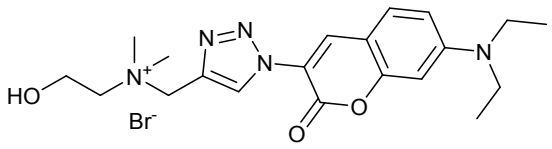
MATERIALS AND METHODS

Chemicals and instruments

All starting materials were obtained from commercial sources and were used without further purification. Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) was purchased by Sigma-Aldrich. NMR spectra were performed for small molecules on a Bruker Advance 400 MHz for ^{1}H-NMR spectra and 100 MHz for ^{13}C-NMR spectra. Chemical shifts are reported in ppm (δ) relative to the solvents: ^{1}H $\delta(CD_3OD)$ = 3.3 ppm, ^{13}C $\delta(CD_3OD)$ = 49.15 ppm. Accurate mass spectra were recorded on a time-of-flight (TOF) spectrometer (Waters, XEVO G2-S QTof). NMR spectroscopic measurements of native LTA or PG-WTA preparations were performed in D_2O at 300 K (LTA) or 307 K (PG-WTA) on a Bruker Avance^{III} 700 MHz spectrometer (equipped with an inverse 5 mm quadruple-resonance Z-grad cyroprobe). Deuterated solvents were purchased from Deutero GmbH (Kastellaun, Germany). Acetone was used as an external standard to calibrate ^{1}H (δ_H 2.225) and ^{13}C (δ_C 30.89) NMR spectra. ^{31}P NMR spectra (δ_P 0.0) were calibrated with 85% phosphoric acid in D_2O as an external standard. ^{1}H NMR assignments were confirmed by two-dimensional ^{1}H, ^{1}H COSY and TOCSY experiments, and ^{13}C NMR assignments were indicated by two-dimensional ^{1}H, ^{13}C HSQC, based on the ^{1}H NMR assignments. Inter-residual connectivity and further evidence for ^{13}C assignment were obtained from two-dimensional ^{1}H, ^{13}C heteronuclear multiple bond correlation and ^{1}H, ^{13}C HSQC-TOCSY experiments. Phosphate group connectivity was assigned by two-dimensional ^{1}H, ^{31}P HMQC and ^{1}H, ^{31}P HMQC-TOCSY. All data were acquired and processed using Bruker TOPSIN V 3.0 or higher.

Compounds: N-(2-hydroxyethyl)-N,N-dimethylbut-3-yn-1-aminium bromide (propargyl-choline) **1**[1] and 3-Azido-7-(diethylamino)-2H-chromen-2-one **2**[2] were obtained according to previous reported syntheses.

N-((1-(7-(diethylamino)-2-oxo-2H-chromen-3-yl)-1H-1,2,3-triazol-4-yl)methyl)-2-hydroxy-N,N-dimethylethan-1-aminium bromide (**3**):



The propargyl-choline **1** (50 mg, 0.24 mmol) and the azido-coumarin **2** (69 mg, 0.27 mmol) were diluted in dry CH_3CN (0.6 mL) and the polymer-supported Amberlyst-A21/CuI catalyst[3] (17 mg) was added in one portion. After stirring overnight, the black solution is filtrated to remove the catalyst. The solvent was evaporated and the product was directly purified (silica gel, 10% MeOH/CH_2Cl_2) to give **3** (107 mg, 0.23 mmol, 95%) as a yellow solid.

^{1}H NMR (400 MHz, CD_3OD): δ 1.19 (t, J = 7.0 Hz, 6H), 3.28 (s, 6H), 3.46 (q, J = 7.0 Hz, 4H), 3.62 (m, 2H), 4.13 (m, 2H), 4.93 (m, 2H), 6.47 (d, J = 2.0 Hz, 1H), 6.73 (dd, J = 9.0, 2.1 Hz, 1H), 7.45 (d, J = 9.0 Hz, 1H), 8.31 (s, 1H), 8.91 (s, 1H).

^{13}C NMR (100 MHz, CD_3OD): δ 12.9 (2×CH_3), 46.0 (2×CH_2), 52.2 (2×CH_3), 57.2 (CH_2), 60.8 (CH_2), 66.7 (CH_2), 97.7 (CH), 108.1 (C), 111.7 (CH), 117.2 (C), 130.4 (CH), 131.9 (CH), 136.9 (C), 138.3 (CH), 153.6 (C), 157.5 (C), 158.7 (C).

HRMS (ESI+) *m/z* calc. for $C_{20}H_{28}N_5O_3$ $[M]^+$ 386.2187, found 386.2185.

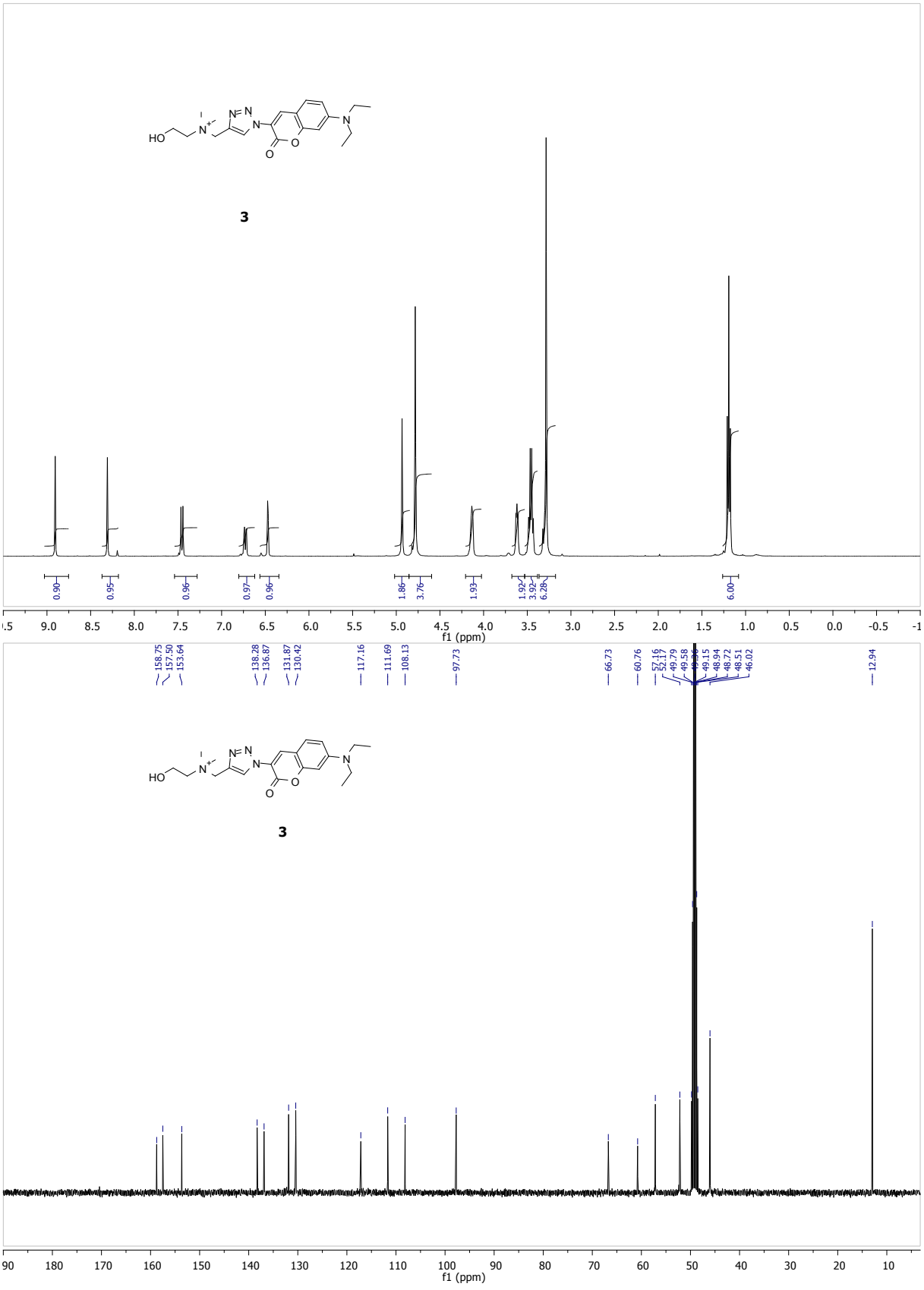


Figure S1: ^1H and ^{13}C NMR spectra of **3** in CD_3OD.

Bacterial growth conditions

Liquid cultures of the unencapsulated pneumococcal strain R6 were grown at 37 °C/5%CO_2 in a chemically defined medium (C-medium), supplemented with 4 µg/mL choline (Cmed-choline). Note that contrary to the original composition, the C-medium used here did not contain neither yeast extract nor albumin. Cells were harvested by centrifugation at 3,320 × *g* for 10 min, washed three times with C-medium without choline, concentrated to OD_{600nm} of 2 and stored at – 80 °C as aliquots containing 15% glycerol (v/v).

For bioorthogonal reactions, 10 mL of Cmed-choline and 10 mL of C-medium containing 4 µg/mL of propargyl-choline **1** were inoculated at OD_{600nm} of 0.05 with aliquots of cells conditioned in C-medium as described above. The growth was pursued for 3 h at 37 °C/5% CO_2 until OD_{600} of 0.2-0.25 was reached, which corresponds to the early exponential growth phase. The cells were pelleted by centrifugation at 3,320 × *g* for 10 min and subsequently incubated with 500 µL of 2% choline chloride (w/v) for 10 min at room temperature to remove the Choline-Binding Proteins (CBPs) that bind to choline residues. In the case of propargyl-choline residues, the presence of CBPs might impair the labeling of those molecules by the pro-fluorescent azide reporter **2**. The cells were washed twice with PBS (1 min centrifugation at 4,500 × *g*) and resuspended in 400 µL of PBS. A volume of 100 µL was used for each bioorthogonal reaction. In pulse experiments, cells were grown in Cmed-choline for 3 h, washed twice in PBS, resuspended in C-medium containing 4 µg/mL of propargyl-choline **1** and incubated at 37 °C/5% CO_2 for 30 min before proceeding to the labeling.

E. coli, *B. subtilis* and *P. aeruginosa* growth conditions in C-medium supplemented with both forms of choline were tested before conducting the click reactions with the same protocol as the one developed for *S. pneumoniae*.

Copper catalyzed click chemistry

Labeling in live cell condition: Labeling was performed on cells grown in presence of choline or propargyl-choline **1** at 4 µg/mL. A volume of 100 µL of cell suspension prepared as described above was incubated with the following reagents, which final concentration is indicated: **2** (1 mM), sodium ascorbate (1 mM), copper (II) sulfate (50 µM), THPTA (tris(3-hydroxypropyltriazolylmethyl)amine) (300 µM) for 30 min at room temperature, under mild agitation and protected from the light. Labeled cells were washed twice with PBS and resuspended in PBS for microscopy observation.

Fluorescence microscopy and image analysis

Pneumococcal cells were transferred to microscope slides and observed using an Olympus BX61 optical microscope equipped with a UPFLN 100× O-2PH/1.3 objective and a QImaging Retiga-SRV 1394 cooled charge-coupled device camera. Image acquisition and analysis were performed using the software packages Volocity and open-source Oufti,[4] respectively and processed with Adobe Photoshop CS5. Cell population demographs were constructed by Oufti which integrates the signal values in each cell. The cells are then sorted by their length value and the fluorescence values are plotted as a heat map.

Isolation and chemical analysis of LTA and WTA preparations

Extraction and isolation of LTA: LTA purification was performed basically as described elsewhere,[5] but to optimize yield of LTA, one specific detail has been modified. Pneumococcal cells were resuspended in citrate buffer (50 mM, pH 4.7) and disrupted three

times by French press (Constant Cell Disruption System, Serial No. 1020) at 10 °C at a pressure of 20 kPSI. SDS was added to a final concentration of 4% to the combined supernatants. The solution was incubated for 30 min at 100 °C and was stirred afterwards overnight at room temperature. The solution was centrifuged at 30,000 × *g* for 15 min at 4 °C. The pellet was washed four times with citrate buffer using the centrifugation conditions as above. The combined LTA-containing supernatants and the resulting sediment, containing the crude PG-WTA complex, were lyophilized separately. The resulting solids were both washed five times with ethanol (centrifugation: 20 min, 20 °C, 10,650 × *g*) to remove SDS and lyophilized (leading to pellet A containing LTA and pellet B containing the PG-WTA complex). For LTA isolation, pellet A was resuspended in citrate buffer and extracted with an equal volume of butan-1-ol at room temperature under vigorous stirring. The phases were separated by centrifugation at 4,000 × *g* for 15 min at 4 °C. The aqueous phase (containing LTA) was collected, and the extraction procedure was repeated twice with the organic phase plus interphase. The combined aqueous phases were lyophilized and subsequently dialyzed for 5 days at 4 °C against 50 mM ammonium acetate buffer (pH 4.7; 3.5 kDa cut-off membrane); the buffer was changed every 24 h. The resulting crude LTA was purified further by hydrophobic interaction chromatography (HIC) performed on a HiPrep Octyl-Sepharose column (GE Healthcare; 16 × 100 mm, bed volume 20 ml). The crude LTA material was dissolved in as little starting buffer (15% propan-1-ol in 0.1 M ammonium acetate (pH 4.7)) as possible and centrifuged at 13,000 × *g* for 5 min at room temperature and the resulting supernatant was lyophilized. The LTA-containing pellet was dissolved in the HIC start buffer at a concentration of 30 mg/mL and purified by HIC using a linear gradient from 15% to 60% propan-1-ol in 0.1 M ammonium acetate (pH 4.7). LTA-containing fractions were identified by a photometric phosphate test.[6] The phosphate-containing fractions were combined, lyophilized and washed with water upon freeze-drying to remove residual buffer.

Extraction and isolation of WTA*:* WTA isolation and extraction was carried out as described elsewhere,[7] but with minor modifications. Pellet B (containing the crude PG-WTA complex), which arose during LTA isolation, was resuspended at a concentration of 10 mg/mL in 100 mM Tris-HCl (pH 7.5) containing 20 mM $MgSO_4$. DNase A and RNase I were added to final concentrations of 10 and 50 µg/mL, respectively. The suspension was stirred for 2 h at 37 °C. Subsequently, 10 mM $CaCl_2$ and trypsin (100 µg/mL) were added and the stirring was continued overnight at 37 °C. SDS at a final concentration of 1% was added, and the mixture was incubated for 15 min at 80 °C to inactivate the enzymes. The cell wall was recovered by centrifugation for 45 min at 130,000 × *g* at 25 °C. The resulting pellet was resuspended in 0.8 mL 8 M LiCl per 1 mL initially used Tris-HCl solution and incubated for 15 min at 37 °C. After another centrifugation using the same conditions as above, the pellet was resuspended in 1 mL 10 mM ethylenediaminetetraacetic acid (EDTA, pH 7.0) per mL of the Tris-HCl solution used initially and this sample was incubated at 37 °C for 15 min. The pellet was washed twice with water. Finally, the pellet was resuspended in 2 to 4 mL of water and lyophilized, yielding the purified PG-WTA complex. To remove all amino acids from the PG, the PG-WTA complex was dissolved in 50 mM Tris-HCl (pH 7.0; 10 mg/mL) and treated with the pneumococcal LytA amidase as described elsewhere.[7] Recombinant His-tagged LytA amidase (1 mg / 10 µg LytA; kindly provided by T. P. Kohler, Univ. Greifswald) was added in three aliquots after 0, 24 and 48 h for a total period of incubation of 72 h at 37 °C. Subsequently, the enzyme was inactivated by boiling for 5 min at 100 °C. After centrifugation (25,000 × *g*, 15 min, 20 °C) the supernatant was collected and lyophilized. The crude LytA-treated PG-WTA complex was further purified by GPC on a Bio-Gel P-30 (45-90 µm, BioRad; column size: 1.5 × 120 cm; buffer: 150 mM ammonium acetate (pH 4.7)) column.

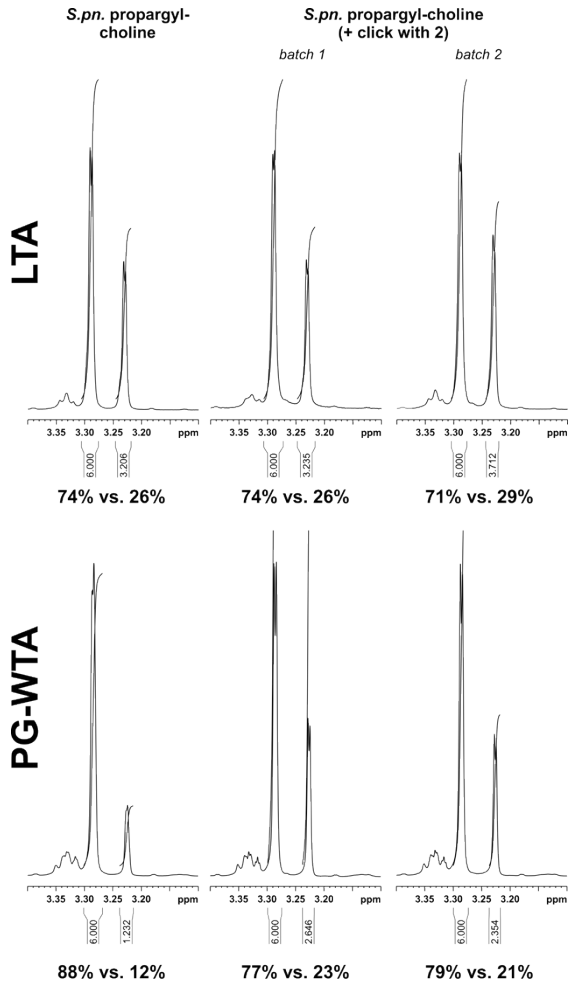


Figure S2: Sections (δ_H 3.40-3.10) of ^{1}H NMR spectra of LTA and PG-WTA preparations, isolated from *S. pneumoniae* grown in the presence of propargyl-choline **1** w/wo performed bioorthogonal reaction with **2**. For the latter, two independent batches (see also Figure S5, panels (iii) and (iv) were prepared and analyzed. Both of these preparations led to virtually identical sets of NMR spectra. The signal at δ_H 3.28 ppm represents the protons of the methyl groups of the $N(CH_3)_2$ moiety in propargyl-choline **1** and its integral has therefore been set to a value of 6.0 (for 6 protons) for calculation. The signal at δ_H 3.22 ppm represents the protons of the methyl groups of the $N(CH_3)_3$ moiety in normal choline (compare Figures 2 and S3) and comprises therefore 9 protons. Rates of incorporated **1** vs. normal choline has been calculated (1 : X/9 with X = integral for signal at δ_H 3.22 ppm), percentage values are given below each spectrum.

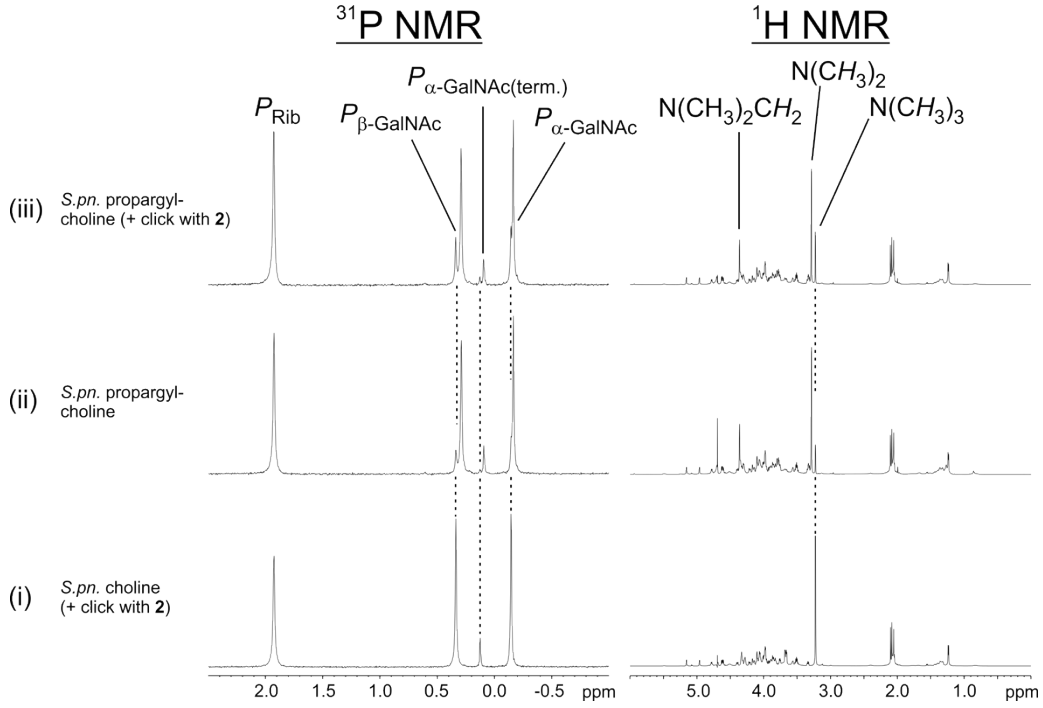


Figure S3: Sections of ^{31}P NMR (δ_P 2.5-(−1.0)) and ^{1}H NMR (water suppressed; δ_H 6.0-0.0)) spectra of PG-WTA preparations after LytA treatment, isolated from *S. pneumoniae* grown in the presence of (i) normal choline and subsequent bioorthogonal reaction with **2**, (ii) propargyl-choline **1** and (iii) propargyl-choline **1** and subsequent bioorthogonal reaction with **2**. Incorporation of **1** in WTA is visible by the occurrence of the additional signals in panels (ii) and (iii). The chemical structure for signal assignment is depicted in Figure S7 (P_{Rib} = P_C; $P_{\beta\text{-GalNAc}}$ = $P_{D/D(term)}$; $P_{\alpha\text{-GalNAc(term)}}$ = $P_{E(term)}$; $P_{\alpha\text{-GalNAc}}$ = P_E; $N(C\mathit{H}_3)_3$ = H-3 of Cho; $N(C\mathit{H}_3)_2$ = H-3 of pCho; $N(CH_3)_2C\mathit{H}_2$ = H-4 of pCho).

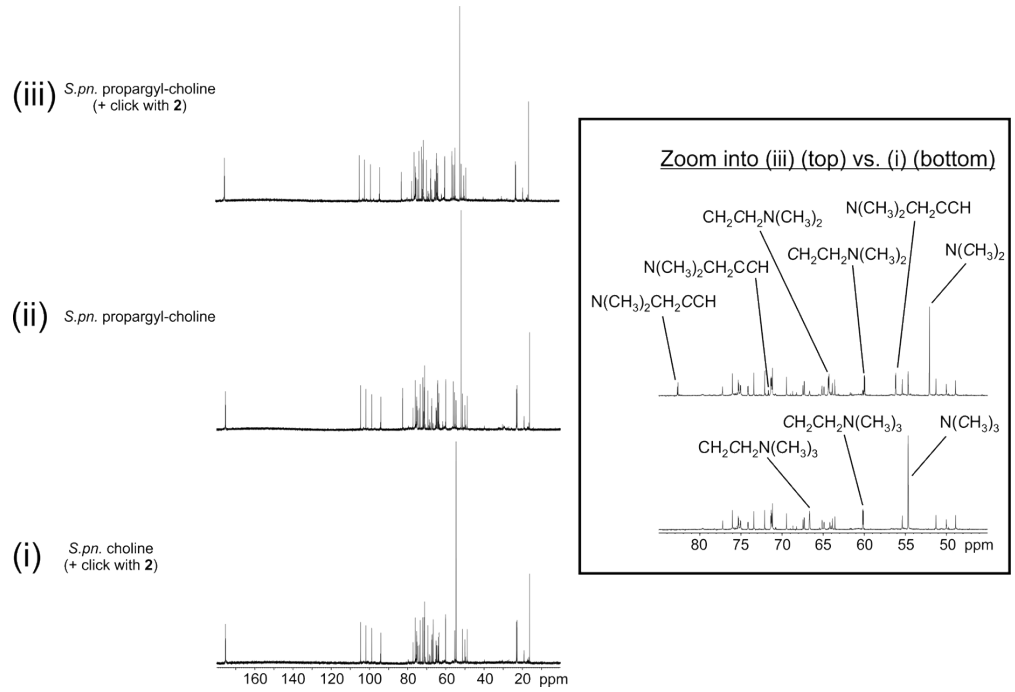


Figure S4: Section of ^{13}C NMR spectra (δ_C 180-0) of PG-WTA preparations after LytA treatment, isolated from *S. pneumoniae* grown in the presence of (i) normal choline and subsequent bioorthogonal reaction with **2**, (ii) propargyl-choline **1** and (iii) propargyl-choline **1** and subsequent

bioorthogonal reaction with **2**. Incorporation of **1** in WTA is visible by the occurrence of the additional signals in panels (ii) and (iii) (see magnified comparison of spectra (iii) and (i) in the box).

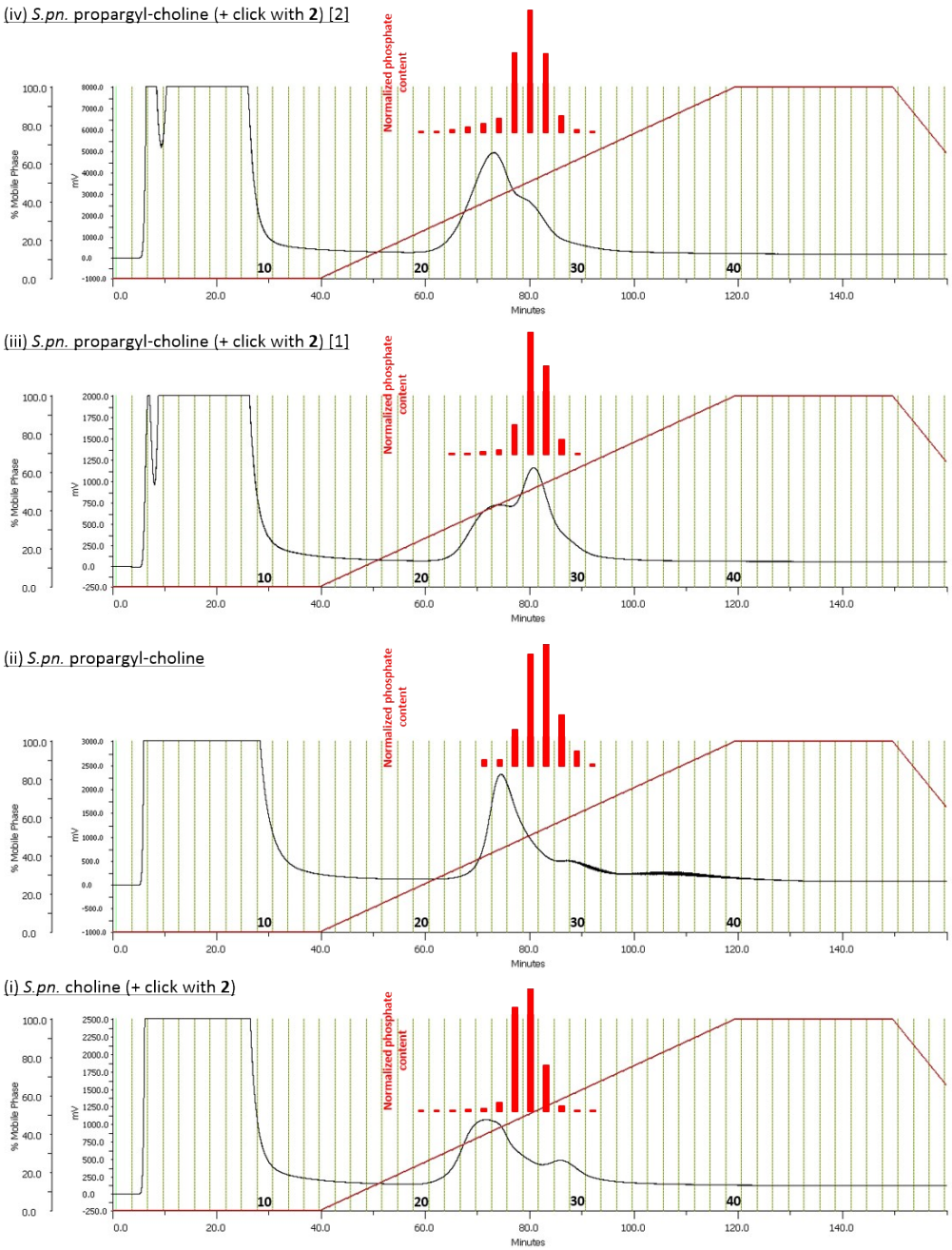


Figure S5: Chromatograms of the hydrophobic interaction chromatography of LTA isolations from *S. pneumoniae* grown in the presence of (i) normal choline (and subsequent bioorthogonal reaction with **2**), (ii) propargyl-choline **1** and (iii, iv) propargyl-choline **1** (and subsequent bioorthogonal reaction with **2**; two independent batches [1] and [2]). The normalized visualization of the phosphate content of selected fractions is included. UV detection was done at $\lambda = 254$ nm, solvent gradient is indicated with the red line. A significant increase in UV signal intensity in the LTA-containing fractions (especially #26-28) in spectra (iii) and (iv) clearly indicates the covalent attachment of the fluorophore. The UV activity (without phosphate positivity) in the earlier eluting fractions is caused by lipopeptides and potentially other co-eluting molecules.

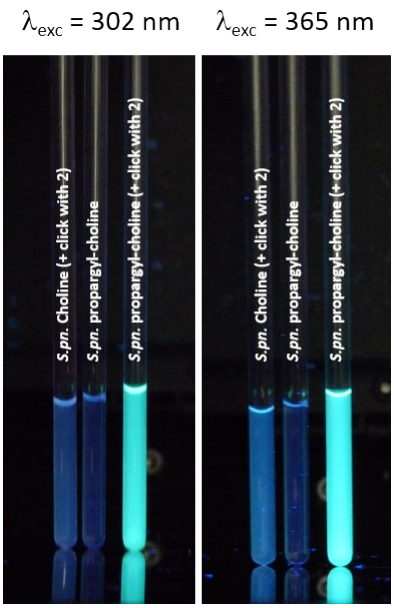


Figure S6: Pictures of LTA samples in NMR tubes (dissolved in equal concentrations in D_2O) from HIC purifications (i), (ii) and (iv) shown in Figure S5 under fluorescence excitation at 302 nm or 365 nm, respectively. The presence of the fluorophore in the sample with incorporated propargyl-choline **1** and performed bioorthogonal reaction with **2** after HIC purification is clearly visible.

Table S1. ^{1}H (700.4 MHz), ^{13}C NMR (176.1 MHz), and ^{31}P NMR (283.5 MHz) chemical shift data (δ, ppm) [*J*, Hz] of WTA bound to the PG sugar chain after LytA-treatment from *S. pneumoniae* grown in the presence of propargyl-choline **1** (corresponding ^{31}P and ^{1}H NMR spectra are shown in Figure S3 panel (ii), the ^{13}C NMR in Figure S4 panel (ii); chemical structure and residue assignment is depicted in Figure S7). *non-resolved multiplet, **multiple signals; without PCho: §4.58 [8.7], 101.9; §§5.08-5.06*, 94.1.

Residue (assignment)	H-1 *C-1*	H-2 *C-2*	H-3 *C-3*	H-4 *C-4*	H-5 *C-5*	H-6 *C-6*	NAc
→3)-α-AATGal*p*-(1→*P* (**A'**)	5.51-5.47* *94.4*	4.21-4.16* *48.9*	4.37-4.32* *75.1*	4.03-3.99* *55.2*	4.54-4.49* *64.2*	1.38-1.30* *16.6*	2.06 *23.0* *175.3*
P→6)-β-D-Glc*p*-(1→ (**B'**)	4.66-4.62* *104.7*	3.35-3.30* *73.4*	3.52-3.47* *76.0*	3.57-3.51* *69.5*	3.58-3.54* *75.0 [7.1]*	4.17-4.12* *64.9 [5.2]*	
→1)-ribitol-(5→ *P* (**C**)	3.97-3.93* 3.88-3.83* *71.3*	4.03-3.98* *71.3*	3.78-3.74* *72.1*	3.92-3.87* *71.4*	4.08-4.03* 4.00-3.95* *67.3 [5.2]*		
→3)-β-D-6-*O*-*P*-(p)Cho-Gal*p*NAc (1→ (**D**)	4.61 [8.7] *101.9*	4.13-4.06* *51.3*	3.88-3.83* *75.3*	4.19-4.15* *63.9*	3.85-3.80* *74.1 [7.8]*	4.09-4.03* *65.2 [4.6]*	2.08 *23.1* *175.4*
→4)-α-D-6-*O*-*P*-(p)Cho-Gal*p*NAc (1→ (**E**)	5.16 [3.1] *94.0*	4.34-4.30* *50.0*	3.94-3.90* *67.5*	4.12-4.08* *77.3*	4.02-3.98* *71.2*	4.06-3.96* *64.4-64.2**	2.05 *22.7* *175.3*
→3)-α-AATGal*p*-(1→ (**A**)	4.96 [2.8] *98.8*	4.23-4.18* *48.9*	4.42-4.37* *75.3*	4.00-3.96* *55.4*	4.80-4.75* *63.6*	1.24 [6.4] *16.1*	2.10 *22.8* *175.1*
P→6)-β-D-Glc*p*-(1→ (**B**)	4.63 [8.2] *104.7*	3.37-3.31* *73.4*	3.52-3.47* *76.0*	3.55-3.49* *69.5*	3.59-3.53* *75.0 [7.1]*	4.12-4.06* *64.9 [5.2]*	
→3)-β-D-6-*O*-*P*-(p)Cho-Gal*p*NAc (1→ (**D^{term}**)	4.63-4.59*,§ *101.9*	4.13-4.06* *51.3*	3.85-3.81* *75.5*	4.15-4.13* *64.0*	3.85-3.80* *74.1 [7.8]*	4.09-4.03* *65.2 [4.6]*	2.08 *23.1* *175.4*
→4)-α-D-6-*O*-*P*-(p)Cho-Gal*p*NAc (**E^{term}**)	5.08 [3.8]§§ *94.2*	4.23-4.20* *50.0*	3.82-3.78* *68.2*	4.05-4.02* *68.7*	4.00-3.96* *70.8 [8.4]*	4.08-4.04* 4.03-3.98* *65.6-65.5**	2.04 *22.7* *175.3*
Cho-*P*-(6-*O*→ @ **D**, **D^{term}**, **E^{term}**	4.35-4.31* *60.2 [5.2]*	3.70-3.66* *66.7-66.5**	3.22 54.7				
Cho-*P*-(6-*O*→ @ **E**	4.30-4.26* *60.1 [5.2]*	3.67-3.64* *66.7-66.5**	3.22 54.7				
pCho-*P*-(6-*O*→ @ **D**, **D^{term}**, **E^{term}**	4.37-4.32* *60.0 [5.2]*	3.82-3.78* *64.4 [8.0]*					
			3.28 52.1	4.38-4.34* 56.2/56.1	- 82.7/82.6	3.34-3.30* 71.7/71.6	
pCho-*P*-(6-*O*→ @ **E**	4.32-4.27* *59.9 [5.1]*	3.79-3.75* *64.4-64.2**					
^{31}P	*P*-5^{C}/6$^{B/B'}$ 1.93; *P*-6$^{D/D(term)}$/CH_2O^{Cho} 0.34; *P*-6$^{D/D(term)}$/CH_2O^{pCho} 0.29; *P*-6$^{E(term)}$/CH_2O^{Cho} 0.12; *P*-6$^{E(term)}$/CH_2O^{pCho} 0.09; *P*-6^{E}/CH_2O^{Cho} −0.15; *P*-6^{E}/CH_2O^{pCho} −0.16; P-1$^{A'}$/6^{MurNAc} −1.00-(−1.40)**.						

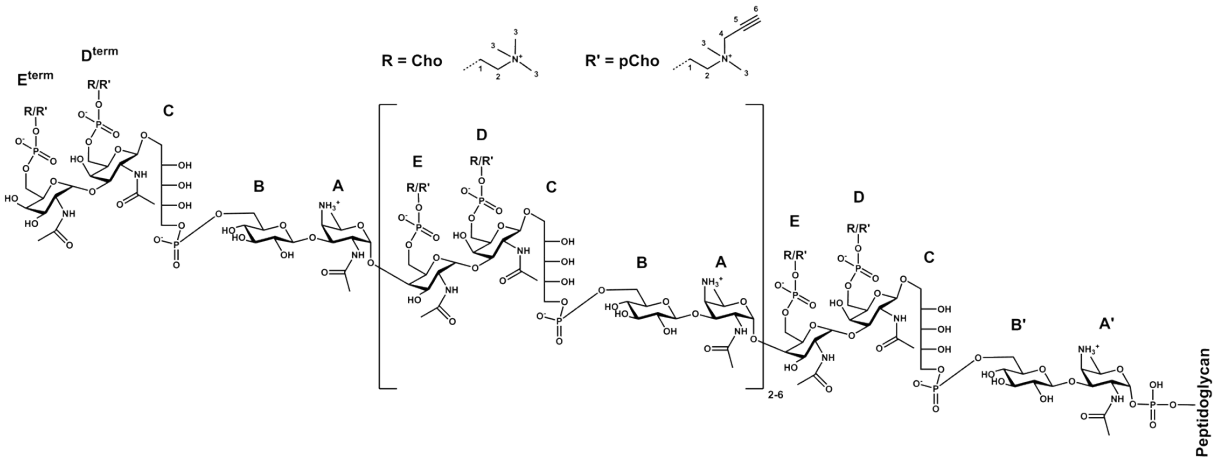


Figure S7: Chemical structure of pneumococcal WTA including residue assignment for NMR interpretation (see Table S1). In pneumococcal LTA the TA chain is identical, but residue A' is β-1,3-linked to the glycolipid anchor α-D-Glc-diacylglycerol.[5]

Supplemental References

1 C. Y. Jao, M. Roth, R. Welti and A. Salic, *Proc. Natl. Acad. Sci. U.S.A.*, 2009, **106**, 15332.

2 K. Sivakumar, F. Xie, B. M. Cash, S. Long, H. N. Barnhill and Q. Wang, *Org. Lett.*, 2004, **6**, 4603.

3 C. Girard, E. Önen, M. Aufort, S. Beauvière, E. Samson and J. Herscovici, *Org. Lett.*, 2006, **8**, 1689.

4 A. Paintdakhi, B. Parry, M. Campos, I. Irnov, J. Elf, I. Surovtsev and C. Jacobs-Wagner, *Mol. Microbiol.*, 2016, **99**, 767.

5 N. Gisch, T. Kohler, A. J. Ulmer, J. Müthing, T. Pribyl, K. Fischer, B. Lindner, S. Hammerschmidt and U. Zähringer, *J. Biol. Chem.*, 2013, **288**, 15654.

6 O. H. Lowry, N. R. Roberts, K. Y. Leiner, M.-L.Wu and A. L. Farr, *J. Biol. Chem.*, 1954, **207**, 1.

7 N. K. Bui, A. Eberhardt, D. Vollmer, T. Kern, C. Bougault, A. Tomasz, J.-P. Simorre, W. Vollmer, *Anal. Biochem.*, 2012, **421**, 657.