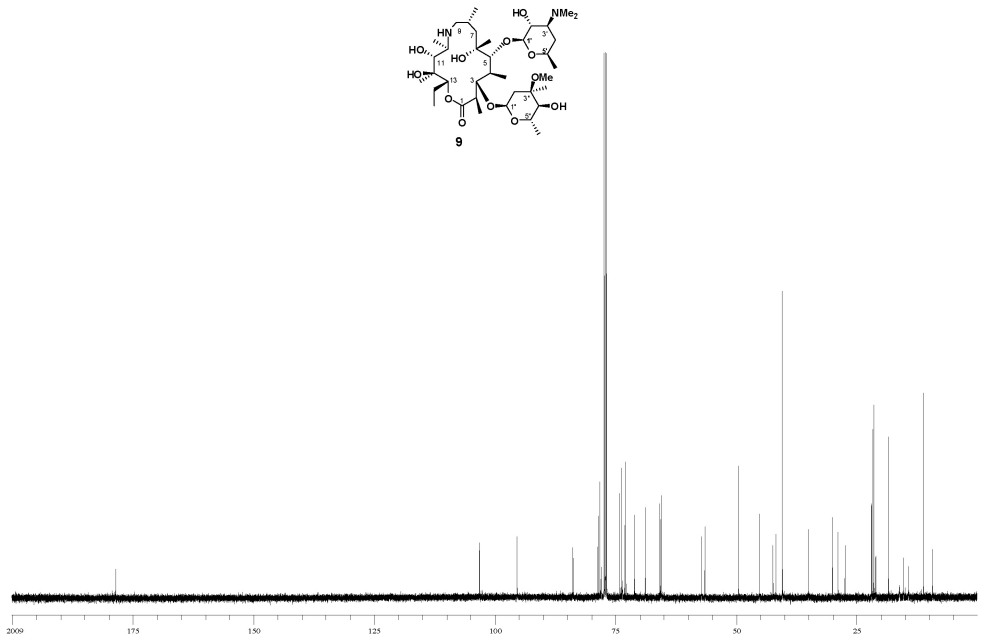
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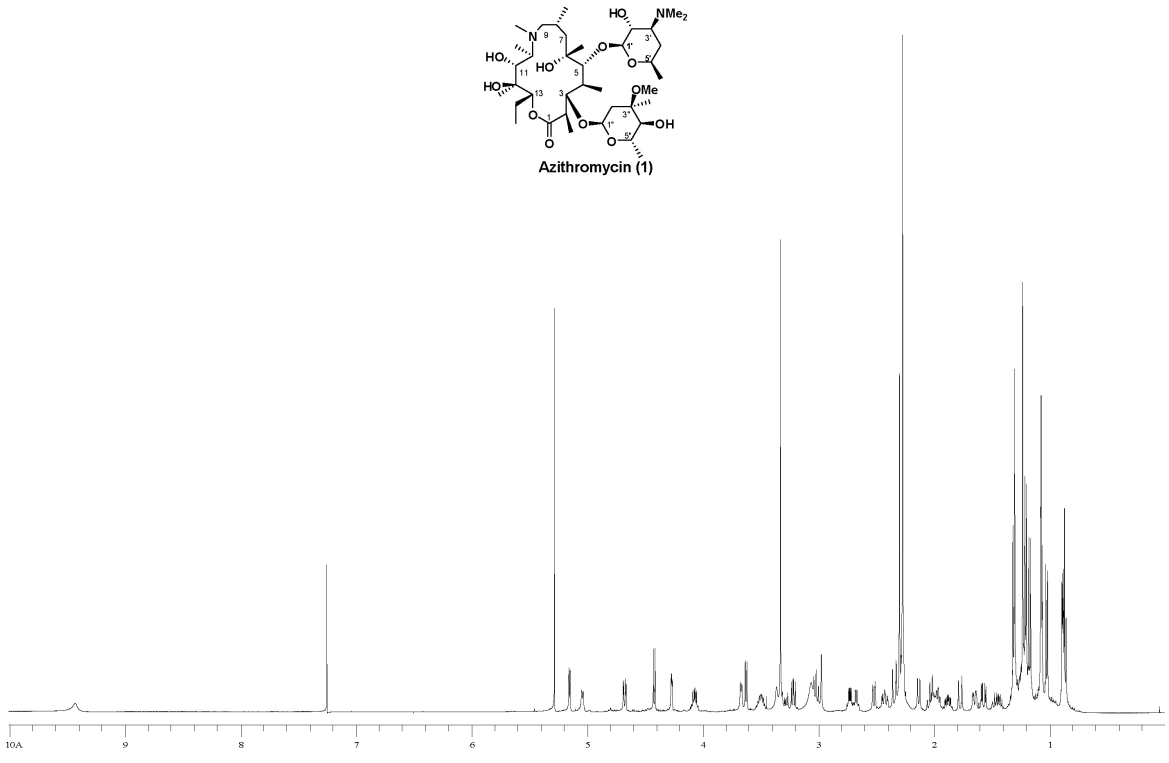
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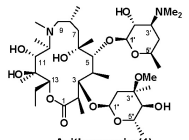
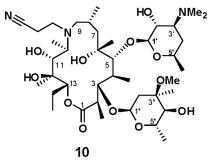


Azithromycin (1)



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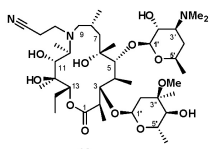

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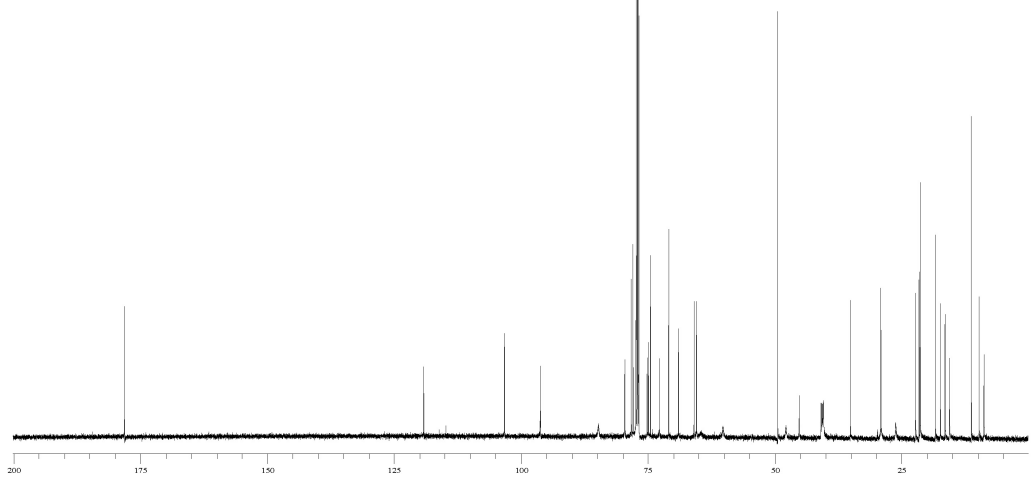

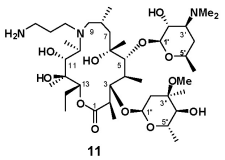

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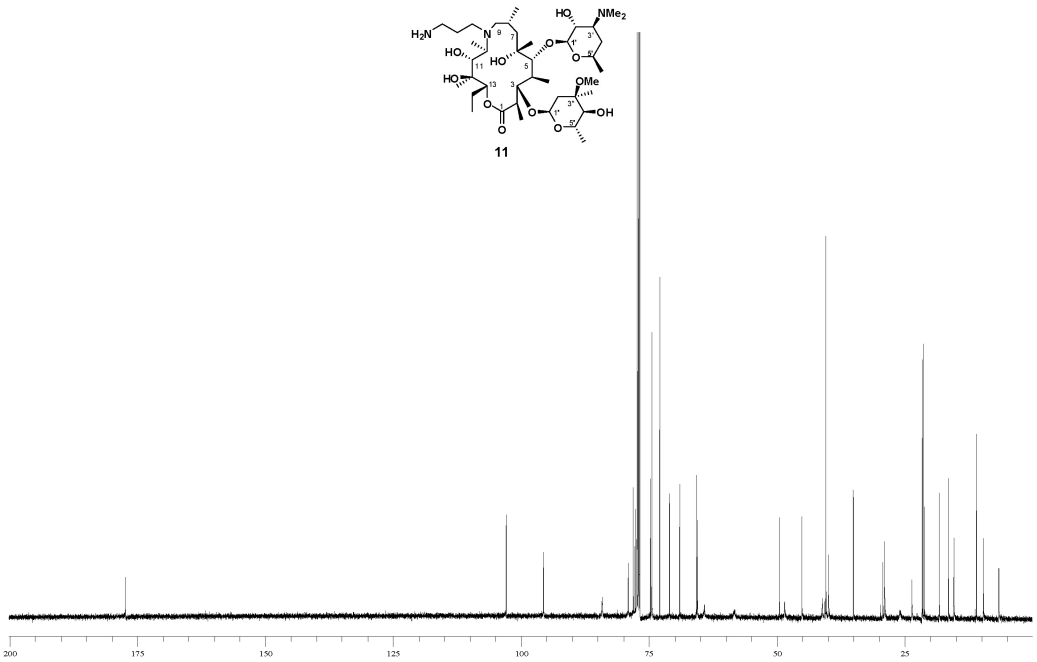

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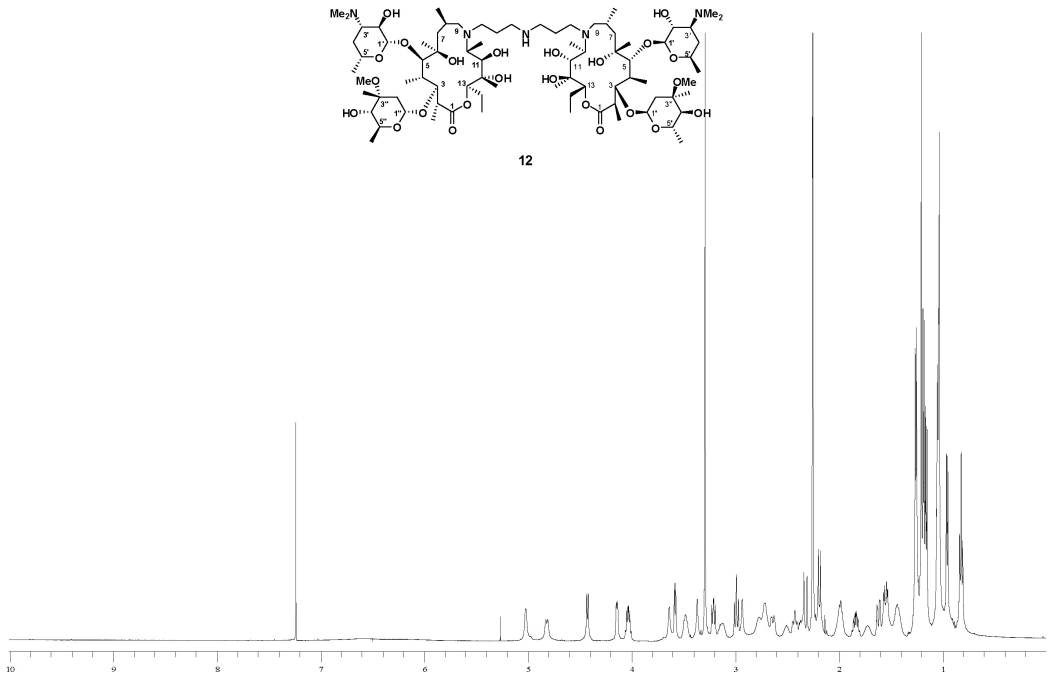

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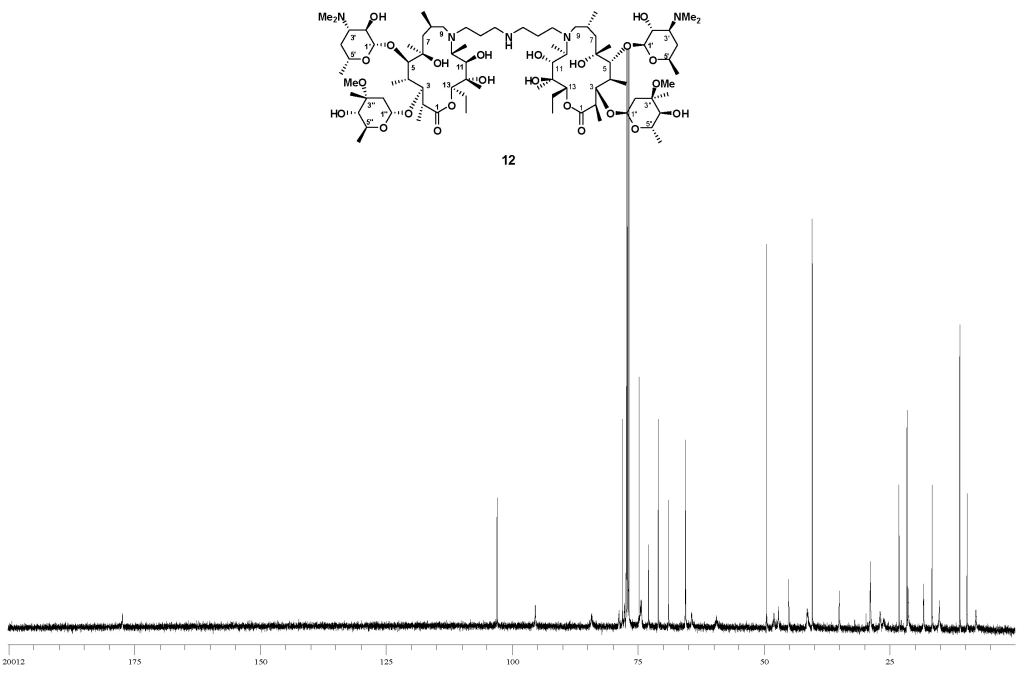

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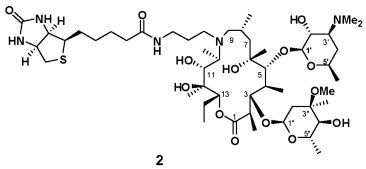

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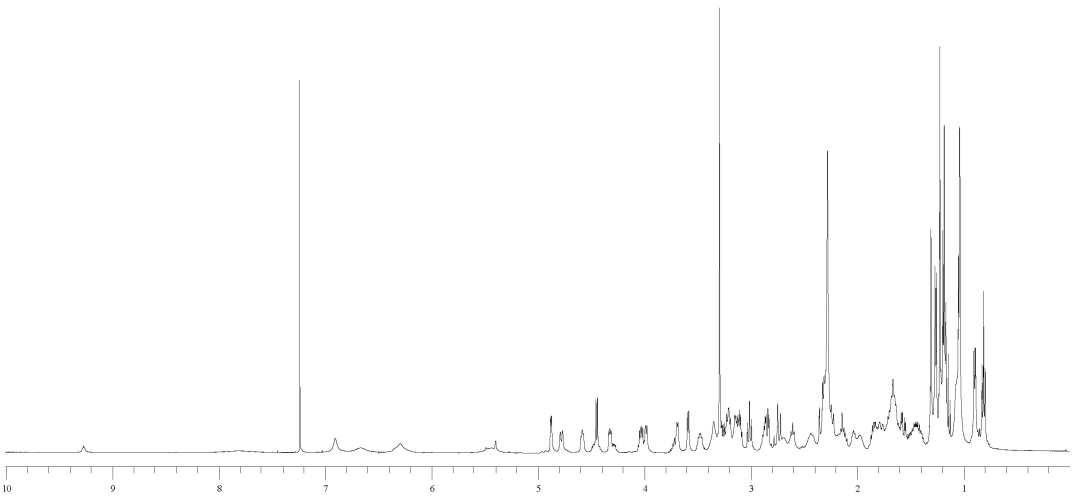

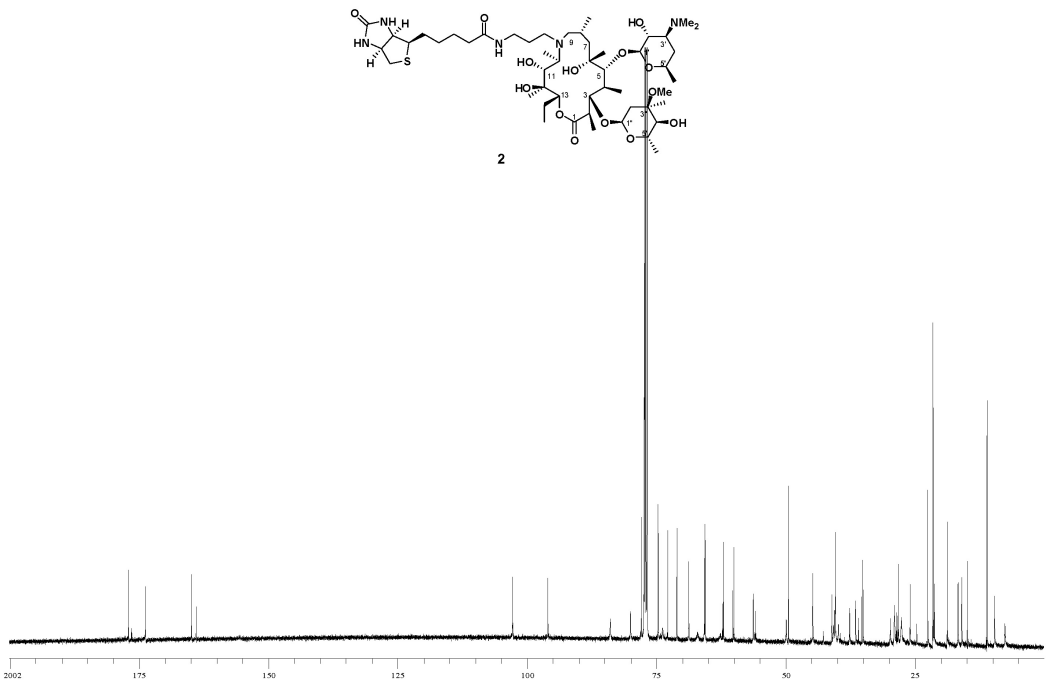
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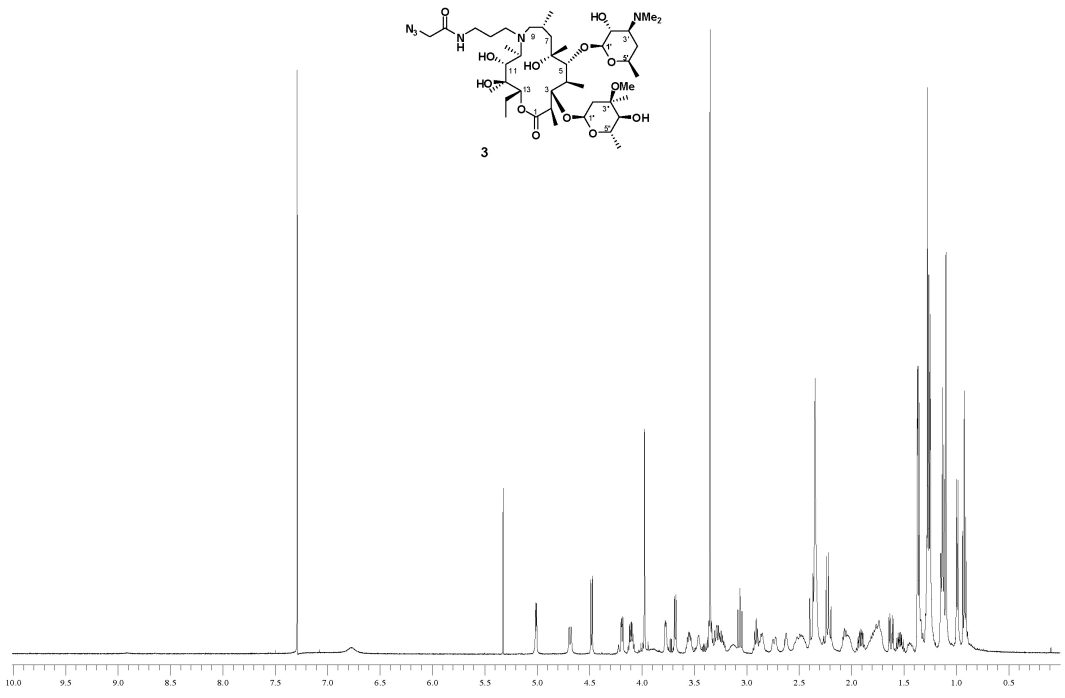
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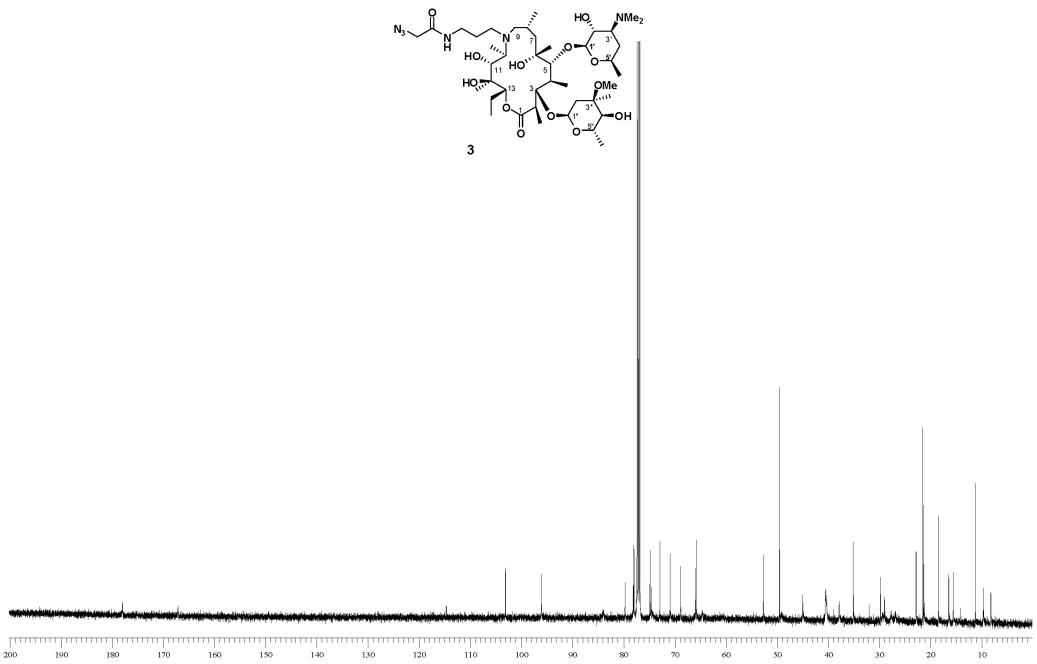

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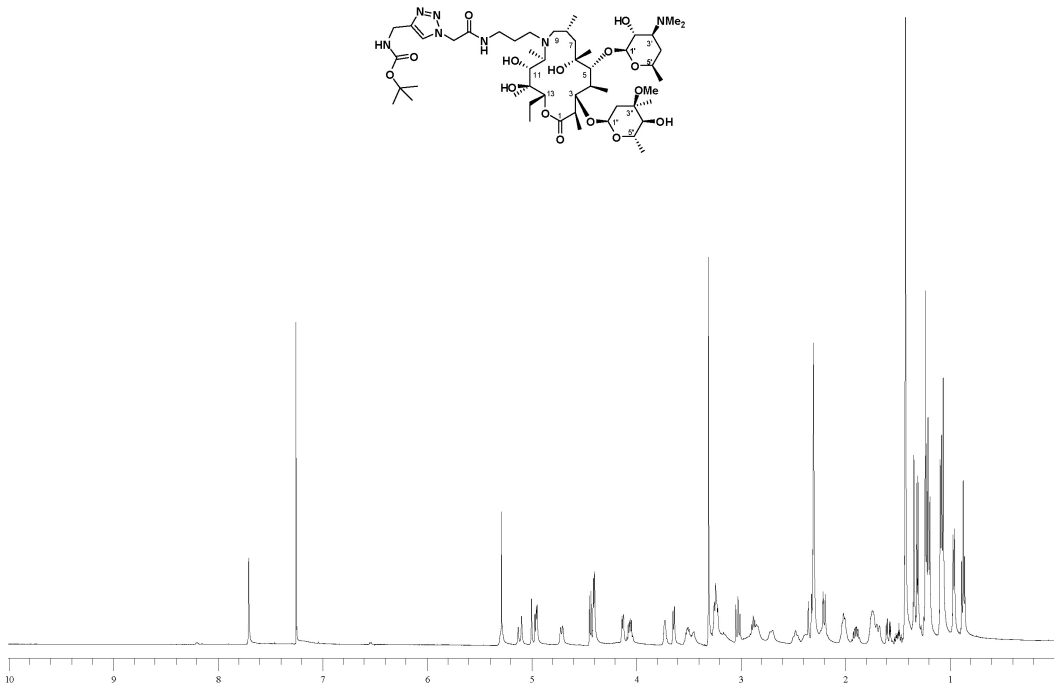

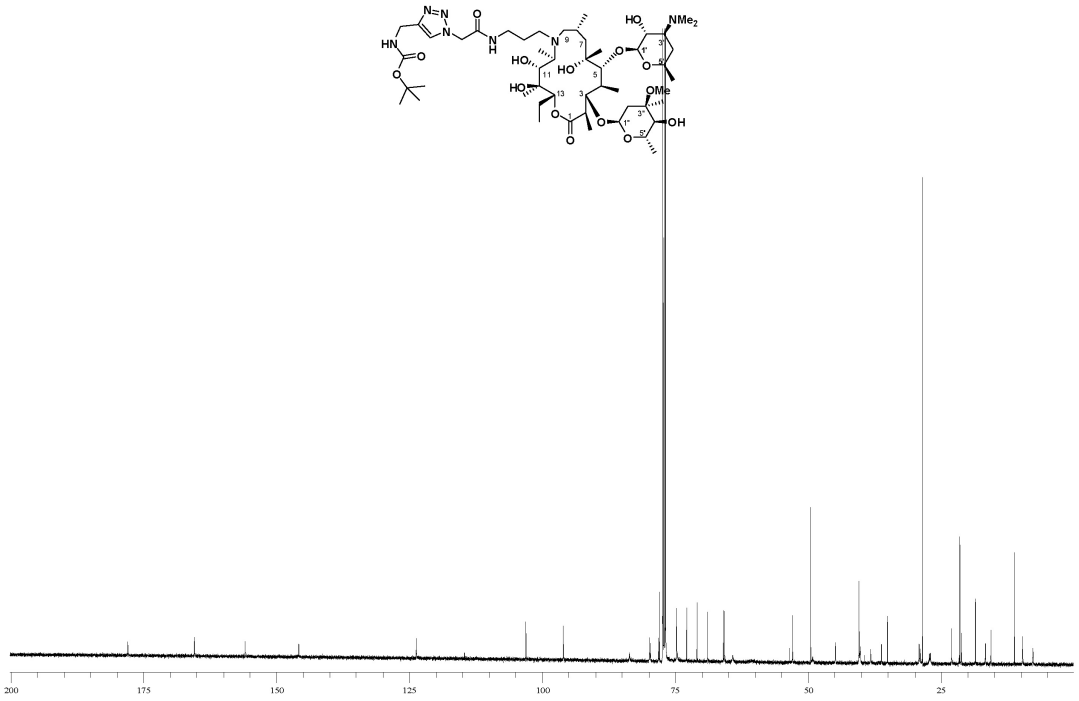
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