Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

Covalent Lectin Inhibition and Application in Bacterial Biofilm Imaging

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Author Contributions

S.W. performed and analyzed microbiology and biofilm experiments, D.H. performed all chemical syntheses except for compound **17**, which was synthesized by R.S., I.J., and R.S. performed the competitive LecA inhibition and binding assays, M.H. and R.M. performed and analyzed protein MS and small molecule HRMS experiments, A.V. and A.I. established and analyzed the crystal structure of **3** in complex with LecA, A.T. conceived and designed the study, A.T. wrote the paper with input from all authors.

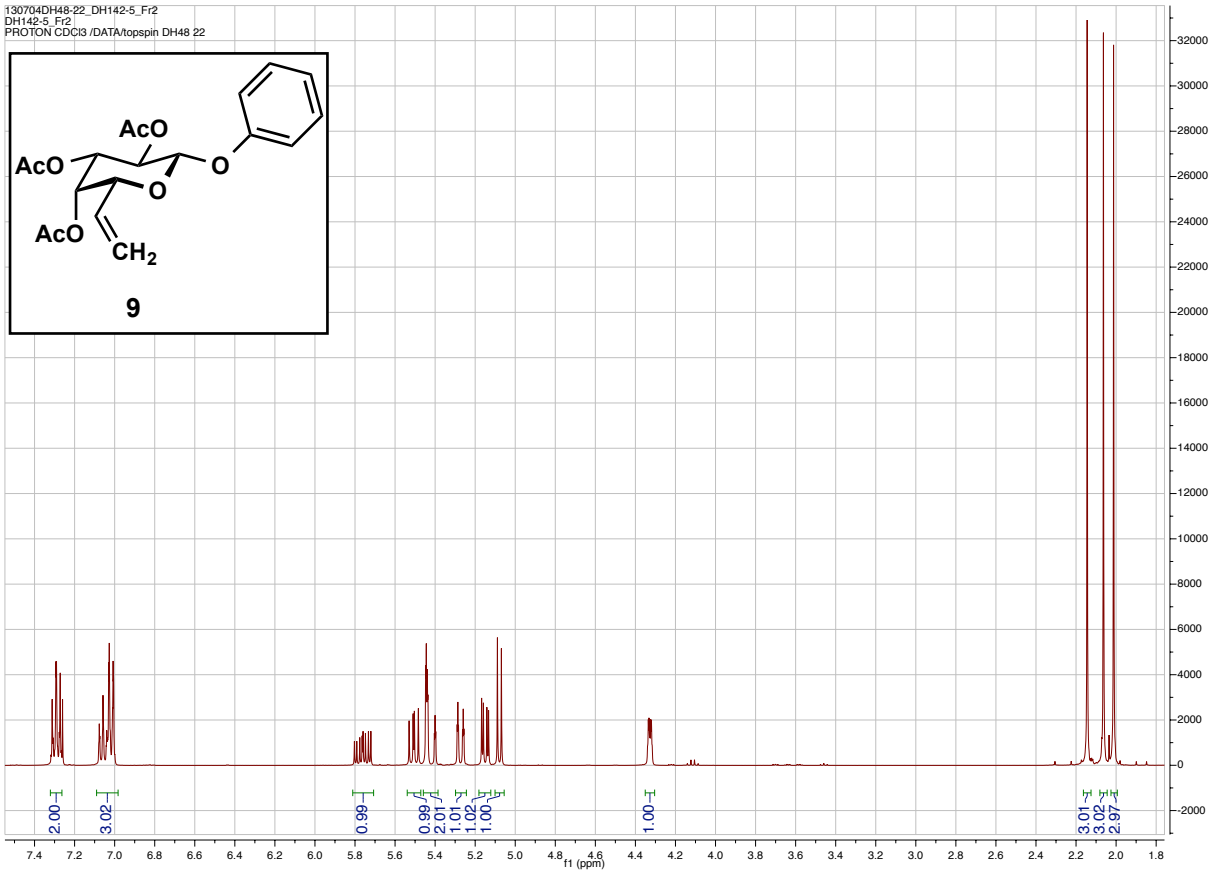

Figure S1: 1H NMR of **9** (DH142-5_Fr2)

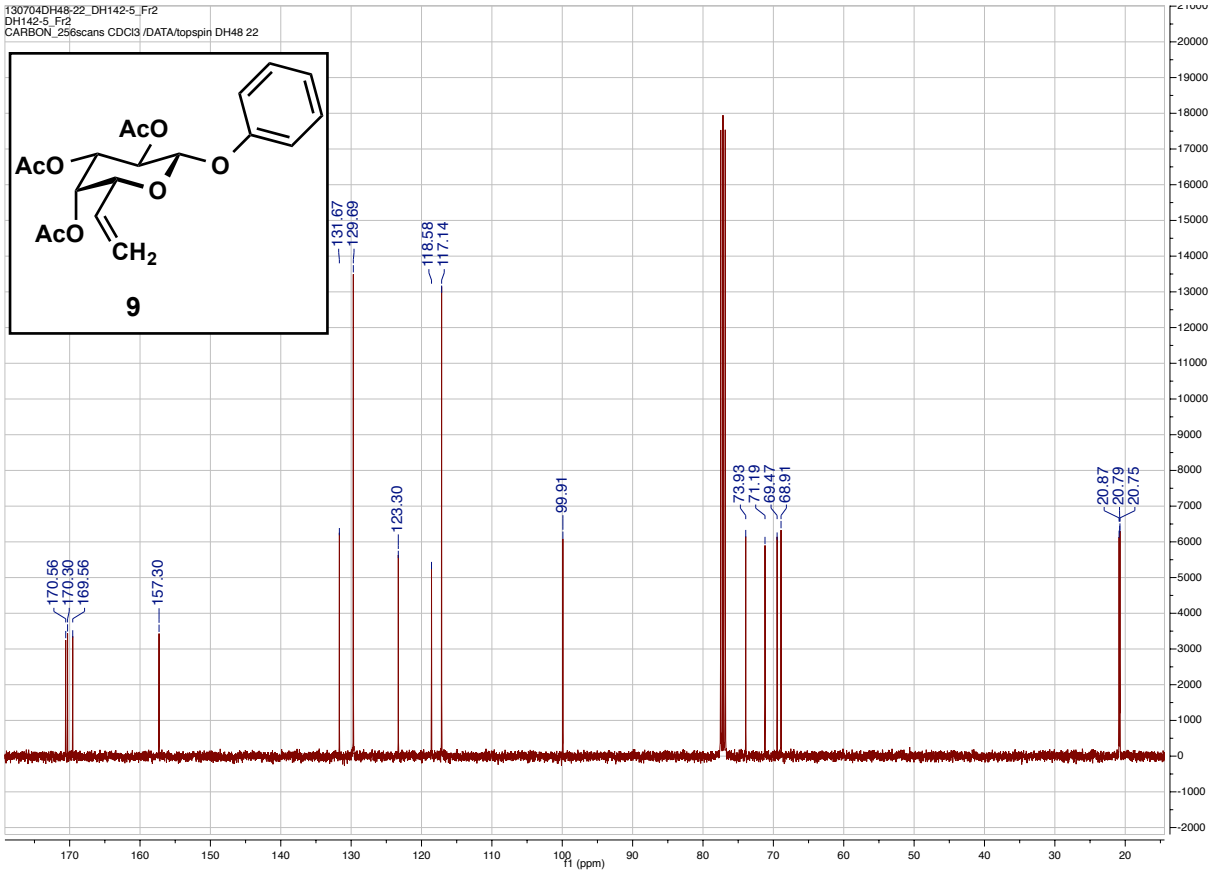

Figure S2: 13C NMR of **9** (DH142-5_Fr2)

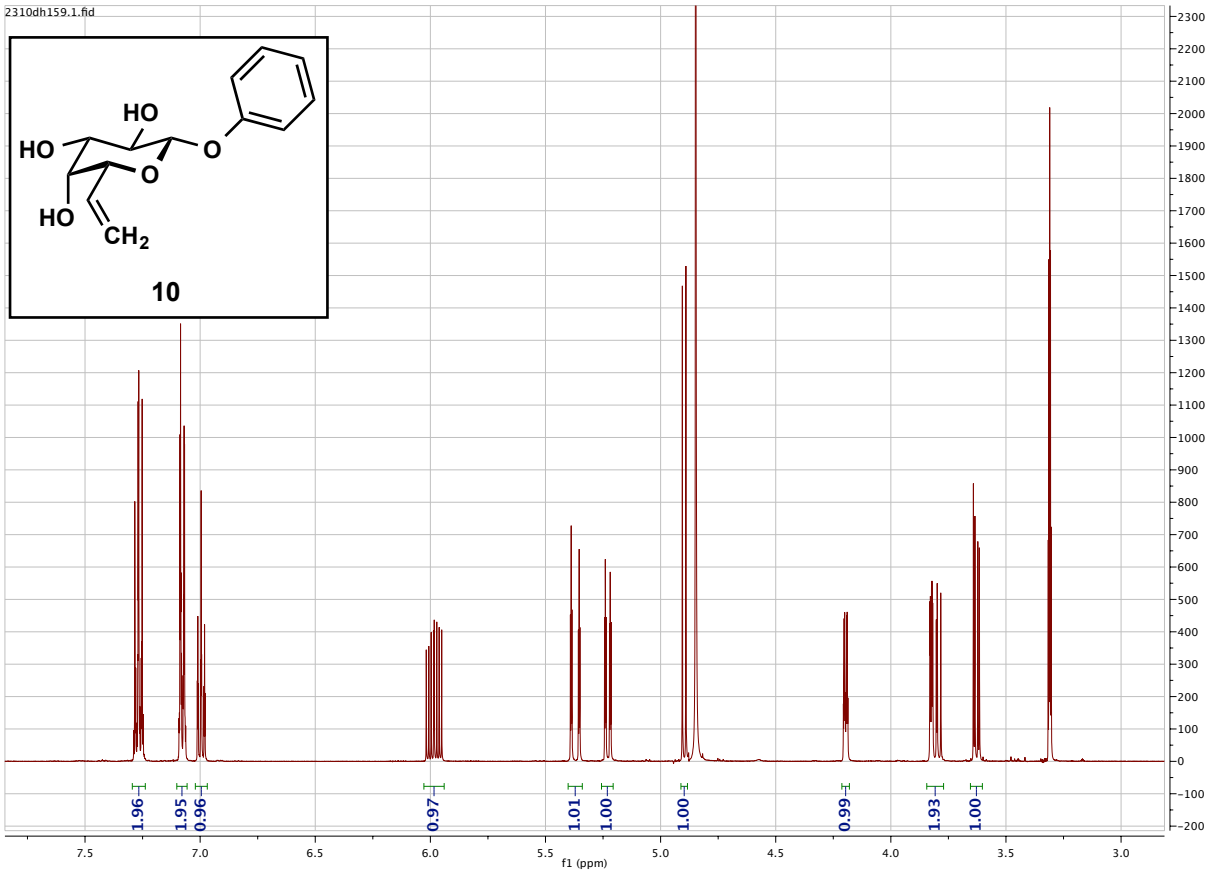

Figure S3: 1H NMR of **10** (DH159)

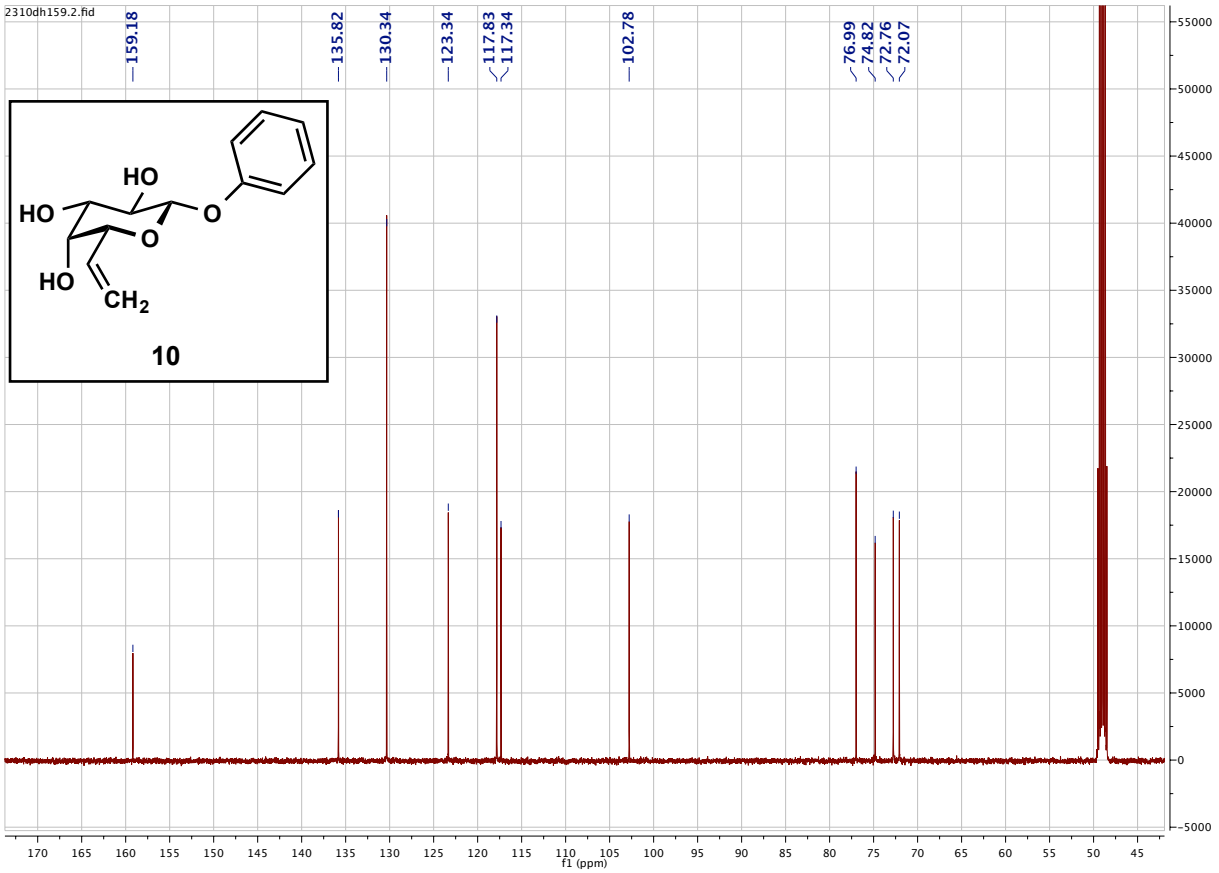

Figure S4: 13C NMR of **10** (DH159)

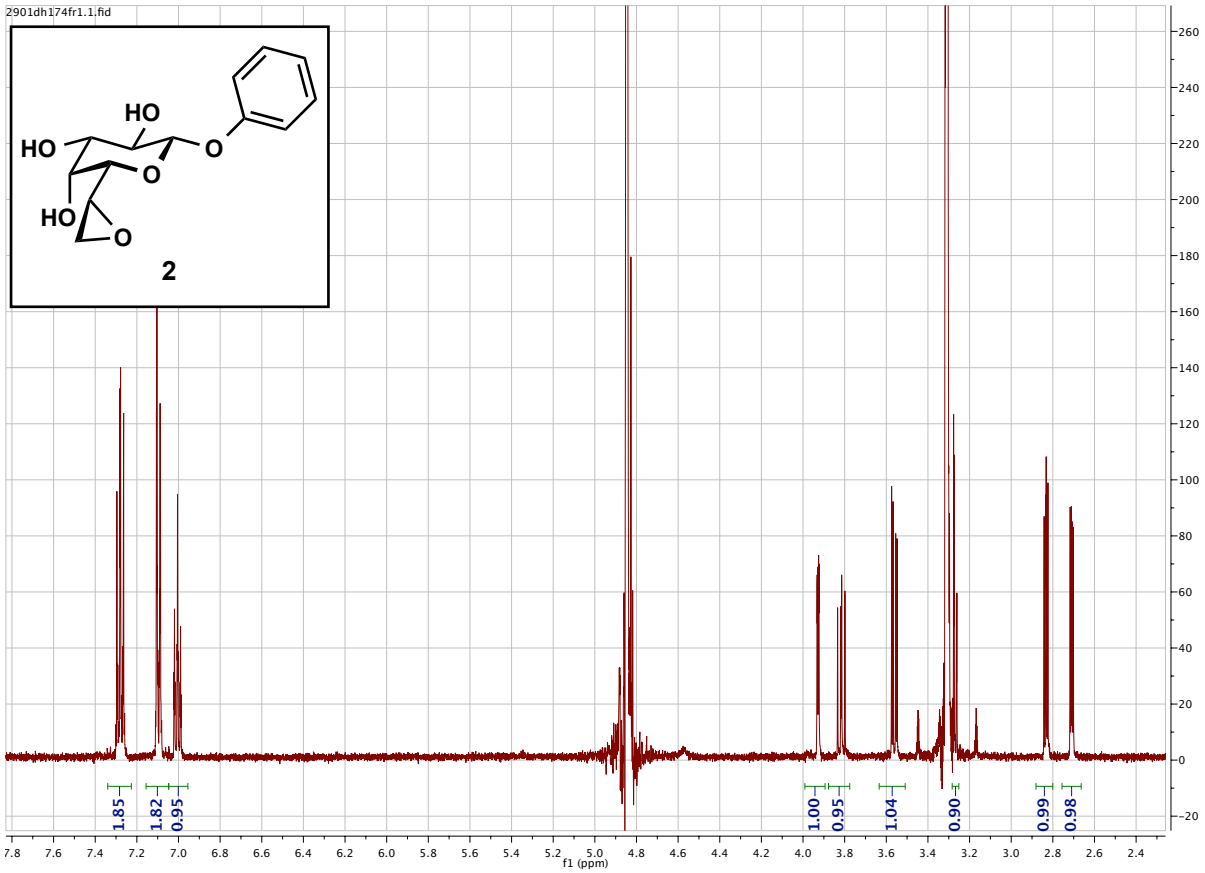

Figure S5: 1H NMR of **2** (DH174fr1)

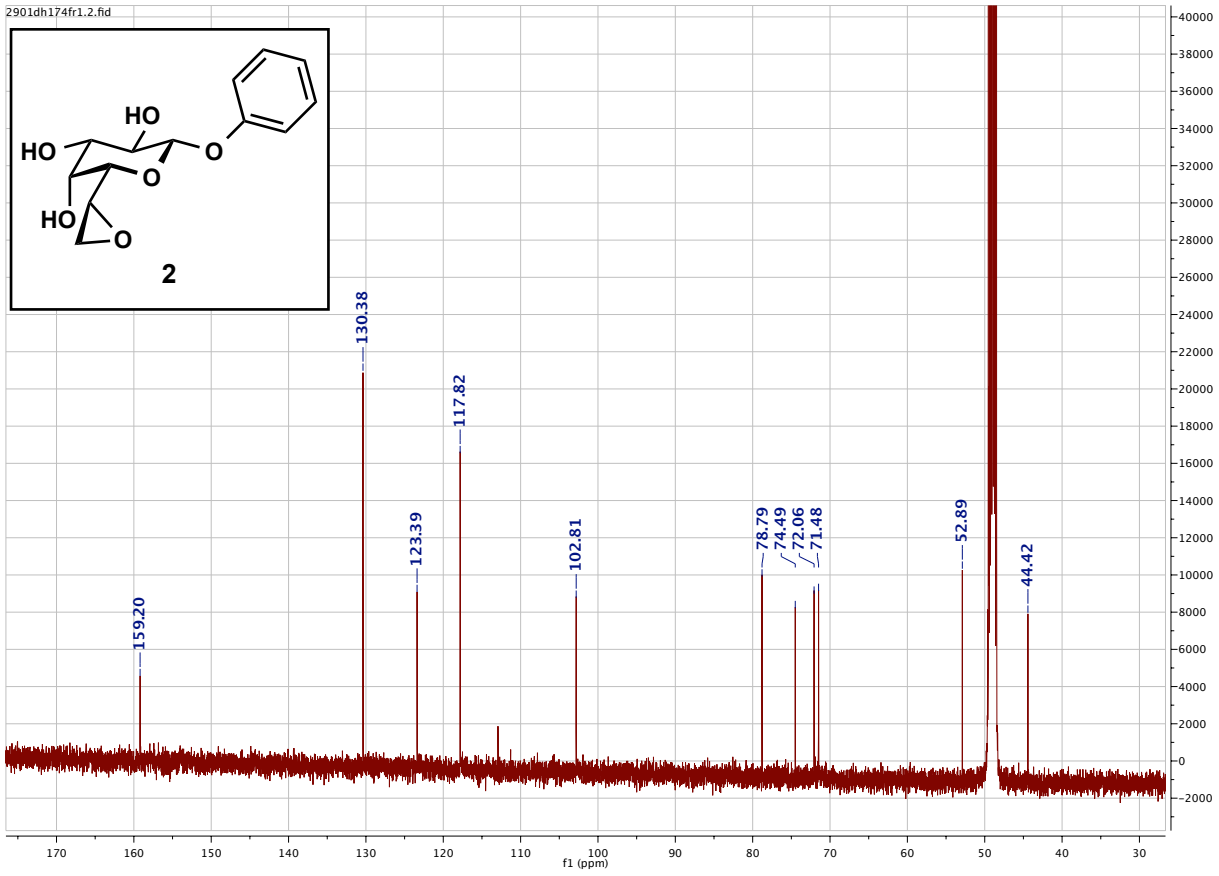

Figure S6: 13C NMR of **2** (DH174fr1)

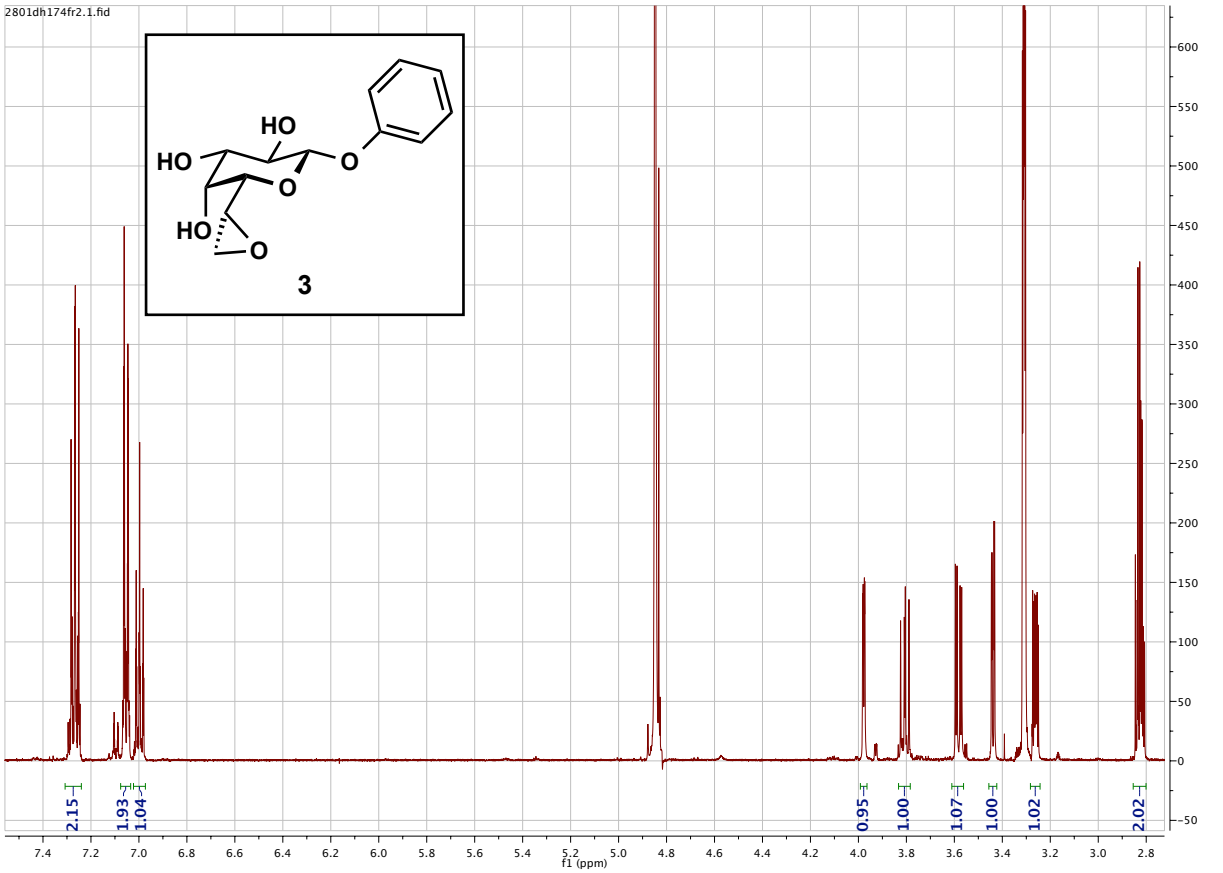

Figure S7: 1H NMR of **3** (DH174fr2)

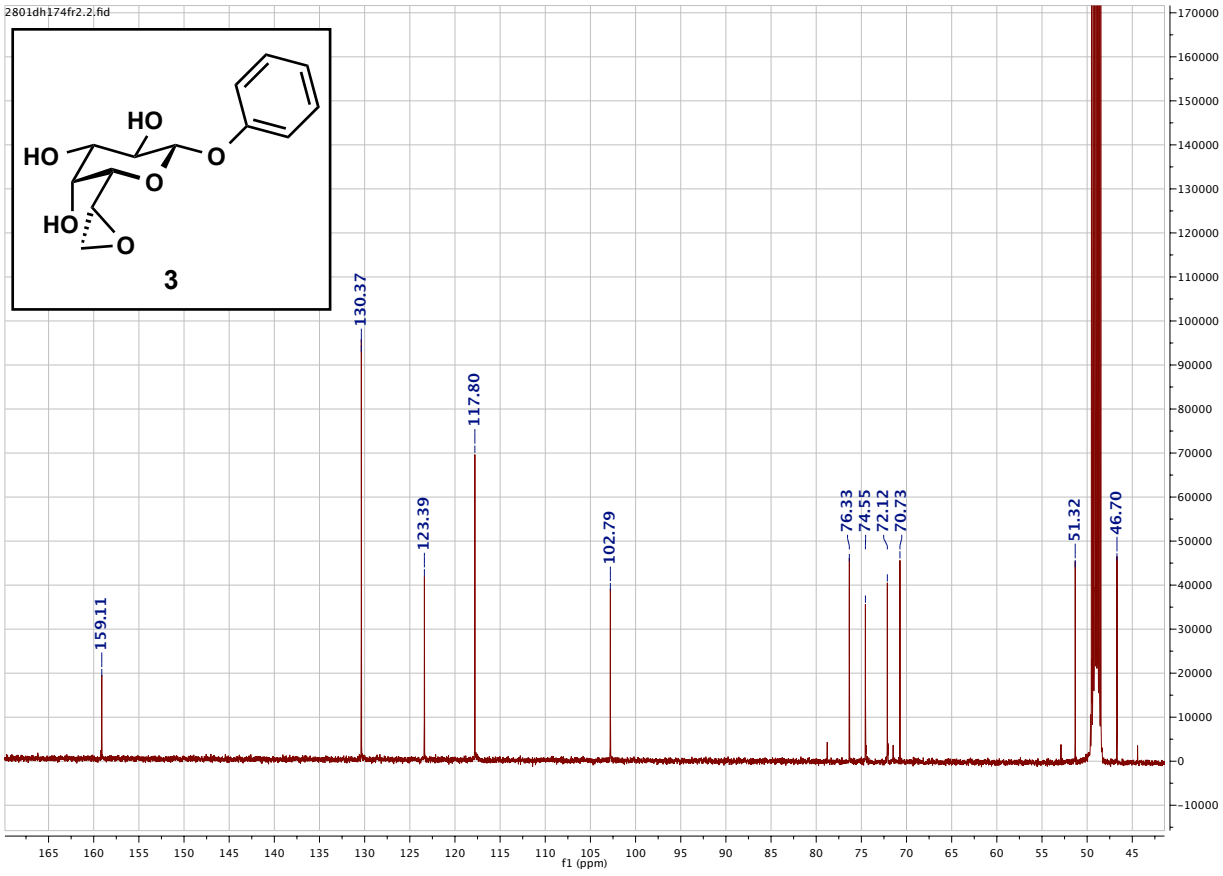

Figure S8: 13C NMR of **3** (DH174fr2)

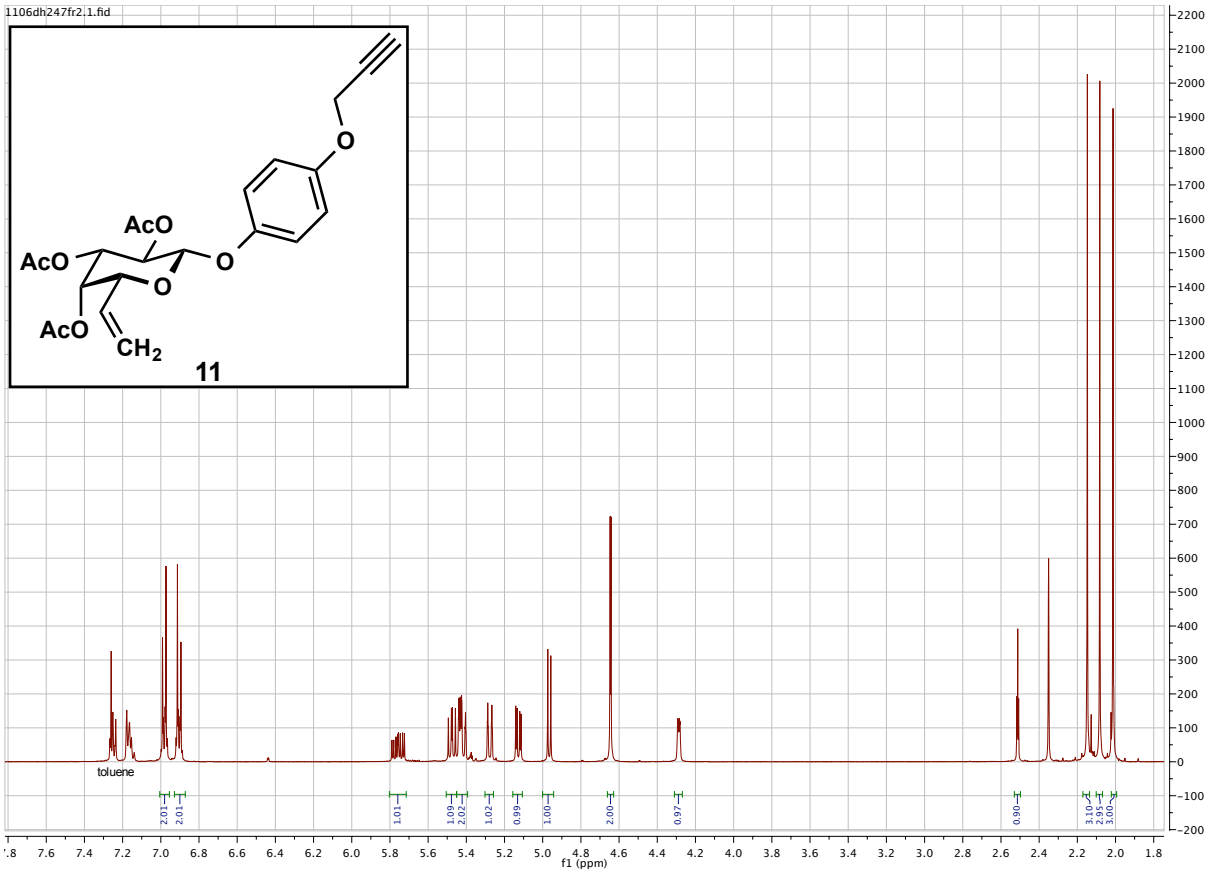

Figure S9: 1H NMR of **11** (DH247fr2)

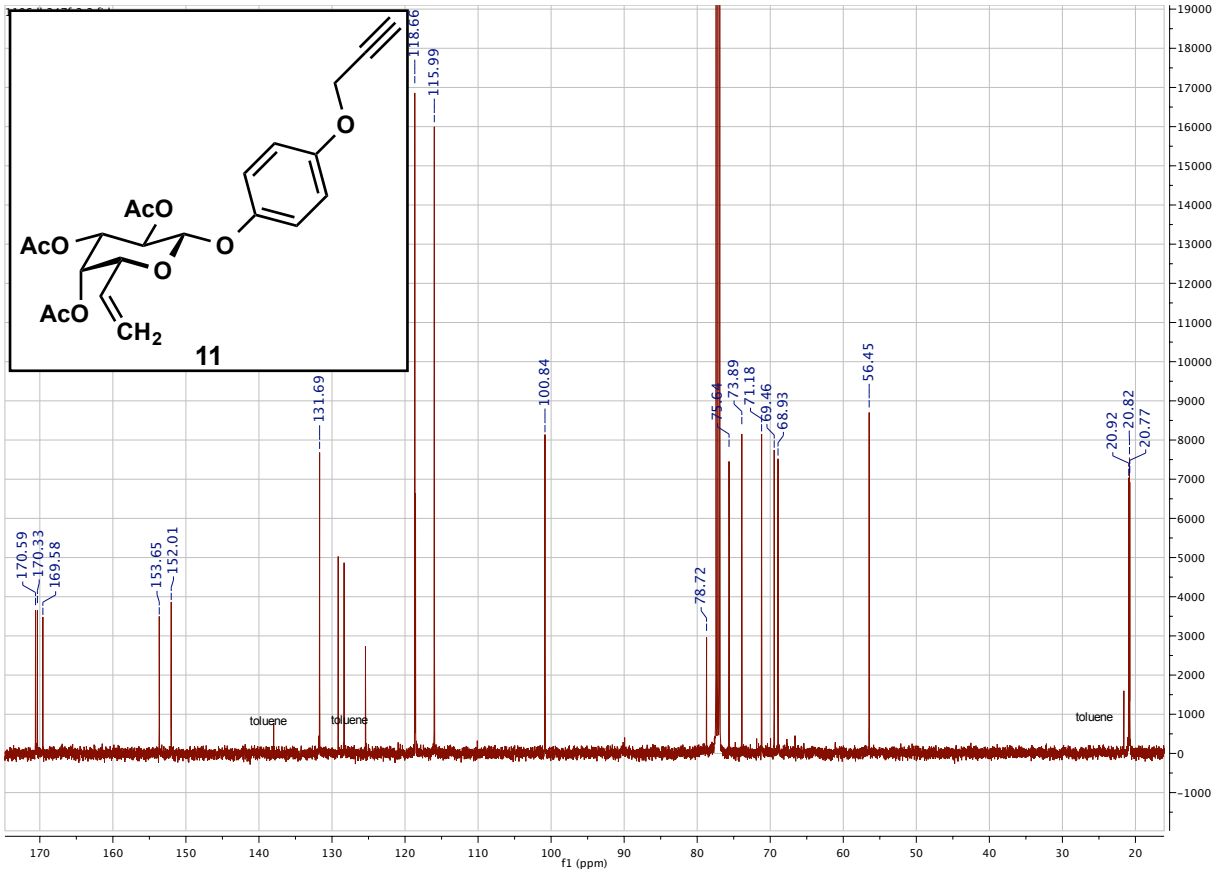

Figure S10: 13C NMR of **11** (DH247fr2)

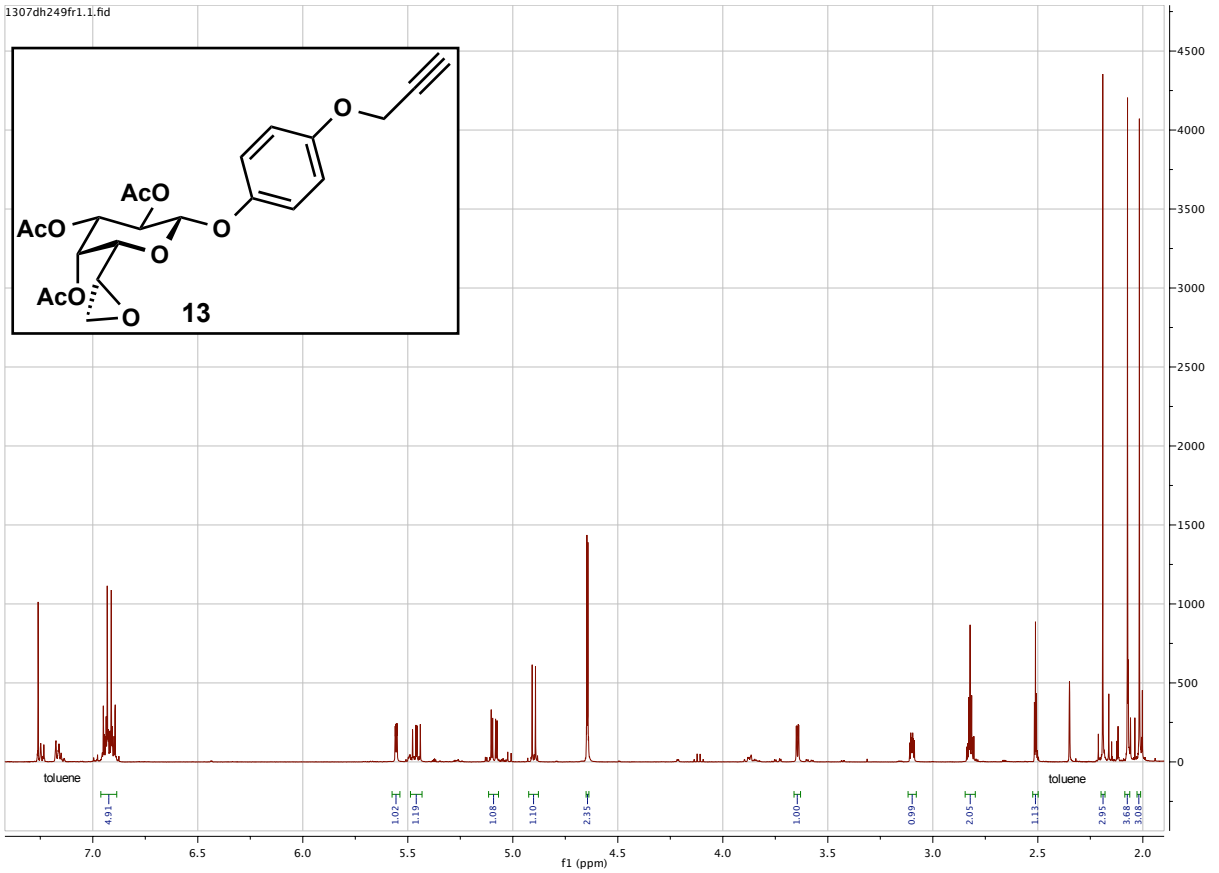

Figure S11: 1H NMR of **13** (DH249fr1)

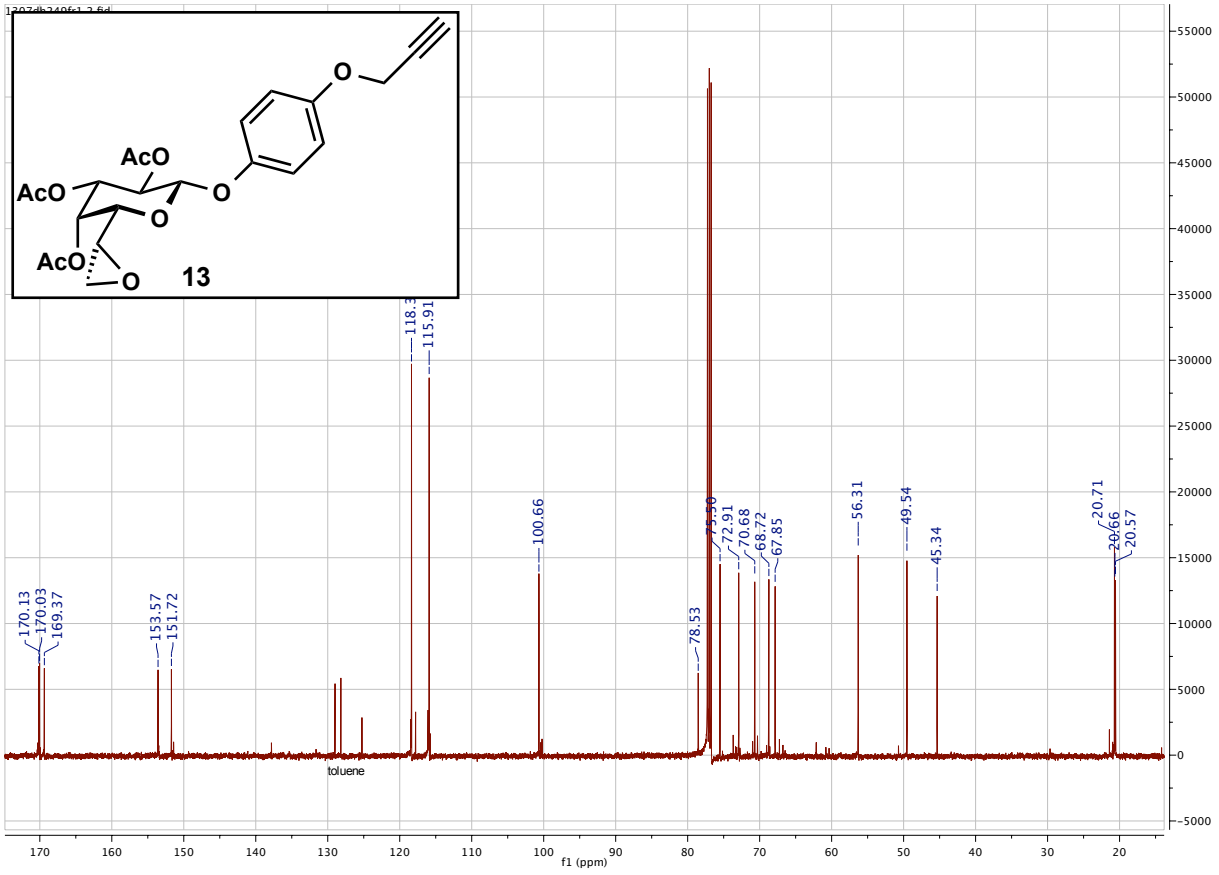

Figure S12: 13C NMR of **13** (DH249fr1)

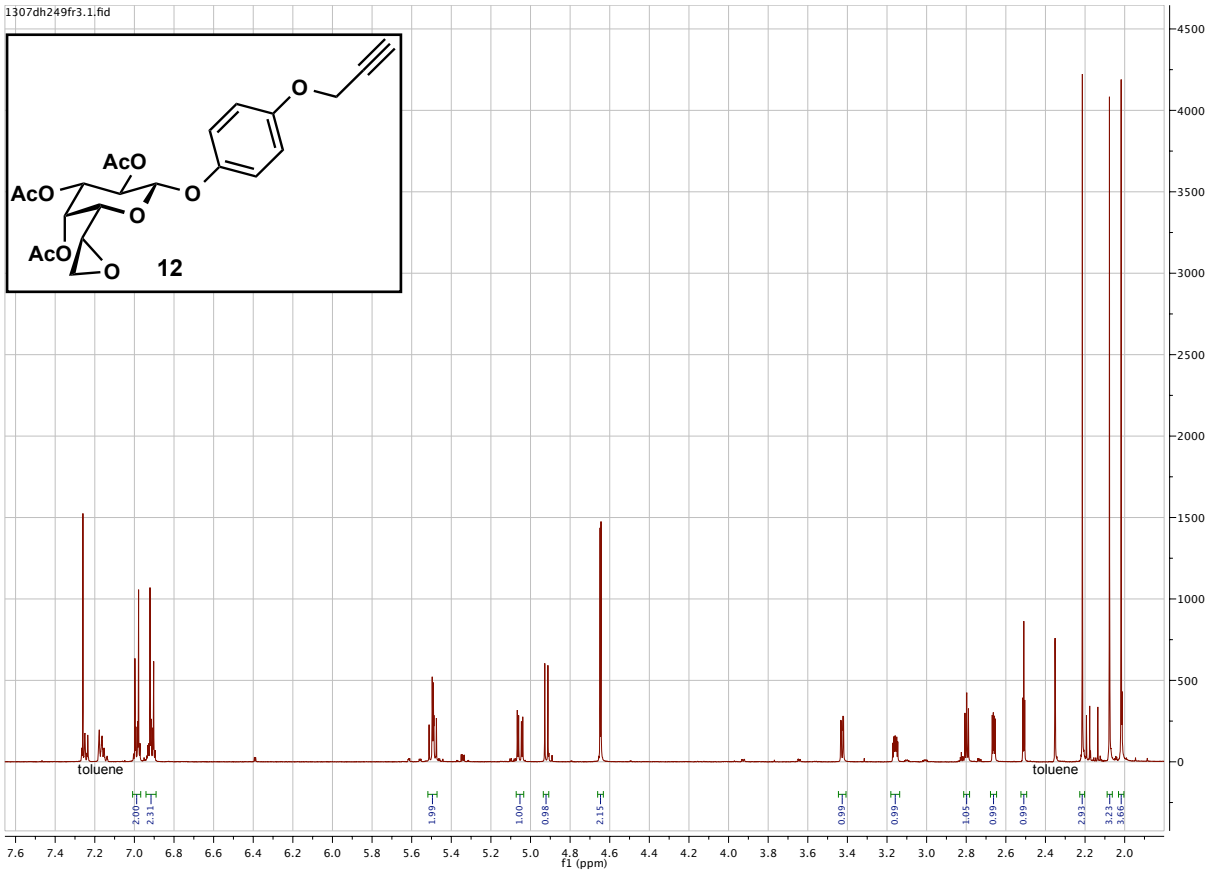

Figure S13: 1H NMR of **12** (DH249fr3)

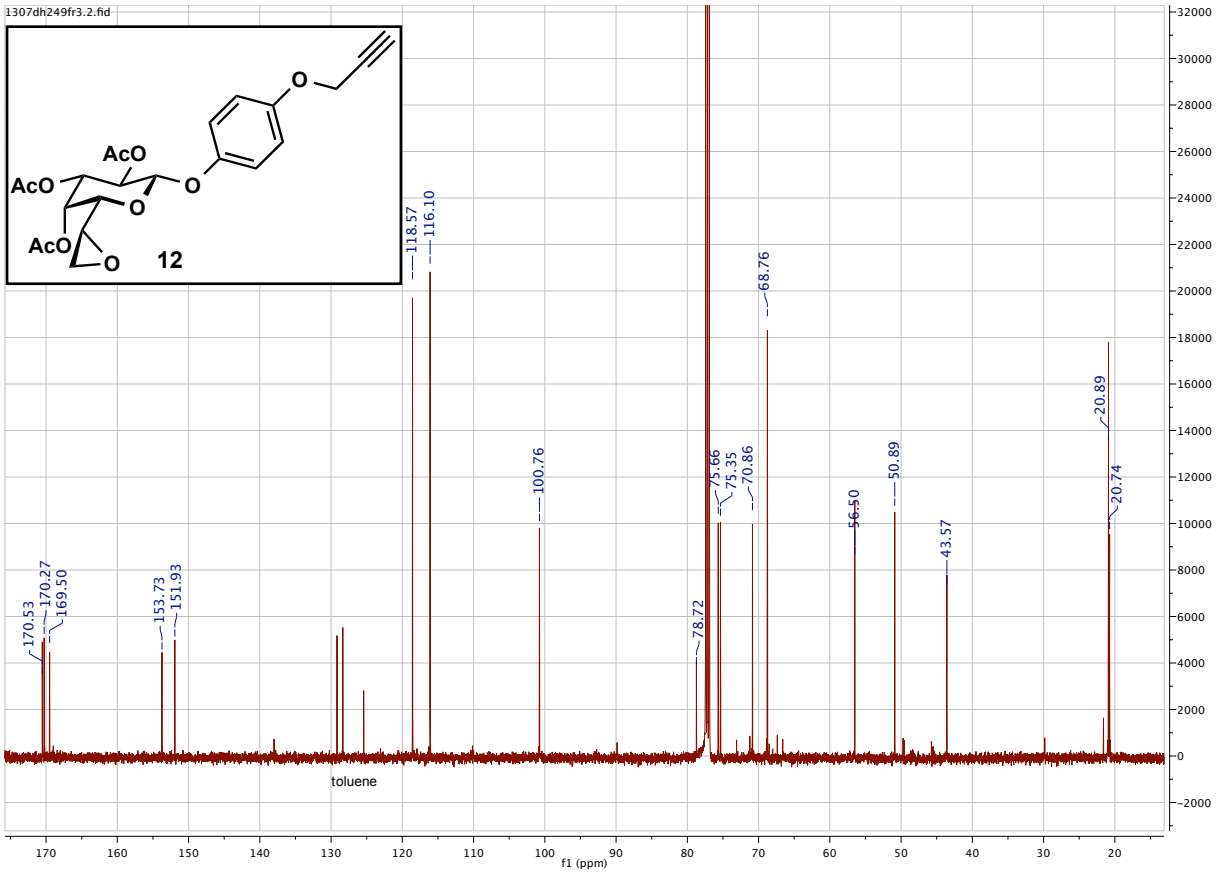

Figure S14: 13C NMR of **12** (DH249fr3)

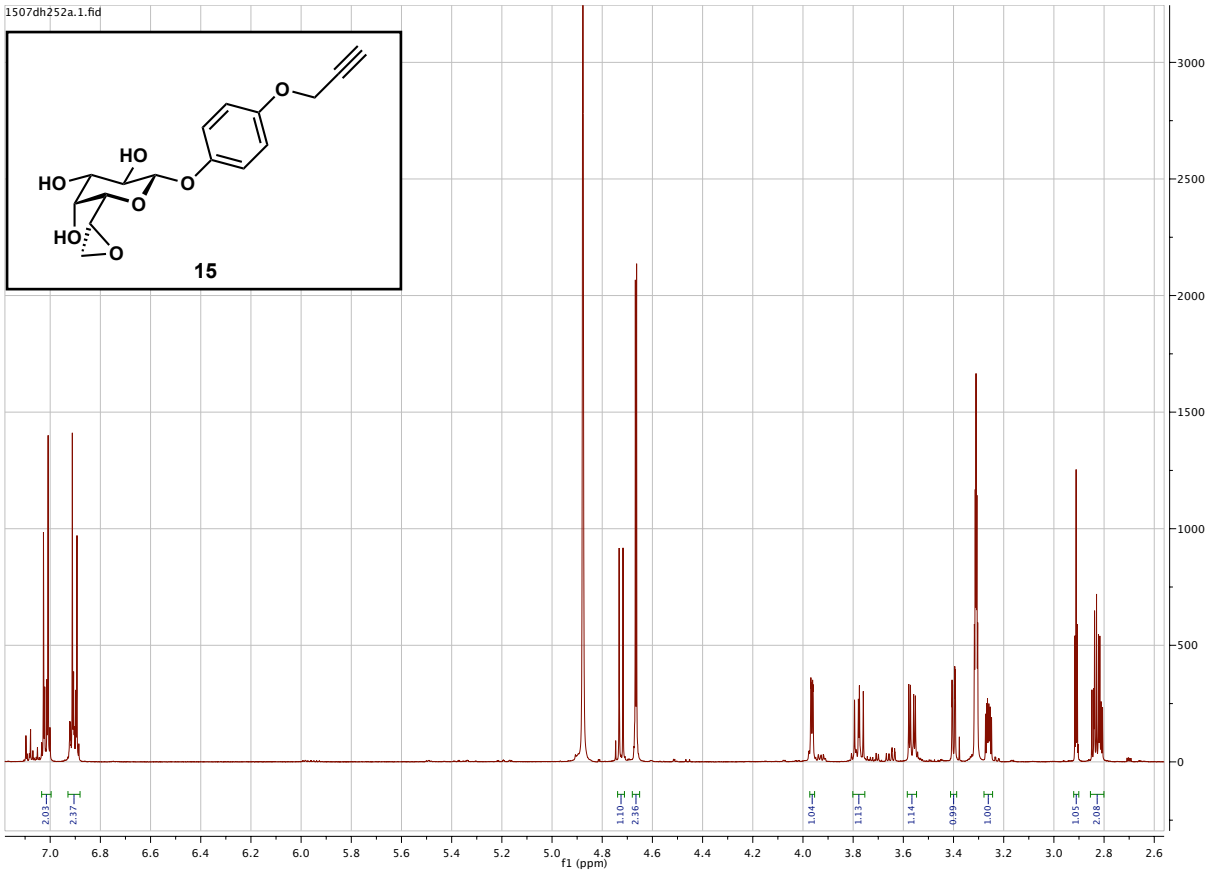

Figure S17: 1H NMR of **15** (DH252A)

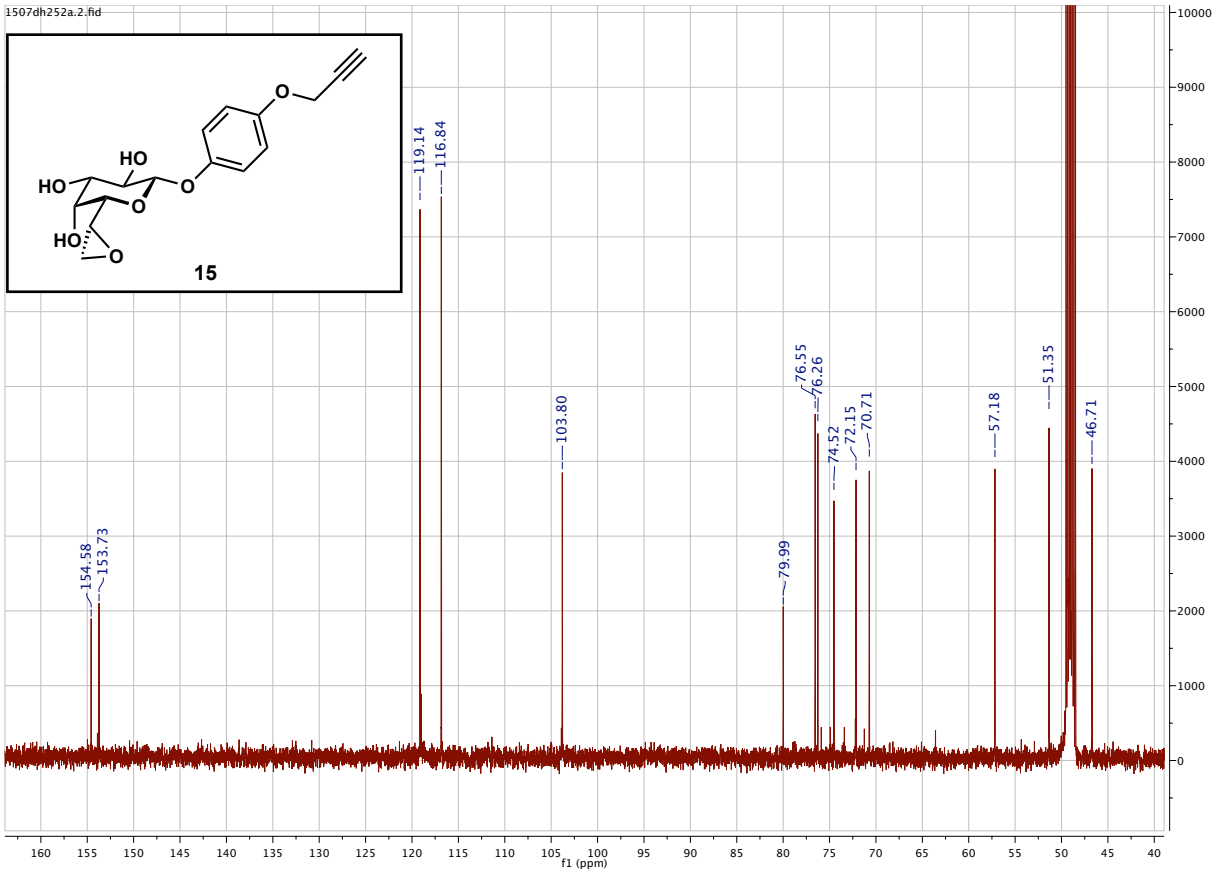

Figure S18: 13C NMR of **15** (DH252A)

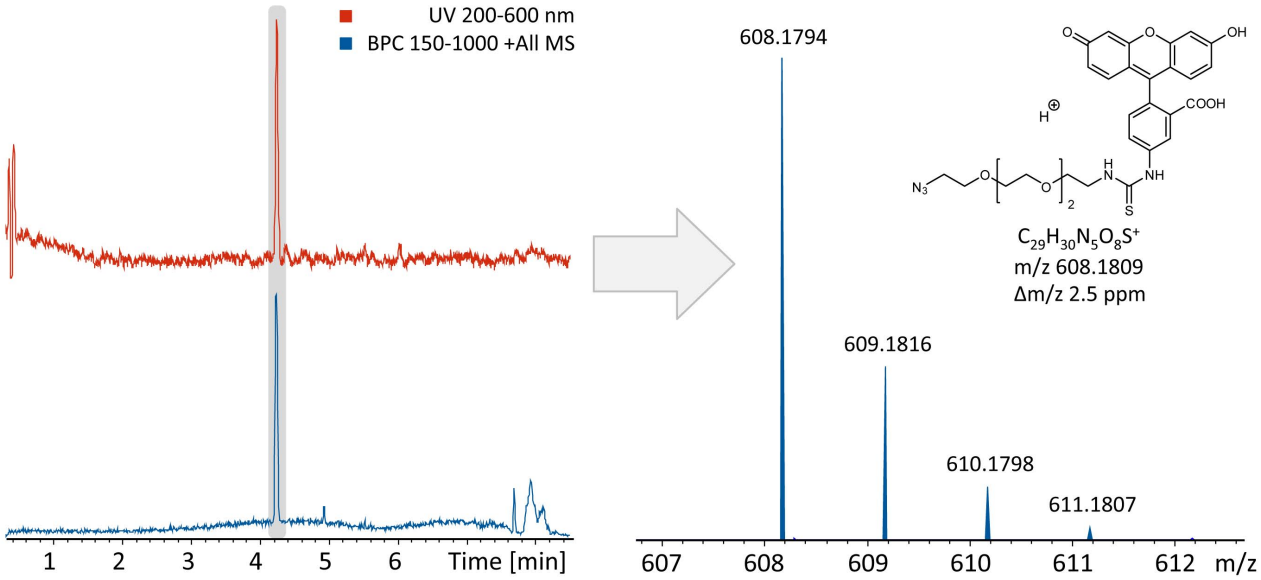

Figure S19: LCMS of FITC-Azide **13**

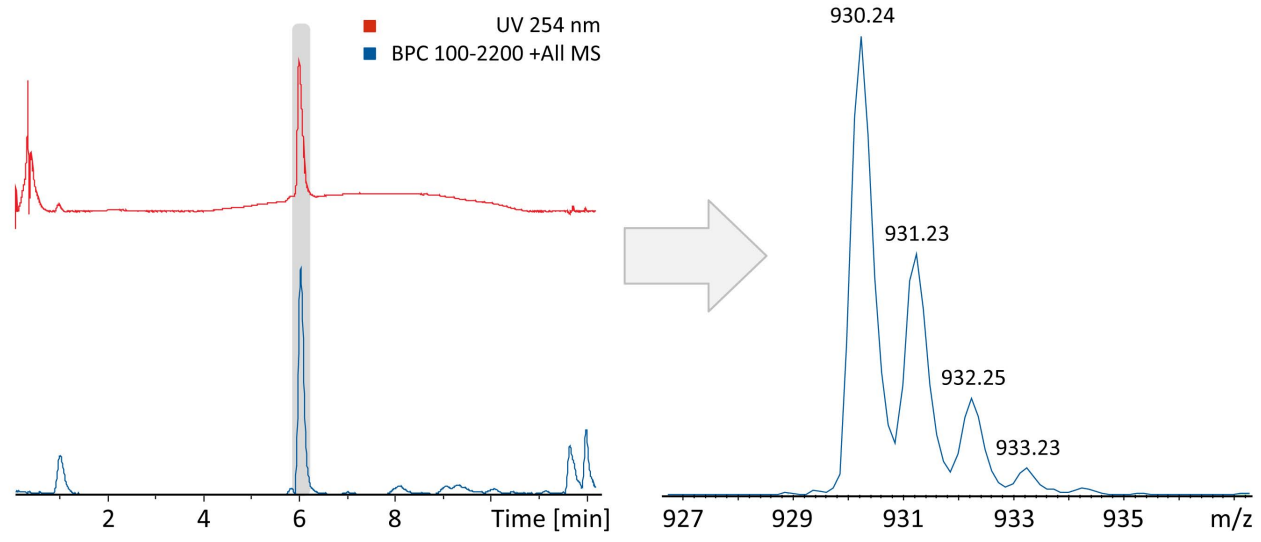

Figure S20: LCMS of FITC labelled epoxide 6D-**17**

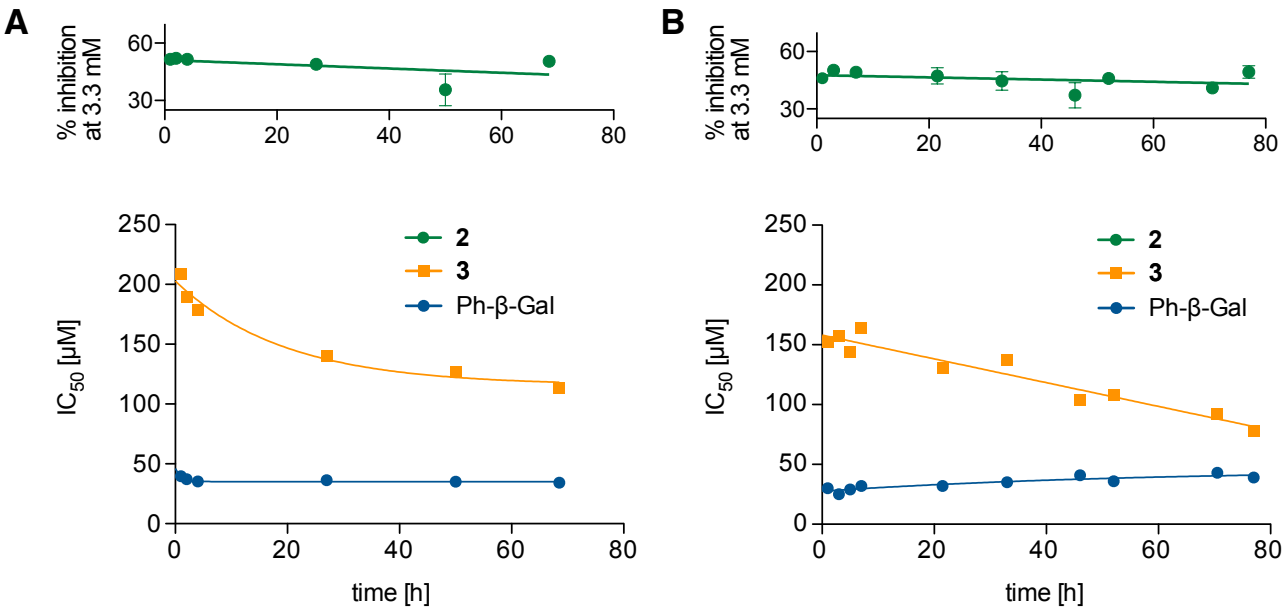

Figure S21: Kinetics of LecA inhibition of the two epoxide diasteromers **2** and **3** and the non-covalent inhibitor phenyl β-D-galactoside at r.t. (A) and 37 °C (B), respectively.

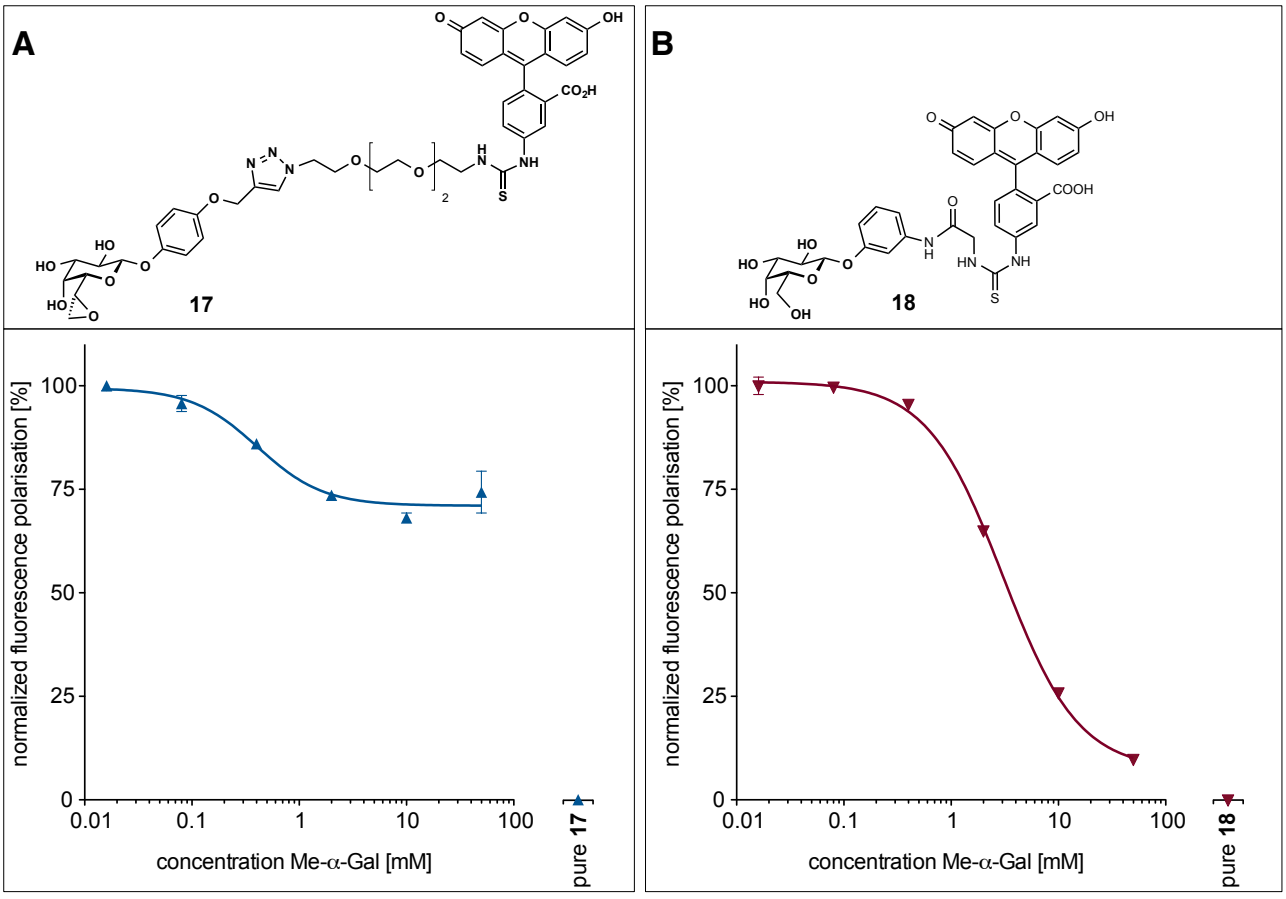

Figure S22: Displacement of **17** (A) or **18** (B) from their complexes with LecA using the competitive inhibitor methyl α-D-galactoside as studied by fluorescence polarization.

Table S1: Data collection and refinement statistics for LecA structure with **3**

	LecA in complex with 3			
Data collection				
Beamline	ESRF BM30-A			
Wavelength	0.979745			
Space group	$P2_1$			
Unit cell dimensions	27.34 73.77 101.45 90.00 93.36 90.00			
Resolution (Å)	27.30-1.80 (1.84-1.80)			
R_{merge}	0.043 (0.193)			
R_{pim}	0.041 (0.183)			
I / σI	19.8 (5.2)			
Completeness (%)	98.9 (96.1)			
Redundancy	3.6 (3.4)			
CC1/2	0.999 (0.954)			
Nb reflections	134653			
Nb uniques reflections	36960			
Refinement				
Resolution (Å)	27.30-1.80			
No. reflections	35035			
No. free reflections	1907			
R_{work} / R_{free}	14.9 / 19.4			
R.m.s Bond lengths (Å)	0.170			
Rmsd Bond angles (°)	1.662			
Rmsd Chiral (Å^3)	0.111			
No. atoms / Bfac (Å^2)	Chain A	Chain B	Chain C	Chain D
Protein	907 / 15.4	922 / 14.6	907 / 14.6	927 / 14.7
Calcium	1 / 14.8	1 / 16.0	1 / 15.2	1 / 11.1
Sugar	19 / 17.1	19 / 22.6	19 / 15.5	19 / 12.9
PEG			14 / 27.5	
Waters	160 / 25.1	180 / 25.4	149 / 23.7	192 / 23.6
Ramachandran Allowed	100			
Favored	97.4			
Outliers	0			
PDB Code	5MIH			

*Values in parentheses are for highest-resolution shell.

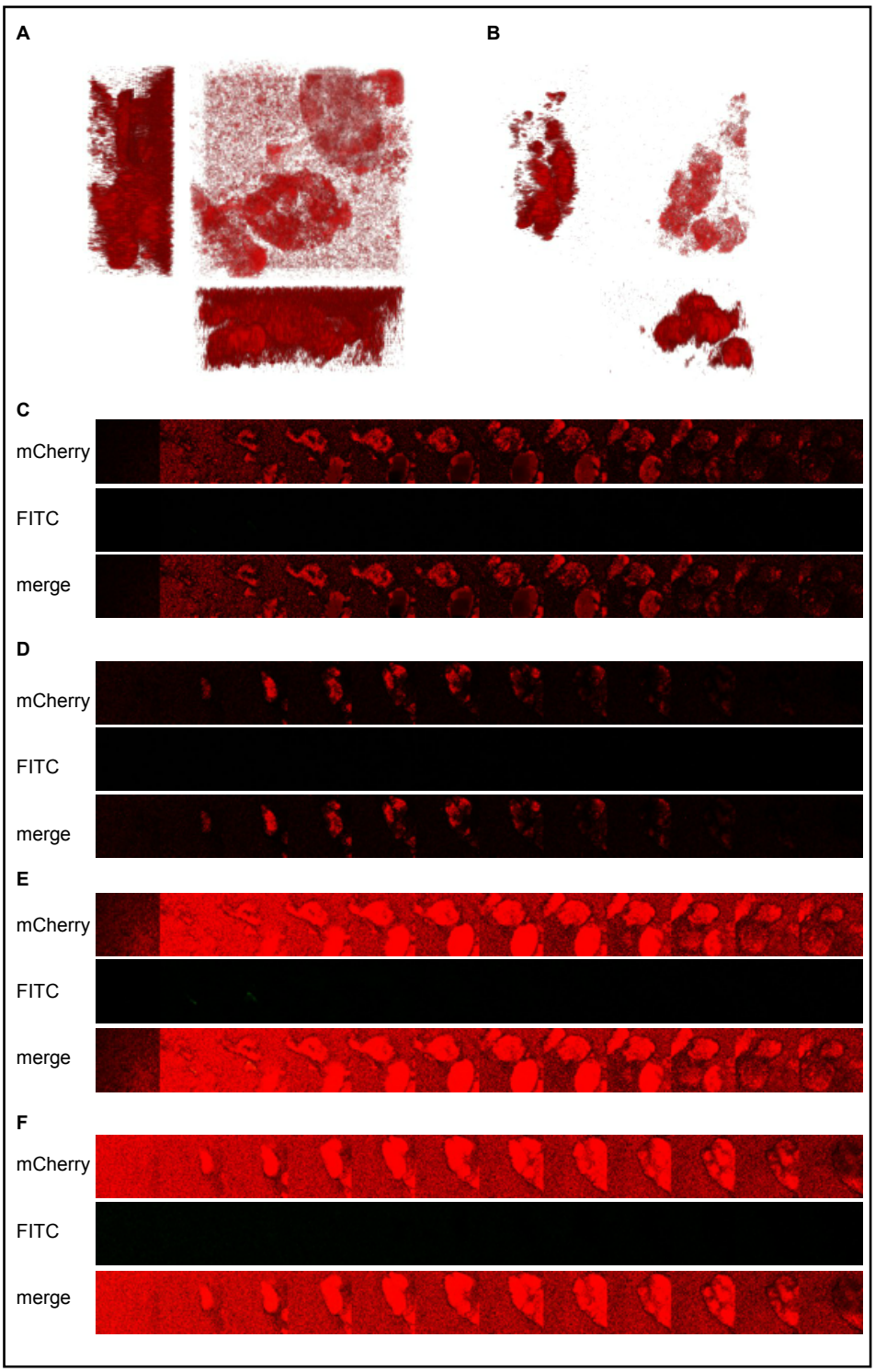

Figure S23: *P. aeruginosa* PAO1 biofilms before LecA-dependent staining.

P. aeruginosa PAO1 wt (A, C, E) or the lecA knockout (ΔlecA) mutant (B, D, F) expressing mCherry from pMP7605 were incubated at 37 °C for 24 hours with agitation (180 rpm). Z-stacks (232 x 232 μm) were recorded every 2 μm at 561 nm for mCherry (red, C-F upper panels) and 488 nm for fluorescein (green, C-F middle panels). The galleries show only every 6th z-stack recorded. Lower panels show merged images of both channels (488 nm and 561 nm). A and B: 3D images show merged images of both channels (488 nm and 561 nm) from top and side views. Images were background corrected and outliers were removed using imageJ software. C and D: Galleries of background corrected and noise reduced z-stacks. E and F: Galleries of raw z-stacks.

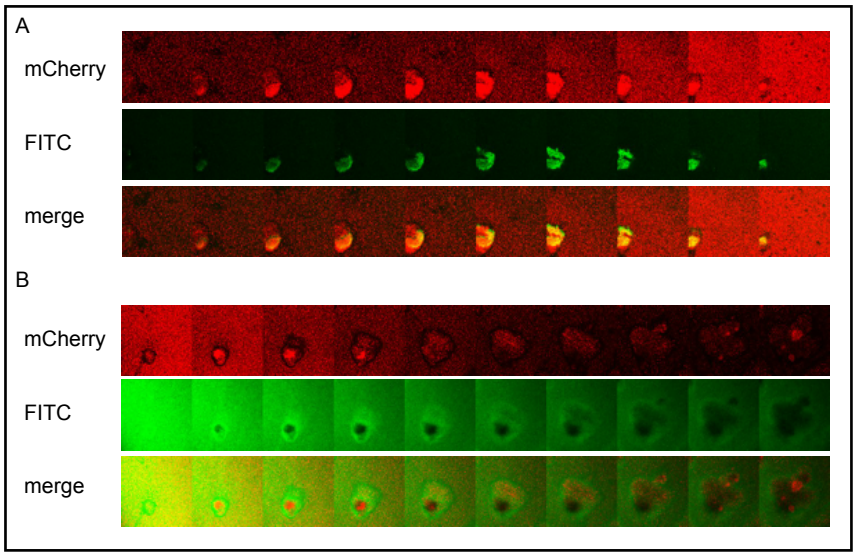

Figure S24: LecA-dependent staining of *P. aeruginosa* PAO1 biofilms - **raw data**

P. aeruginosa PAO1 wt (A) or the lecA knockout (ΔlecA) mutant (B) expressing mCherry from pMP7605 were incubated at 37 °C for 24 hours with agitation (180 rpm). Biofilms were stained with the covalent LecA ligand **17** for 10 - 30 min. Z-stacks (232 x 232 μm) were recorded every 2 μm at 561 nm for mCherry (red, A and B, upper panels) and 488 nm for fluorescein (green, A and B middle panels). The galleries show only every 4th z-stack recorded. Lower panels show merged images of both channels (488 nm and 561 nm). Images show raw data.

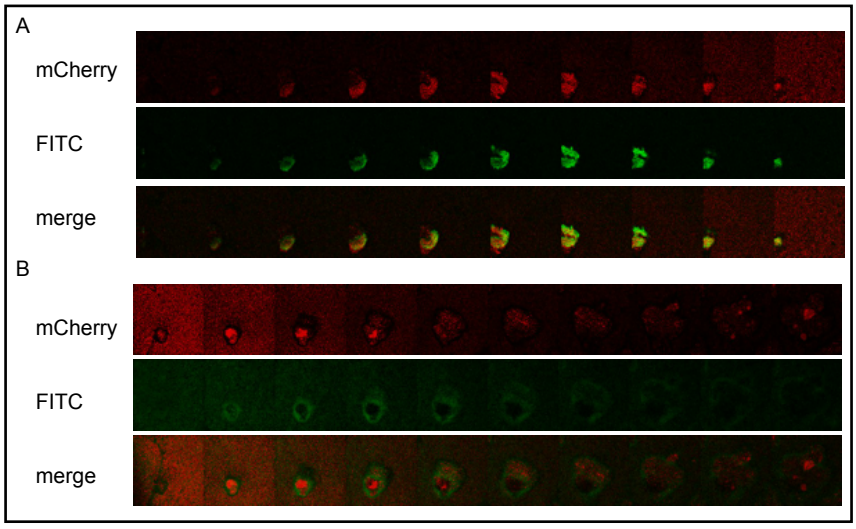

Figure S25: Galleries of LecA-dependent staining of *P. aeruginosa* biofilms with **17**. *P. aeruginosa* PAO1 wt (A, identical image to Figure 5A for comparison) or the lecA knockout (ΔlecA) mutant (B, largest identified aggregate of the knockout strain culture) expressing mCherry from pMP7605 were incubated at 37 °C for 24 hours with agitation (180 rpm). Biofilms were stained with the covalent LecA ligand fused to fluorescein (**17**) for 10 - 30 min. Z-stacks (232 x 232 μm) were recorded every 2 μm at 561 nm for mCherry (red, A and B, upper panels) and 488 nm for fluorescein

(green, A and B middle panels). The galleries show every 4th z-stack recorded. Lower panels show merged images of both channels (488 nm and 561 nm).

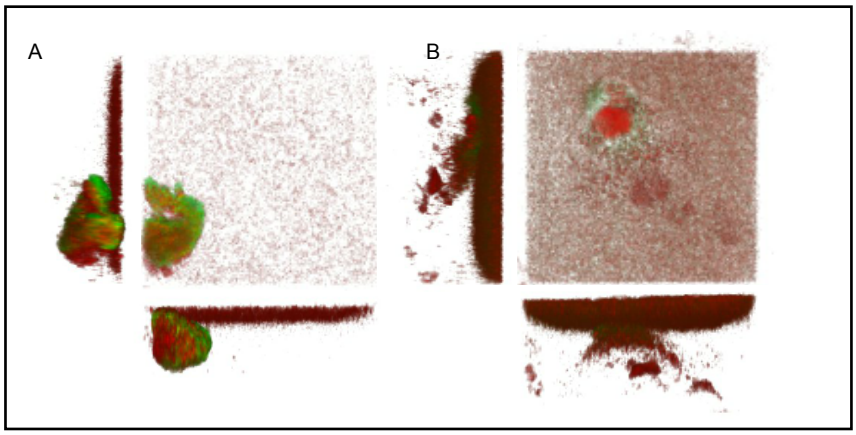

Figure S26: Three-dimensional imaging of LecA-dependent staining of *P. aeruginosa* PAO1 biofilms with **17**. *P. aeruginosa* PAO1 wt (A, identical image to Figure 6A for comparison) or the lecA knockout (ΔlecA) mutant (B, largest identified aggregate of the knockout strain culture) expressing mCherry from pMP7605 were incubated at 37 °C for 24 hours with agitation (180 rpm). Biofilms were stained with **17** for 10 - 30 min. Z-stacks (232 x 232 μm) were recorded every 2 μm at 561 nm for mCherry (red) and 488 nm for fluorescein (green). The 3D images show merged images of both channels (488 nm and 561 nm) from top and side views.

Experimental

General Experimental Details

Commercial chemicals and solvents were used without further purification. D-galactose was purchased from Dextra Laboratories (Reading, UK) and fluorescein isothiocyanate isomer I (FITC) from Sigma-Aldrich (Munich, Germany). Deuterated solvents were from Eurisotop (Saarbrücken, Germany). Thin layer chromatography (TLC) was performed using silica gel 60 coated aluminum sheets containing fluorescence indicator (Macherey & Nagel, Düren, Germany) using UV light (254 nm) and by charring either in aqueous $KMnO_4$ solution or in a molybdate solution (a 0.02 M solution of ammonium cerium sulfate dihydrate and ammonium molybdate tetrahydrate in aqueous 10% H_2SO_4) with heating. Medium pressure liquid chromatography (MPLC) was performed on a Teledyne Isco Combiflash Rf200 system using pre-packed silica gel 60 columns from Teledyne Isco, SiliCycle or Macherey-Nagel. Nuclear magnetic resonance (NMR) spectroscopy was performed on a Bruker Avance III 500 UltraShield spectrometer at 500 MHz (^{1}H) or 126 MHz (^{13}C). Chemical shifts are given in ppm and were calibrated on residual solvent peaks.[1] Multiplicities were specified as s (singlet), m (multiplet) or interpreted according to 1st order and higher order where possible. The signals were assigned with the help of ^{1}H,^{1}H-COSY, DEPT-135-edited ^{1}H,^{13}C-HSQC and ^{1}H,^{13}C-HMBC experiments. Preparative HPLC-MS was performed on a Thermo Dionex Ultimate 3000 HPLC with UV detection. Analytical HPLC-MS was performed on a Thermo Dionex Ultimate 3000 HPLC coupled to a Bruker amaZon SL for low resolution mass spectra or on a Bruker maxis 4G hr-QqToF spectrometer for high resolution, and the data were analyzed using DataAnalysis (Bruker Daltonics, Bremen, Germany).

1,2:3,4-Di-O-isopropylidene-α-D-galacto-hexodialdo-1,5-pyranose (**6**). **6** was synthesized from 1,2:3,4-Di-O-isopropylidene-α-D-galactopyranose[2] (**5**, 500 mg, 1.92 mmol) by Swern oxidation following the procedure by Streicher and Wünsch.[3] After chromatography, **6** (348 mg, 1.35 mmol) was obtained as colorless amorphous solid. The NMR of **6** corresponded to the one given in the literature.[4]

6,7-Dideoxy-1,2:3,4-di-O-isopropylidene-α-D-galacto-hept-6-enopyranose (**7**). **7** was synthesized by treating **6** with a Wittig reagent in analogy to Lehmann and Schäfer.[5] In contrast to the literature, we used methyltriphenylphosphonium iodide and sodium hydride in DMSO to generate

the ylid. Crude olefin **7** was obtained after extraction and was used without purification in the next step. The NMR of the crude product corresponded to literature values.[4]

6,7-Dideoxy-1,2,3,4-tetra-O-acetyl-α/β-D-galacto-hept-6-enopyranose (**8**). **α-8** was first described by Lee *et al*. and the NMR corresponded to literature values.[4]

Phenyl 2,3,4-tri-O-acetyl-6,7-dideoxy-β-D-galacto-hept-6-enopyranoside (9).

8 (1.24 g, 3.6 mmol) and phenol (678 mg, 7.2 mmol) were dissolved in dry CH_2Cl_2 (24 mL) and added to a round bottom flask with powdered activated molecular sieves (3Å, 600 mg) under argon. After cooling to 0 °C, BF_3•Et_2O (1.77 mL, 14.4 mmol) was added in four portions over 1 h. Then, the reaction was stirred for 50 min at 0 °C after which the reaction was allowed to warm to r.t. during 20 min. The reaction was stopped by pouring on cold aqueous satd. $NaHCO_3$, after addition of CH_2Cl_2 (60 mL) the phases were separated and the organic layer was washed with cold aqueous satd. $NaHCO_3$ (2 x 60 mL). The combined aqueous layers were extracted with CH_2Cl_2 (3 x 60 mL). The combined organic layers were dried over anhydrous Na_2SO_4, filtered and the volatiles were removed *in vacuo*. The residue was purified by MPLC (SiO_2, petrol ether/EtOAc gradient of 5-50%) to elute first the anomer **α-9** (55 mg, 4%), then the title compound **9** (760 mg, 56%) and then recovered starting material **8** (358 mg, 26%). Analytical data for **9**: ^{1}H NMR (400 MHz, $CHCl_3$-*d1*) δ 7.34 – 7.25 (m, 2H, ArH), 7.10 – 6.99 (m, 3H, ArH), 5.76 (ddd, *J* = 17.3, 10.7, 4.8 Hz, 1H, H6), 5.51 (dd, *J* = 10.5, 8.0 Hz, 1H, H2), 5.46 – 5.37 (m, 2H, H4, H7a), 5.27 (dt, *J* = 10.7, 1.4 Hz, 1H, H7b), 5.15 (dd, *J* = 10.4, 3.4 Hz, 1H, H3), 5.08 (d, *J* = 7.9 Hz, 1H, H1), 4.33 (dq, *J* = 4.7, 1.5 Hz, 1H, H5), 2.14 (s, 3H, CH_3CO), 2.06 (s, 3H, CH_3CO), 2.01 (s, 3H, CH_3CO). ^{13}C NMR (101 MHz, $CHCl_3$-*d1*) δ 170.56 (CO), 170.30 (CO), 169.56 (CO), 157.30 (ArC), 131.67 (C6), 129.69 (2C, ArCH), 123.30 (ArCH), 118.58 (C7), 117.14 (2C, ArCH), 99.91 (C1), 73.93 (C5), 71.19 (C3), 69.47 (C4), 68.91 (C2), 20.87 (CH_3CO), 20.79 (CH_3CO), 20.75 (CH_3CO). LC-HRMS: $[C_{19}H_{22}O_8+Na]^+$ calcd: 401.1207, found: 401.1206.

Phenyl 6,7-dideoxy-β-D-galacto-hept-6-enopyranoside (10). Triacetate **9** (300 mg, 0.79 mmol) was dissolved in dry MeOH (8 mL) under nitrogen. A solution of NaOMe in MeOH (1M, 0.24 mL) was added and the reaction was stirred at r.t. for 2 h. The reaction was neutralized with acidic anion exchange resin (Amberlite IR120/H^+), filtered and the volatiles were removed *in vacuo*. **10** (199 mg, 97%) was obtained as analytically pure colorless amorphous solid. ^{1}H NMR (500 MHz, MeOH-*d4*) δ 7.31 – 7.23 (m, 2H, ArH), 7.10 – 7.05 (m, 2H, ArH), 7.00 (tt, *J* = 7.4, 1.1 Hz, 1H, ArH), 5.98 (ddd, *J* = 17.4, 10.7, 5.5 Hz, 1H, H6), 5.37 (dt, *J* = 17.3, 1.7 Hz, 1H, H7a), 5.23 (dt, *J* = 10.7, 1.6 Hz, 1H, H7b), 4.90 (d, *J* = 7.7 Hz, 1H, H1), 4.20 (dq, *J* = 5.5, 1.4 Hz, 1H, H5), 3.85 – 3.76

(m, 2H, H4, H2), 3.63 (dd, *J* = 9.7, 3.4 Hz, 1H, H3). ^{13}C NMR (126 MHz, MeOH-*d4*) δ 159.18 (ArC), 135.82 (C6), 130.33 (2C, ArCH), 123.34 (ArCH), 117.83 (2C, ArCH), 117.34 (C7), 102.78 (C1), 76.99 (C5), 74.82 (C3), 72.76 (C4), 72.07 (C2). LC-HRMS: $[C_{13}H_{16}O_5+HCOO]^-$ calcd: 297.0980, found: 297.0977.

Phenyl 6,7-dideoxy-6,7-epoxy-β-D-galacto-heptopyranoside (*6L*)-2 and (*6D*)-3

Olefin **10** (55 mg, 0.22 mmol) was dissolved in dry CH_2Cl_2 (6 mL) containing 3% MeOH and $NaHCO_3$ (80 mg, 0.95 mmol) was added. After cooling to 0 °C, mCPBA (135 mg, 0.78 mmol) was added portionwise under stirring. Stirring was continued for 10 min and the reaction was allowed to warm to r.t.. After 3 d, mCPBA (135 mg, 0.78 mmol) and $NaHCO_3$ (80 mg, 0.95 mmol) was added. After a reaction time of 10 d, the reaction was diluted with EtOAc (25 mL) and washed with brine (2 x 3 mL). The organic layer was dried over Na_2SO_4, filtered and the volatiles were removed *in vacuo*. The aqueous phase contained the desired products and was therefore lyophilized and the combined residues were purified by normal phase flash chromatography to give impure 19 mg of **3** and 17 mg of **2**. Both were then separately purified by reversed phase (C18) flash chromatography to give **2** (5.0 mg, 9%) and its epimer **3** (11.0 mg, 19%). The stereochemistry of **2** and **3** was assigned by co-crystallization of the active diastereomer **3** with LecA (*vide infra*). Analytical data for **(*6L*)-2**: ^{1}H NMR (500 MHz, MeOH-*d4*) δ 7.32 – 7.25 (m, 2H, ArH), 7.13 – 7.07 (m, 2H, ArH), 7.00 (tt, *J* = 7.5, 1.1 Hz, 1H, ArH), 4.83 (d, *J* = 7.8 Hz, 1H, H1, signal overlap with residual water), 3.93 (dd, *J* = 3.4, 1.1 Hz, 1H, H4), 3.82 (dd, *J* = 9.8, 7.7 Hz, 1H, H2), 3.56 (dd, *J* = 9.7, 3.4 Hz, 1H, H3), 3.35 – 3.29 (m, 1H, H6, signal overlap with residual solvent), 3.27 (dd, *J* = 6.7, 1.2 Hz, 1H, H5), 2.83 (dd, *J* = 4.9, 4.2 Hz, 1H, H7a), 2.71 (dd, *J* = 5.0, 2.7 Hz, 1H, H7b). ^{13}C NMR (126 MHz, MeOH-*d4*) δ 159.19 (ArC), 130.38 (2C, ArCH), 123.39 (ArCH), 117.82 (2C, ArCH), 102.81 (C1), 78.79 (C5), 74.49 (C3), 72.06 (C2), 71.48 (C4), 52.89 (C6), 44.42 (C7). LC-HRMS: $[C_{13}H_{16}O_2+HCOO]^-$ calcd: 313.0929, found: 313.0928. Analytical data for **(*6D*)-3**: ^{1}H NMR (500 MHz, MeOH-*d4*) δ 7.32 – 7.22 (m, 2H, ArH), 7.08 – 7.03 (m, 2H, ArH), 7.00 (tt, *J* = 7.4, 1.1 Hz, 1H, ArH), 4.85 – 4.83 (m, 1H, H1, signal overlap with residual water), 3.98 (dd, *J* = 3.4, 1.1 Hz, 1H, H4), 3.81 (dd, *J* = 9.8, 7.7 Hz, 1H, H2), 3.58 (dd, *J* = 9.8, 3.4 Hz, 1H, H3), 3.44 (dd, *J* = 5.5, 1.2 Hz, 1H, H5), 3.26 (ddd, *J* = 5.4, 3.8, 2.7 Hz, 1H, H6), 2.88 – 2.79 (m, 2H, H7a,b). ^{13}C NMR (126 MHz, MeOH-*d4*) δ 159.11 (ArC), 130.37 (2C, ArCH), 123.39 (ArCH), 117.80 (2C, ArCH), 102.79 (C1), 76.33 (C5), 74.55 (C3), 72.12 (C2), 70.73 (C4), 51.32 (C6), 46.70 (C7). LC-HRMS: $[C_{13}H_{16}O_2+HCOO]^-$ calcd: 313.0929, found: 313.0930.

p-Propargyloxyphenyl 2,3,4-tri-O-acetyl-6,7-dideoxy-β-D-galacto-hept-6-enopyranoside 11. 11 was prepared from **8** (800 mg, 2.32 mmol) and hydroquinone monopropargyl ether[6] (688 mg, 4.64

mmol) as described for **9**. Purification of the crude product yielded **11** (627 mg, 62%) as an oil and small amounts of its α-anomer and recovered **α-8**. Analytical data for **11**: ^{1}H NMR (500 MHz, $CHCl_3$-*d1*) δ 7.03 – 6.95 (m, 2H, ArH), 6.95 – 6.86 (m, 2H, ArH), 5.76 (ddd, *J* = 17.2, 10.7, 4.8 Hz, 1H, H6), 5.48 (dd, *J* = 10.5, 8.0 Hz, 1H, H2), 5.45 – 5.39 (m, 2H, H7a, H4), 5.28 (dt, *J* = 10.8, 1.3 Hz, 1H, H7b), 5.13 (dd, *J* = 10.5, 3.4 Hz, 1H, H3), 4.97 (d, *J* = 8.0 Hz, 1H, H1), 4.64 (d, *J* = 2.4 Hz, 2H, HCCC*H*$_2$-OR), 4.29 (dq, *J* = 4.6, 1.4 Hz, 1H, H5), 2.51 (t, *J* = 2.4 Hz, 1H, *H*CCCH$_2$-OR), 2.15 (s, 3H, CH_3CO), 2.08 (s, 3H, CH_3CO), 2.01 (s, 3H, CH_3CO); ^{13}C NMR (126 MHz, $CHCl_3$-*d1*) δ 170.59 (CO), 170.33 (CO), 169.58 (CO), 153.65 (ArC), 152.01 (ArC), 131.69 (C6), 118.66 (2C, ArCH), 118.57 (C7), 115.99 (2C, ArCH), 100.84 (C1), 78.72 (HC*C*CH$_2$-OR), 75.64 (H*C*CCH$_2$-OR), 73.89 (C5), 71.18 (C3), 69.46 (C4), 68.93 (C2), 56.45 (HCC*C*H$_2$-OR), 20.92 (*C*H$_3$CO), 20.82 (*C*H$_3$CO), 20.77 (*C*H$_3$CO); LC-HRMS: $[C_{22}H_{24}O_9+Na]^+$ calcd: 455.1313, found: 455.1309.

p-Propargyloxyphenyl 2,3,4-tri-O-acetyl-6,7-dideoxy-6,7-epoxy-β-D-galacto-heptopyranoside (*6L*)-12 and (*6D*)-13.

Olefin **11** (256 mg, 0.59 mmol) was dissolved in CH_2Cl_2 (12 mL) and cooled to 0 °C. mCPBA (511 mg, 2.96 mmol) was added portionwise under stirring and cooling. Then, the reaction was allowed to warm to r.t. and stirring was continued for 6 d when the reaction was diluted with CH_2Cl_2 (60 mL) and extracted with satd aq. $Na_2S_2O_3$, $NaHCO_3$, H_2O. The organic layer was dried over Na_2SO_4, filtered and the volatiles were removed *in vacuo*. The residue was purified by MPLC (silica, PhMe/ EtOAc 5-15%) to give first **13** (119 mg, 46%) and then **12** (55 mg, 21%). Analytical data for **(*6D*)-13**: ^{1}H NMR (500 MHz, $CHCl_3$-*d1*) δ 7.00 – 6.83 (m, 4H, ArH), 5.55 (dd, 1H, *J* = 3.4, 1.0 Hz, H4), 5.46 (dd, *J* = 10.5, 7.9 Hz, 1H, H2), 5.09 (dd, *J* = 10.4, 3.4 Hz, 1H, H3), 4.90 (d, *J* = 7.9 Hz, 1H, H1), 4.64 (d, *J* = 2.4 Hz, 2H, HCCC*H*$_2$-OR), 3.64 (dd, *J* = 4.7, 1.2 Hz, 1H, H5), 3.10 (ddd, *J* = 4.7, 3.7, 2.7 Hz, 1H, H6), 2.83 (dd, 1H, *J* = 5.3, 2.6 Hz, H7a), 2.81 (dd, 1H, *J* = 5.2, 3.8 Hz, H7b), 2.51 (t, *J* = 2.4 Hz, 1H, *H*CCCH$_2$-OR), 2.19 (s, 3H, CH_3CO), 2.07 (s, 3H, CH_3CO), 2.02 (s, 3H, CH_3CO); ^{13}C NMR (126 MHz, $CHCl_3$-*d1*) δ 170.30 (CO), 170.20 (CO), 169.53 (CO), 153.74 (ArC), 151.88 (ArC), 118.53 (2C, ArCH), 116.08 (2C, ArCH), 100.83 (C1), 78.70 (HC*C*CH$_2$-OR), 75.67 (H*C*CCH$_2$-OR), 73.07 (C5), 70.85 (C3), 68.89 (C2), 68.02 (C4), 56.47 (HCC*C*H$_2$-OR), 49.71 (C6), 45.51 (C7), 20.88 (*C*H$_3$CO), 20.83 (*C*H$_3$CO), 20.74 (*C*H$_3$CO). LC-HRMS: $[C_{22}H_{25}O_{10}]^+$ calcd: 449.1442, found: 449.1447. Analytical data for **(*6L*)-12**: ^{1}H NMR (500 MHz, $CHCl_3$-*d1*) δ 7.01 – 6.97 (m, 2H, ArH), 6.94 – 6.89 (m, 2H, ArH), 5.53 – 5.45 (m, 2H, H2+H4), 5.05 (dd, *J* = 10.4, 3.5 Hz, 1H, H3), 4.92 (d, *J* = 7.9 Hz, 1H, H1), 4.65 (d, *J* = 2.4 Hz, 2H, HCCC*H*$_2$-OR), 3.43 (dd, *J* = 5.9, 1.2 Hz, 1H, H5), 3.16 (ddd, *J* = 5.9, 4.2, 2.6 Hz, 1H, H6), 2.80 (dd, *J* = 4.6, 4.3 Hz, 1H, H7a), 2.66 (dd, *J* = 4.7, 2.7 Hz, 1H, H7b), 2.51 (t, *J* = 2.4 Hz, 1H, *H*CCCH$_2$-OR), 2.21 (s, 3H, CH_3CO), 2.08 (s, 3H, CH_3CO), 2.02 (s, 3H, CH_3CO); ^{13}C NMR (126 MHz, $CHCl_3$-*d1*) δ 170.53

(CO), 170.27 (CO), 169.50 (CO), 153.73 (ArC), 151.93 (ArC), 118.57 (2C, ArCH), 116.10 (2C, ArCH), 100.76 (C1), 78.72 (HC*C*CH_2-OR), 75.66 (H*C*CCH_2-OR), 75.35 (C5), 70.86 (C3), 68.76 (C2+C4), 56.50 (HCC*C*H_2-OR), 50.89 (C6), 43.57 (C7), 20.89 (2C, *C*H_3CO), 20.74 (*C*H_3CO); LC-HRMS: $[C_{22}H_{25}O_{10}]^+$ calcd: 449.1442, found: 449.1439.

p-Propargyloxyphenyl 6,7-dideoxy-6,7-epoxy-β-D-galacto-heptopyranoside (*6L*)-14.

Acetylated **(*6L*)-12** (34 mg, 76 μmol) was dissolved in dry MeOH (380 μL) and cooled to 0 °C. Freshly prepared NaOMe (60 mM in MeOH, 570 μL) was added dropwise and the reaction was stirred for 1.5 h at 0 °C. Then, Amberlite IR120/H^+ was added, the reaction was filtered and the volatiles were removed *in vacuo*. Analytically pure **(*6L*)-14** (21 mg, 86%) was obtained. ^{1}H NMR (500 MHz, MeOH-*d4*) δ 7.06 (d, *J* = 9.2 Hz, 2H, ArH), 6.91 (d, *J* = 9.1 Hz, 2H, ArH), 4.72 (d, *J* = 7.8 Hz, 1H, H1), 4.67 (d, *J* = 2.4 Hz, 2H, HCCC*H*$_2$-OR), 3.91 (dd, *J* = 3.5, 1.1 Hz, 1H, H4), 3.79 (dd, *J* = 9.8, 7.7 Hz, 1H, H2), 3.54 (dd, *J* = 9.8, 3.4 Hz, 1H, H3), 3.33 – 3.29 (m, 1H, H6), 3.23 (dd, *J* = 6.7, 1.2 Hz, 1H, H5), 2.91 (t, *J* = 2.4 Hz, 1H, *H*CCCH_2-OR), 2.83 (dd, *J* = 4.9, 4.2 Hz, 1H, H7a), 2.70 (dd, *J* = 4.9, 2.7 Hz, 1H, H7b); ^{13}C NMR (126 MHz, MeOH-*d4*) δ 154.58 (ArC), 153.83 (ArC), 119.12 (2C, ArCH), 116.86 (2C, ArCH), 103.79 (C1), 79.99 (HC*C*CH_2-OR), 78.74 (C5), 76.54 (H*C*CCH_2-OR), 74.47 (C3,) 72.08 (C2), 71.47 (C4), 57.19 (HCC*C*H_2-OR), 52.92 (C6), 44.44 (C7); LC-HRMS: $[C_{16}H_{18}O_7$+HCOO$]^-$ calcd: 367.1035, found: 367.1031.

p-Propargyloxyphenyl 6,7-dideoxy-6,7-epoxy-β-D-galacto-heptopyranoside (*6D*)-15.

Acetylated **(*6L*)-13** (70 mg, 156 μmol) was was treated with NaOMe as described before for its diastereomer. Analytically pure **(*6D*)-15** (47 mg, 94%) was obtained. ^{1}H NMR (500 MHz, MeOH-*d4*) δ 7.04 – 6.99 (m, 2H, ArH), 6.96 – 6.87 (m, 2H, ArH), 4.72 (d, *J* = 7.7 Hz, 1H, H1), 4.67 (d, *J* = 2.5 Hz, 2H, HCCC*H*$_2$-OR), 3.96 (dd, *J* = 3.4, 1.2 Hz, 1H, H4), 3.78 (dd, *J* = 9.8, 7.7 Hz, 1H, H2), 3.57 (dd, *J* = 9.8, 3.5 Hz, 1H, H3), 3.40 (dd, *J* = 5.4, 1.1 Hz, 1H, H5), 3.26 (ddd, *J* = 5.4, 3.9, 2.6 Hz, 1H, H6), 2.91 (t, *J* = 2.4 Hz, 1H, *H*CCCH_2-OR), 2.84 (dd, *J* = 5.3, 3.9 Hz, 1H, H7a), 2.81 (dd, *J* = 5.3, 2.7 Hz, 1H, H7b); ^{13}C NMR (126 MHz, MeOH-*d4*) δ 154.58 (ArC), 153.73 (ArC), 119.14 (2C, ArCH), 116.84 (2C, ArCH), 103.80 (C1), 79.99 (HC*C*CH_2-OR), 76.55 (C5), 76.26 (H*C*CCH_2-OR), 74.52 (C3), 72.15 (C2), 70.71 (C4), 57.18 (HCC*C*H_2-OR), 51.35 (C6), 46.71 (C7); LC-HRMS: $[C_{16}H_{18}O_7$+HCOO$]^-$ calcd: 367.1035, found: 367.1033.

Fluoresceine coupled 6,7-dideoxy-6,7-epoxy-β-D-galacto-heptopyranoside (*6D*)-17.

Propargylated epoxide **(*6D*)-15** (1.28 mg, 4 μmol) and azide modified FITC **16**[7] (2.8 mg, 4 μmol) were dissolved in DMF (120 μL). After the addition of $CuSO_4$*$5H_2O$ (0.3 mg, 1.2 μmol) and sodium ascorbate (80 μL, 100 mM in H_2O, 8 μmol), the reaction was stirred at r.t. for 3 h. After

lyophilization, the residue was purified by preparative HPLC (C18, H_2O/MeCN, gradient of 20-60%) to give pure ***(6D)*-17** (2.8 mg, 76%). LC-MS: $[C_{45}H_{47}N_5O_{15}S+H]^+$ calcd: 930.3, found: 930.3.

Competitive binding to LecA using fluorescence polarization

The protein LecA was expressed and purified as described previously.[8,9] The competitive binding assay was performed as described previously.[9] In brief, to 20 μL of a stock solution of LecA and fluorescent ligand (final assay concentration 10 nM) in TBS/Ca were added 10 μL serial dilutions of test compounds in TBS/Ca in triplicates in black 384-well microtiter plates (Greiner Bio-One, Germany, cat no 781900). After addition of the reagents, the microtiter plates were centrifuged at 800 rpm for 1 min at 23 °C and subsequently incubated for 4-6 h at r.t. in a humidity chamber on a rocking table. Fluorescence was measured on a PheraStar FS plate reader (BMG Labtech GmbH, Germany) with excitation filters at 485 nm and parallel and perpendicular emission filters at 535 nm. The measured intensities were reduced by the values of only LecA in buffer. The fluorescence polarization data were calculated and analyzed with MARS Data Analysis Software (BMG Labtech GmbH, Germany) and fitted according to the four parameter variable slope model. Bottom and top plateaus were defined by the standard compounds included as controls in each assay (methyl α-D-galactoside and phenyl β-D-galactoside, respectively) and the data was reanalyzed with these values fixed. A minimum of three independent measurements on three plates was performed for each inhibitor. For binding kinetics, fluorescence polarization was measured over a period of 3 days at r.t. or 37 °C.

Complex formation of LecA with covalent fluorescent reporter ligand 17 and the non covalent control 18 and displacement using a competitive inhibitor

A stock solution of LecA (600 μM) was incubated with **17** (100 μM) or with the previously described galactose-based ligand **18**[9] (100 μM) in TBS/Ca (20 mM Tris, 137 mM NaCl, 2.6 mM KCl at pH 7.4 supplemented with 1 mM $CaCl_2$ and 2.5% DMSO) at 25 °C for 48 h (Figure S22). The resulting complexes were distributed in a black 384-well microtiter plate, 20 μL per well and mixed with with 10 μL of serial dilutions (150 mM to 48 μM) of methyl α-D-galactoside in TBS/Ca in duplicates. After incubation for 1 h at r.t., fluorescence polarization was determined as described above. Fluorescence polarization was normalized where values of unbound ligand **17** or **18**, respectively, were set to 0% effect and those wells with the lowest methyl α-D-galactoside content were set to 100% effect. The data was fitted according to the four parameter variable slope model.

LecA and the complex of LecA with **17** was analyzed after heat denaturation in Lämmli buffer (5 min, 95 °C) by 17% SDS-PAGE. Gels were analyzed by fluorescence scanning on a Typhoon 9400 device (GE Healthcare) at 488 nm and subsequent Coomassie staining.

Mass spectrometry

Analyzed LecA samples with inhibitors **1** or **3** were taken from the competitive binding assay containing 3 mM (intact protein) or 1 mM (MALDI) inhibitor after 24 h incubation. All intact protein ESI-MS-measurements were performed on a Dionex Ultimate 3000 RSLC system using an Aeris Widepore XB-C8, 150 x 2.1 mm, 3.6 µm dp column (Phenomenex, USA). Separation of 0.5 µL sample was achieved by a linear gradient from (A) H_2O + 0.1% HCOOH to (B) MeCN + 0.1% HCOOH at a flow rate of 300 µL/min and 45 °C. The gradient was initiated by a 1.0 min isocratic step at 2% B, followed by an increase to 75% B in 10 min to end with a 3 min step at 75% B before reequilibration with initial conditions. UV spectra were recorded by a DAD in the range from 200 to 600 nm. The LC flow was split to 75 µL/min before entering the maXis 4G hr-ToF mass spectrometer (Bruker Daltonics, Bremen, Germany) using the standard Bruker ESI source. In the source region, the temperature was set to 180 °C, the capillary voltage was 4000 V, the dry-gas flow was 6.0 L/min and the nebulizer was set to 1.1 bar. Mass spectra were acquired in positive ionization mode ranging from 600-1800 m/z at 2.5 Hz scan rate. Protein masses were deconvoluted by using the Maximum Entropy algorithm (Spectrum Square Associates, Inc.).

All MALDI-ToF measurements were acquired on a Bruker ultrafleXtreme MALDI-ToF/ToF mass spectrometer (Bruker Daltonics, Germany) equipped with a smartbeam II solid state 1 kHz laser. In-source decay (ISD) experiments for top-down sequencing were performed in positive ion reflectron mode ranging from 4000-9000 m/z. The acceleration voltage was set to 25.00 kV, extraction voltage to 22.55 kV, lens voltage was held at 7.5 kV and reflector voltage was set to 26.45 kV. The ISD spectra comprised 80000 accumulated laser shots (arbitrary laser power of 20%) and were externally calibrated using c-type fragment ions generated from intact ubiquitin. All ISD samples were prepared by pre-mixing sample solution (250 µM protein in H_2O) and matrix solution (1,5-diaminonaphthalene saturated in MeCN - H_2O + 0.1% TFA (50:50)) in a ratio of 1:2 (v:v) and spotted 0.5 µL onto a ground steel target.

Crystallization and structure determination

LecA dissolved in water to 10 mg/mL was co-crystallized with 2 mM ligand **3** after incubation during one day at room temperature. Crystallization screening was performed using the vapor

diffusion method. Sitting drops of 200 nl drops containing a 1/1 (v/v) mix of protein and reservoir solution at 20 °C were made using the robot of the HTXlab, Grenoble, France. Crystal clusters were obtained in five days from solution 6 from the PEGs-I screen (Qiagen): 25% peg 2KMME and 0.1M sodium acetate pH 4.6. A broken part of the cluster was directly mounted in a cryoloop and flash-freezed in liquid nitrogen. Diffraction data were collected at 100 K at the European Synchrotron Radiation Facility (Grenoble, France) on BM30A-FIP using an ADSC Q315r detector. The data were processed using XDS.[10] All further computing was performed using the CCP4 suite. [11] Data quality statistics are summarized in Table S1. The structure was solved by molecular replacement using PHASER and the tetramer coordinates of PDB-ID 1OKO as search model.[12] This model was initially rebuild using ARP/WARP[13] and then the structure was refined with restrained maximum likelihood refinement using REFMAC 5.8 and local NCS restrains[14] iterated with manual rebuilding in Coot.[15] Five percent of the observations were set aside for cross-validation analysis, and hydrogen atoms were added in their riding positions and used for geometry and structure-factor calculations. Incorporation of the ligand was performed after inspection of the ARP/WARP 2Fo-DFc weighted maps. Water molecules, introduced first with ARP/WARP and then automatically using Coot, were inspected manually. The model was validated with the wwPDB Validation server: http://wwpdb-validation.wwpdb.org. The coordinates were deposited in the Protein Data Bank under code 5MIH.

Microbiology

Generation of fluorescent P. aeruginosa strains

Bacterial cultures were grown in LB medium at 37 °C and 180 rpm. *P. aeruginosa* PAO1 wt (DSM19880) and *P. aeruginosa* PAO1 ΔlecA (source Bodo Philipp, University of Münster) were transformed with mCherry expressing plasmid pMP7605[16] by three parental mating using *E. coli* DH5α (source Rolf Müller lab) as donor strain and *E. coli* HB101 [RK 600] (source Bodo Philipp, University of Münster) as helper strain. Bacterial pre-cultures were inoculated from single colonies in 5 mL LB with appropriate antibiotics (30 μg/mL chloramphenicol for bacteria containing pRK600; 15 μg/mL gentamicin for for bacteria containing pMP7605) and grown at 37 °C and 180 rpm overnight to stationary phase. For each mating, 100 μL pre-culture was combined and washed twice with pre-warmed LB medium. Strain mixtures were resuspended in 30 μL pre-warmed LB medium and spotted onto pre-warmed LB agarose plates and incubated overnight at 37 °C. After 24 hours from each plate all growth was resuspended in 1 mL LB per plate. 100 μL of the bacterial suspension and 100 μL of a 1:10 dilution were plated on LB-gentamicin (120 μg/mL) agar plates

and incubated at 37 °C for 24 hours. Successfully transformed bacteria were gentamicin-resistant and the colonies were identified by their pink color and restreaked.

Biofilm experiments

Bacterial pre-cultures of *P. aeruginosa* PAO1 w.t. pMP7605 or ΔlecA pMP7605 were inoculated from single colonies in 5 mL LB and grown at 37 °C and 180 rpm overnight to stationary phase. For the biofilm assay the bacterial pre-cultures were diluted to an OD600nm of 0.02 in fresh LB medium. 400 μL bacterial culture were transferred to each well of a 24-well imaging plate (cat no 3231, zell-kontakt GmbH, Germany). Plates were incubated at 37 °C and shaking at 180 rpm for 20 - 24 hours.

FITC-labelled epoxide **17** was suspended in H_2O/10% DMSO (0.84 mg/mL), centrifuged and the supernatant was used in the staining experiments. Biofilms were stained for the presence of LecA by the addition of 44 μL FITC-labelled epoxide **17** to each well. After an incubation time of 10 - 30 min at r.t., bacterial biofilms were directly visualized by measuring the fluorescence of the fluorescein-labeled LecA ligand and mCherry using a confocal laser scanning microscope (Leica TCS Sp8 CLSM). Fluorescein was excited with a 488-nm laser and mCherry at 561 nm. Focal planes were acquired starting from the bottom of the plate (position 0) with an interplane distance (z-step size) of 2 µm using a 25× numerical-aperture water objective. Images were batch-processed using the ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2016.) for background correction and noise filter ("remove outliers").

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