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Graphical Abstract

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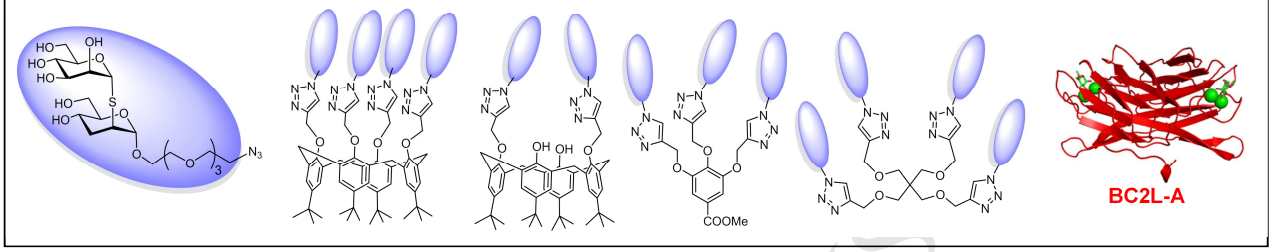

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Tri- and tetravalent mannoclusters cross-link and aggregate BC2L-A lectin from *Burkholderia cenocepacia*

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ABSTRACT

The opportunistic Gram-negative bacterium *Burkholderia cenocepacia* causes lethal infections in cystic fibrosis patients. Multivalent mannoside derivatives were prepared as potential inhibitors of lectin BC2L-A, one of the virulence factors deployed by *B. cenocepacia* in the infection process. An (α1→2)-thio-linked mannobioside mimic bearing an azide functionalized aglycon was conjugated to different multivalent scaffolds such as propargylated calix[4]arenes, methyl gallate and pentaerythritol by azide-alkyne 1,3-dipolar cycloaddition. The interaction between the glycoclusters and the mannose binding BC2L-A lectin from *B. cenocepacia* was examined by isothermal microcalorimetry, surface plasmon resonance, inhibition of yeast agglutination and analytical ultracentrifugation.

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1. Introduction

Burkholderia cenocepacia is a Gram-negative bacterium with the ability to form a biofilm. It is recognized as an opportunistic human pathogen causing lung infections in immunocompromised individuals, especially in cystic fibrosis patients, with significant mortality and morbidity.[1] This bacterium produces three soluble lectins, named BC2L-A, BC2L-B and BC2L-C, which are homologues of the lectin PA-IIL from *Pseudomonas aeruginosa*, the most widespread pathogen attacking humans suffering from cystic fibrosis.[2] These lectins are significant virulence factors of *B. cenocepacia* and are hypothesized to play an important role in host cell recognition and adhesion and in biofilm formation.[3-5] Therefore, they are considered novel therapeutic targets.

The shortest, soluble *Burkholderia* lectin, BC2L-A, consisting of one PA-IIL like domain, associates as a dimer and displays a strict specificity for oligomannose-type *N*-glycans.[2] Titration microcalorimetry measurements demonstrated that Me α-D-mannoside as well as α-(1-2)-, α-(1-3)- and α-(1-6)-linked mannobiosides are high-affinity ligands for BC2L-A with micromolar dissociation constants.[6] On the other hand, BC2L-A can bind directly to bacterial lipopolysaccharides (LPSs) on the outer membrane through L-*glycero*-D-*manno*-heptoses which are abundant in the LPSs of *Burkholderia.*[3] It was thus proposed that this lectin can act as a cross-linker between bacterial cells and influence the social life of the bacteria (i.e. biofilm formation).[3] However, the strong affinity of BC2L-A for D-mannose, which can be found on epithelial cells, suggests that this lectin could be also directly involved in adhesion to the host cells. Consequently, competitive inhibitors which can strongly bind to the lectin can disrupt the colonization of the host by *B. cenocepacia*.

With the goal of obtaining high affinity ligands of BC2L-A, which are potentially useful to block bacterial adhesion and are stable *in vivo*, we envisioned the synthesis of multivalent thio-mannoside derivatives. Due to the cluster glycoside effect, multimeric carbohydrates generally exhibit an enhanced activity toward lectins compared to the corresponding monovalent ligand on a valence-corrected basis.[7] Generally, replacement of the glycosidic oxygen with a sulfur atom improves the biological stability of the planned analogues against chemical and enzymatic hydrolysis. Furthermore, it has been reported that many lectins tolerate the thioglycosidic linkage, and some mannose- and galactose-specifis lectins show similar or even stronger affinity toward *S*-glycosides than the corresponding *O*-glycosides.[8]

We recently reported a rapid and efficient synthesis of spacer-armed (α1→2)-thio-linked mannobioside mimics *via* photoinduced hydrothiolation of the corresponding 2,3-unsaturated glycoside.[9] Having the azido-functionalized pseudomannobiosides **1**[9b] and **2**[9b] to hand, we envisioned their conjugation to multivalent scaffolds bearing terminal alkyne moieties by Cu(I)-catalyzed azide-alkyne cycloaddition reaction.[10] To obtain glycoclusters displaying the mannobioside units in different spatial arrangements, various core compounds equipped with propargyl ether groups were used such as calix[4]arene derivatives **3**[11] and **4**,[12] as well as perpropargylated methyl gallate **5**[13] and pentaerythritol **6**[14] (Figure 1).

Here, we present the preparation of di- tri- and tetravalent thiomannobioside clusters and their interaction with the BC2L-A lectin isolated from *Burkholderia cenocepacia.*

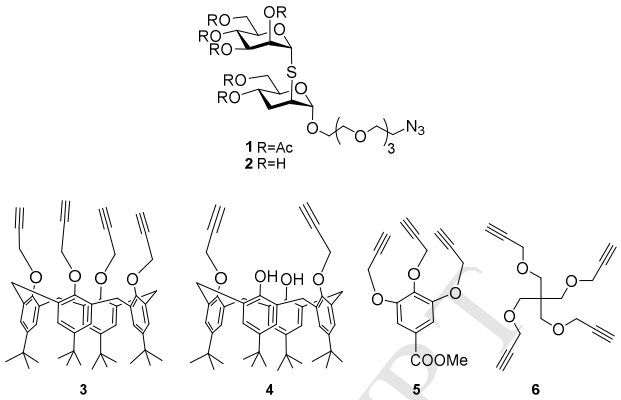
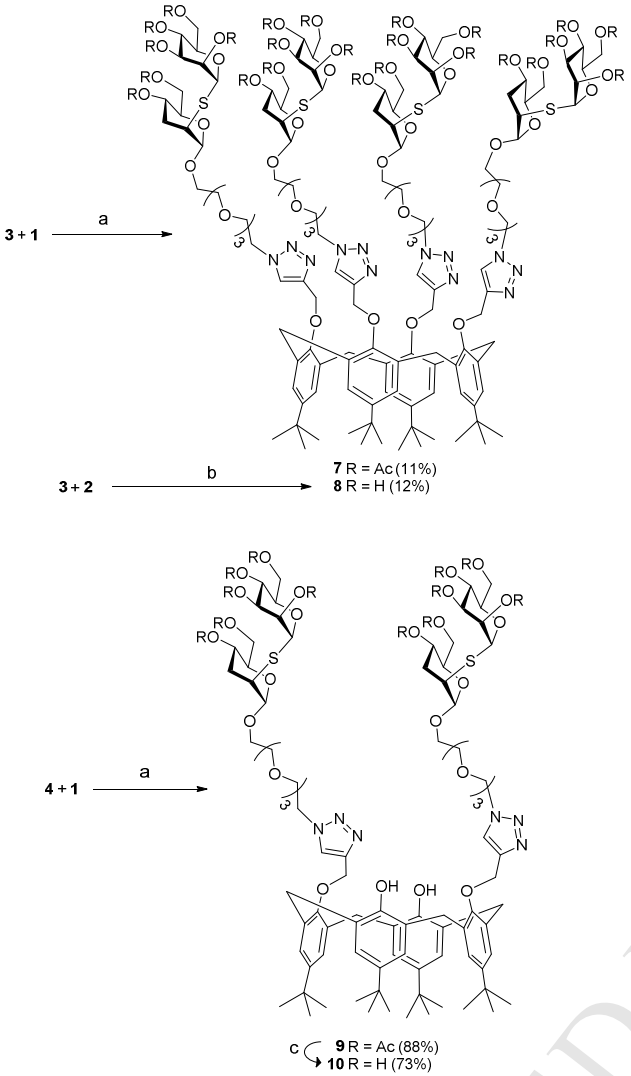


Figure 1. Spacer-linked (α1→2)-thio-linked pseudomannobiosides (**1** and **2**) and propargylated scaffolds (**3**-**6**) as starting materials for the targeted glycoclusters.

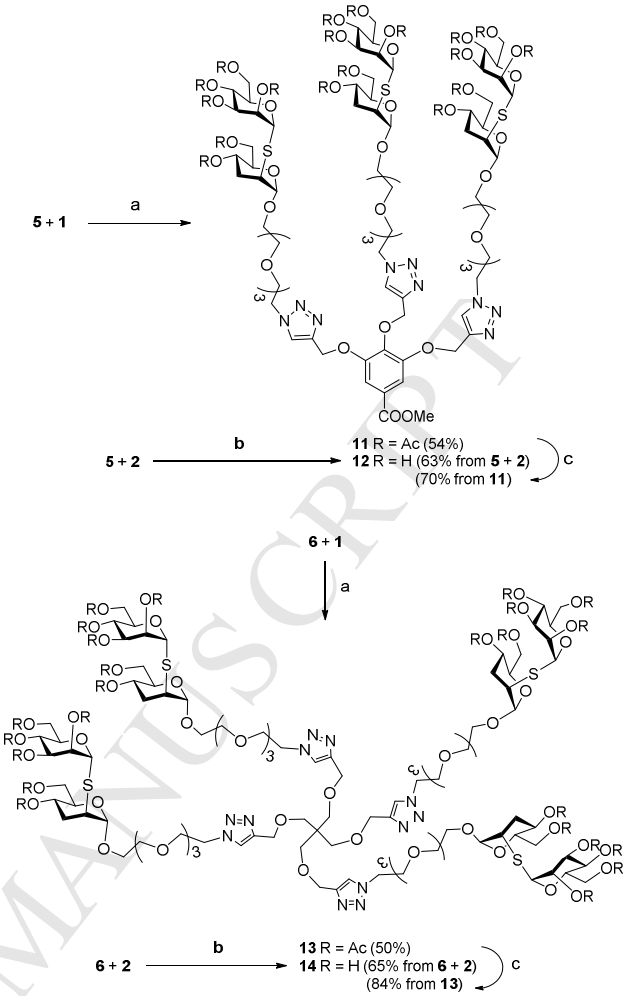
2. Results and discussion

2.1. Synthesis

The azide-alkyne cycloaddition reactions were performed with both the protected and unprotected pseudomannobiosides **1** and **2**, respectively, using copper(I) iodide as the catalyst in the presence of triethylamine, under an argon atmosphere. We first coupled the peracetylated **1** with the tetra-*O*-propargylated scaffold **3** which could be prepared in one step from commercially available *tert*-butylcalix[4]arene. The reaction proceeded with moderate conversion within 24 h at room temperature to result in the protected tetravalent mannoside derivative **7** with an isolated yield of 11%. The cycloaddition took place in a similar manner using unprotected **2** as the azide reactant to give the desired mannocluster **8** in a 12% yield (Scheme 1). The limited efficacy of tetra-*O*-propargylated calix[4]arenes in a click reaction has already been described and is attributed to the high density of alkyne which promotes the side reaction, the homocoupling of scaffolded alkynes.[11,15]



Scheme 1. Synthesis of di- and tetravalent clusters with a calix[4]arene scaffold. Reagents and conditions: (a) Cu(I)I, Et_3N, CH_3CN, rt; (b) Cu(I)I, Et_3N, DMF, rt; (c) NaOMe, MeOH, rt.



Scheme 2. Synthesis of methyl gallate- and pentaerythritol-based clusters. Reagents and conditions: (a) Cu(I)I, Et_3N, CH_3CN, rt; (b) Cu(I)I, Et_3N, DMF, rt; (c) NaOMe, MeOH, rt.

Conjugation of the protected sugar **2** to the calix[4]arene derivative **4** bearing only two propargyl moieties occurred with an excellent 88% yield, and subsequent Zemplén deacetylation of the resulting **9** yielded the bivalent thiomannobioside **10** in a 73% yield.

The synthesis of the pseudomannobioside-appended methyl gallate conjugate **12** and pentaerythritol conjugate **14** occurred with good yields, using either the protected or the unprotected sugar derivatives, as shown in Scheme 2. Interestingly, slightly higher yields could be achieved if the unprotected pseudomannobioside **2** was used in the click reaction, despite the difficult separation of the polar products.

2.2. Binding of BC2L-A to multivalent thiomannosides

Investigations of the interaction of the multivalent thiomannobioside derivatives with mannose-binding lectin BC2L-A were carried out using a simple microscopy method for the inhibition of agglutination, isothermal titration calorimetry (ITC), surface plasmon resonance (SPR) and analytical ultracentrifugation (AUC). These methods can provide information about the binding affinity, inhibition potency and effect of cross-linking.

The activity of BC2L-A was verified via its interaction with methyl α-D-mannopyranoside, measured by ITC. Results comparable with those previously published were obtained.[2]

We attempted to incorporate all multivalent mannoside derivatives in the biological investigations. Although the utility of calixarene-based glycoconjugates in sugar-lectin binding assays has already been demonstrated,[16] the amphiphilic calix[4]arene clusters **8** and **10**, unfortunately, could not be used in this study because of their low solubility in water and water−DMSO mixture. Therefore, we focused on the interaction of compounds **12** and **14** with BC2L-A.

As for **12** and **14**, the interactions with BC2L-A observed by ITC resulted in exothermic peaks, which is typical for

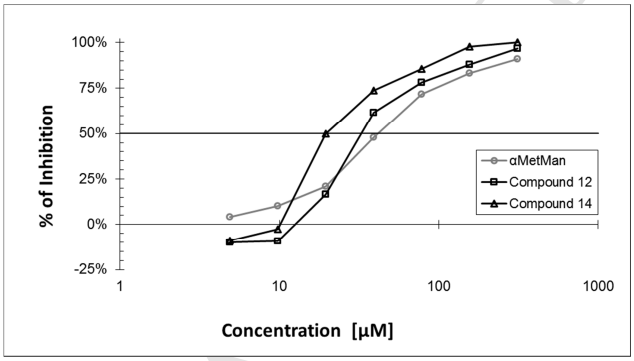
interactions of BC2L-A with carbohydrates.[2,3] However, the peaks were significantly (10x) higher than typical interactions of BC2L-A, and saturation was not reached. Therefore reliable evaluation of the interactions was not possible. These problems probably result from the interaction itself and are not caused simply by the behaviour of **12** and **14**, because they persisted even after the blank measurement (injection of **12** or **14** to the buffer) subtraction. A possible explanation could be cross-linking of BC2L-A molecules with multivalent compounds and subsequent aggregation.

The potential inhibitory activity of **12** and **14** toward BC2L-A was tested via a biosensor with SPR detection. Both compounds were used as inhibitors of BC2L-A binding to the immobilized α-D-mannopyranoside in the verified set-up, with methyl α-D-mannopyranoside as a control.[2,4] Both **12** and **14** were able to inhibit the binding of BC2L-A to the glycosylated surface with the inhibition potency of **12** being only slightly higher (1.3) than that of methyl α-D-mannopyranoside. The inhibition potency of **14** was more than double that of methyl α-D-mannopyranoside (2.17, see Table 1).

Table 1. IC_{50} values obtained by SPR for the inhibition of binding of BC2L-A to immobilized α-D-mannopyranoside.

Inhibitor	IC_{50}(μM)	Potency
αMeMan	42.45	1
Compound **12**	32.65	1.3
Compound **14**	19.53	2.17

Figure 2. Inhibitory effects of **12** and **14** on BC2L-A binding to immobilized α-D-mannopyranoside with methyl α-D-mannopyranoside used as a control and standard



Non-standard behaviour was again observed. The binding responses of BC2L-A mixed with the two lowest concentrations (4.88 μM and 9.65 μM) of **12** and **14** were actually higher than the response of mere BC2L-A, resulting in negative inhibition (i.e. promotion of the interaction, Figure 2). Again, this behaviour could be caused by cross-linking of the BC2L-A molecules. The SPR detects the mass bound to the surface. Therefore, the binding of cross-linked molecules of BC2L-A via one single active binding site could actually generate the same response as the binding of several molecules of active BC2L-A and cause falsely increased responses.

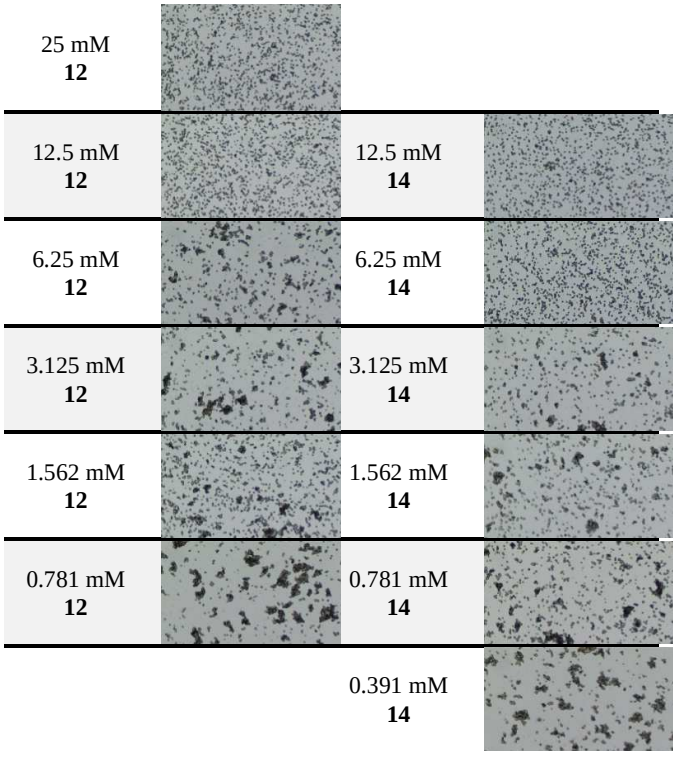
Therefore, the inhibitory activity of **12** and **14** was also tested using a yeast agglutination inhibition assay. BC2L-A as a mannose-specific lectin can agglutinate yeasts. Both **12** and **14** were able to inhibit this agglutination, with results corresponding to that obtained via SPR (Table 2).

Table 2. The values obtained for the inhibition of yeasts agglutination caused by BC2L-A.

Inhibitor	The lowest concentration able to inhibit agglutination	Potency	Note
αMeMan	12.5 mM	1	Standard
D-Man	25 mM	0.5	
D-Gal	Not detected		Negative control
12	12.5 mM	1	
14	6.25 mM	2	

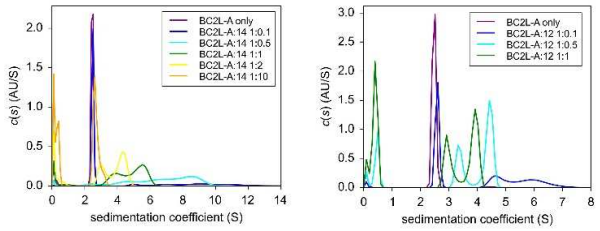
The inhibitory effect of **12** was the same as for methyl α-D-mannopyranoside, whereas **14** was twice as effective as methyl α-D-mannopyranoside (see Table 3). Also, the yeast agglutination inhibition assay with detection with an optical microscope proved to be a simple and inexpensive method for testing newly synthesized compounds. Using this assay, even the differences between the inhibition potencies of D-mannose and methyl α-D-mannopyranoside could be observed, and the results were in agreement with the previously published data (see Table 2).[2]

Table 3. Influence of **12** and **14** on yeast agglutination caused by lectin BC2L-A. Several pictures were taken for each concentration of inhibitor. Compounds **12** and **14** were able to inhibit agglutination at concentrations above 12.5 mM and 6.25 mM, respectively.



The expected cross-linking of BC2L-A via compounds **12** and **14** was verified using analytical ultracentrifugation. BC2L-A itself sediments at 2.5 S and forms a dimer with a globular shape (with an estimated molecular weight of 27.5 kDa and frictional ratio of 1.28). Mixing BC2L-A with compound **14** resulted in the formation of a wide range of small oligomers/aggregates (Figure 3). Since the reaction scheme of the system (divalent lectin, tetravalent ligand) can be quite complicated, and we cannot exclude the possibility that the interaction is governed by fast reaction kinetics (relative to the time-scale of sedimentation) where the positions of the peaks shift in a concentration-dependent manner, no attempts were made to identify the nature of the peaks observed in the molar ratios 1:1 and 1:2. The ratio 1:10 resulted mainly in the dimeric form of BC2L-A, suggesting that all binding sites of the protein are saturated and cross-linking is not possible. No precipitation visible to the naked eye was observed. The addition of compound **12** to BC2L-A also caused the formation of higher oligomers (Figure 3).

Figure 3. Continuous c(s) distributions for BC2L-A with **14** (left) and **12** (right). The figures were created in the program GUSSI.[17]



Again the reaction scheme seems to be quite complex. Slight precipitation was visible in samples with a BC2L-A:compound **12** ratio of 1:2 and 1:10. High concentrations of compound **12** in these samples resulted in very high absorbance (due to the aromatic core) and the data from the 1:2 and 1:10 ratios could not be evaluated. Nevertheless, analytical ultracentrifugation gave clear evidence of the cross-linking of BC2L-A with compound **12**.

3. Conclusion

In conclusion, we have demonstrated a simple and highly efficient approach to produce various glycoclusters with terminal 1,2-thio-linked pseudomannobiosides. The azide-functionalized pseudodisaccharides, both in their protected and unprotected forms, could be conjugated to the propargyl-containing scaffolds with similar efficacy. Investigations of the interactions of the derivatives with mannose-specific lectin BC2L-A were carried out and resulted in confirming that compounds **12** and **14** are able to inhibit the binding activity of the lectin, although the inhibitory effect was not as high as expected. Compounds **12** and **14** are probably unable to chelate both binding sites of the BC2L-A dimer and achieve increased affinity. The compounds instead cross-link and aggregate BC2L-A molecules. This behaviour has been observed previously in interactions of BC2L-A with multivalent inhibitors.[18] Those mannosylated inhibitors were too short to connect the two binding sites of BC2L-A that are 40 Å apart. In the present study, compounds **12** and **14** possess sufficient span but are still unable to achieve significantly increased inhibitory effect. Therefore, this phenomenon is probably not a simple problem of the length but involves possibly also steric hindrances and conformations of compounds **12** and **14** in the solution. However, cross-linking of surface lectins can cause an agglutination of bacteria presenting these lectins, as was observed for the lectin PA-IIL from *Pseudomonas aeruginosa*.[19] This process resulted in interference with biofilm production and bacteria-host cell interactions. Therefore, compounds **12** and **14** are suitable candidates to investigate in terms of their ability to interfere with *Burkholderia cenocepacia* functions at the cell level. Also, a study is planned of the interaction of these mannoclusters with BC2L-C,[4] another lectin of *B. cenocepacia*.

4. Experimental

4.1. General methods

Optical rotations were measured at room temperature with a Perkin-Elmer 241 automatic polarimeter. TLC analysis was performed on Kieselgel 60 F_{254} (Merck) silica gel plates with visualization by immersing in a sulfuric-acid solution (5% in EtOH) followed by heating. Column chromatography was performed on silica gel 60 (Merck 0.063–0.200 mm), flash column chromatography was performed on silica gel 60 (Merck 0.040–0.063 mm). Gel filtration was performed on Sephadex G-25, using methanol as the eluent. Organic solutions were dried over $MgSO_4$ and concentrated under vacuum. The ^1H (400 and 500 MHz) and ^{13}C NMR (100.28, 125.76 MHz) spectra were recorded with Bruker DRX-360, Bruker DRX-400 and Bruker Avance II 500 spectrometers. Chemical shifts are referenced to Me_4Si or DSS (0.00 ppm for ^1H) and to solvent signals ($CDCl_3$: 77.00 ppm, CD_3OD: 49.15 ppm, DMSO-d_6: 39.51 ppm for ^{13}C). MS (MALDI-TOF) analysis was carried out in positive reflectron mode with a BIFLEX III mass spectrometer (Bruker, Germany) with delayed-ion extraction. The matrix solution was a saturated solution of 2,4,6-trihydroxy-acetophenone (THAP) in MeCN. The recombinant protein BC2L-A was cloned using the previously described procedure and purified by affinity chromatography[2]. Isothermal titration calorimetry (ITC) experiments were measured using Auto-iTC200 (Malvern Instruments). Surface plasmon resonance (SPR) experiments were performed using a BIAcore 3000 (GE Healthcare) at 25 °C. Agglutination experiments were carried out using commercially available baker's yeast (Uniferm) and agglutination/inhibition of agglutination was determined with an optical microscope, a Levenhuk D2L NG Digital Microscope (Levenhuk). Analytical ultracentrifugation experiments were performed using a ProteomeLab XL-I analytical ultracentrifuge (Beckman Coulter) equipped with an An-60 Ti rotor.

4.2. Protein production and purification

Protein was produced in *Escherichia coli* BL21(DE3) as previously described.[2] Transformed cells were cultured in LB broth medium containing ampicillin (100 µg.ml^{-1}) at 37 °C. When the culture reached an OD_{600} of 0.5, cells were induced by isopropyl 1-thio-β-D-galactopyranoside (IPTG) added to a final concentration of 0.5 mM. Cells were incubated at 27 °C for 4 hours, harvested by centrifugation and resuspended in equilibrating buffer (20 mM Tris/HCl, 100 mM NaCl, pH 7.4). Cells were then disintegrated by sonication and the cytosolic fraction containing soluble BC2L-A was separated by centrifugation. BC2L-A was purified by affinity chromatography on a D-mannose-agarose column and eluted with 20 mM Tris/HCl, 150 mM NaCl, 10 mM EDTA, pH 7.4. Purified protein was dialysed (against 10 mM Tris/HCl, 50 mM NaCl, 1 mM $CaCl_2$ for 2 days, then against 50 mM NH_4HCO_3 for 2 days), freeze-dried and stored at -20 °C.

4.3. Isothermal titration calorimetry

Freeze-dried BC2L-A was dissolved in 0.1 M Tris/HCl, 500 μM $CaCl_2$, pH 7.5, and equilibrated at room temperature for 1 h before ITC measurement. All experiments were performed in an Auto-iTC200 calorimeter (Malvern Instruments) at 25°C. Compounds **12** and **14** were used at a concentration of 2 mM. Aliquots of 2 μl were added automatically to the 0.2 mM BC2L-A in the calorimeter cell. Blank experiments (injections of **12** and **14** into the buffer) were performed, and heat responses were subtracted. Control experiments performed by injections of the buffer to the protein solution yielded insignificant heats of dilution. Integrated heat effects were analysed using Microcal Origin 7 software (Malvern Instruments). The activity of freeze-dried BC2L-A was tested via interaction with methyl α-D-mannopyranoside.

4.4. Surface plasmon resonance

Surface plasmon resonance (SPR) experiments were performed on a BIAcore 3000 instrument (GE Healthcare) at 25 °C, using 10 mM HEPES, 150 mM NaCl, 0.005 % TWEEN 20, pH 7.4 as a running buffer. Two different carbohydrates were immobilized onto a CM5 sensor chip (GE Healthcare) covered with a carboxymethylated dextran matrix: α-D-galactopyranoside and α-D-mannopyranoside. The sensor chip surface was first coated with streptavidin using a standard amine coupling method. The carboxymethytaled surface was activated with an N-hydroxysuccinimide/N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride solution (NHS/EDC, GE Healthcare) and streptavidin diluted in 10 mM sodium acetate, pH 5.0 to a concentration of 100 $\mu g.ml^{-1}$ was injected onto the CM5 chip. Unreacted groups were blocked with 1 M ethanolamine-HCl, pH 8.5. The volume of all solutions was 50 μl for one channel at a flow rate of 5 μl/min. Saccharides bound to the biotinylated polyacrylamide (biotin-PAA-monosaccharides, Lectinity) were then used for the immobilization. Each biotin-PAA-monosaccharide was diluted in the running buffer to a concentration of 200 $\mu g.ml^{-1}$, and 50 μl of this mixture was injected onto one particular channel. The flow rate was 5 μl/min. Protein BC2L-A at a concentration of 500 $\mu g.ml^{-1}$ was mixed with 10x concentrated inhibitors in a 9:1 ratio. 30 μl of this mixture was injected onto the CM5 chip with immobilized carbohydrates at a flow rate of 5 μl/min. The sensor chip was washed with 100 mM EDTA. IC_{50} (concentration of inhibitor resulting in 50% inhibition of binding) was determined. As IC_{50} is not a constant and depends on the experimental set-up, a parameter called potency was used for characterization. The potency of a certain inhibitor is the ratio of IC_{50} of a chosen standard inhibitor (in this case methyl α-D-mannopyranoside) and the inhibitor in question. As BC2L-A does not bind to α-D-galactopyranoside, the corresponding channel was used as a blank.

4.5. Agglutination inhibition assay

Freeze-dried lectin BC2L-A, carbohydrate inhibitors and yeast cells (Uniferm) were dissolved in the working buffer (20 mM Tris/HCl, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.5). The lectin (2.5 $mg.ml^{-1}$) was mixed with serially diluted carbohydrate inhibitors in a 5 μl:5 μl ratio. The final (working) concentration of the lectin was therefore 1.25 $mg.ml^{-1}$ and the final concentration of carbohydrate inhibitors varied from 100 mM to 0.391 mM. 10 μl of 5% yeast was then added, the mixture was thoroughly mixed and incubated for 10 minutes at room temperature. After incubation, the mixture was again mixed, transferred to a microscope slide and examined. The examination was conducted using the Levenhuk D2L NG Digital Microscope (Levenhuk). Images were obtained with a Levenhuk D2L digital camera (Levenhuk) using the software ToupView for Windows (Levenhuk). The positive (experiment without inhibitor) and negative control (experiment without lectin) were prepared and processed in the same way using the appropriate volume of dissolving buffer instead of the omitted components. The lowest concentration of inhibitor able to inhibit agglutination was determined and compared with the standard (methyl α-D-mannopyranoside). As BC2L-A cannot recognize D-galactose, this carbohydrate was used as a negative control.

4.6. Analytical ultracentrifugation

Analytical ultracentrifugation experiments were performed using a ProteomeLab XL-I analytical ultracentrifuge (Beckman Coulter) equipped with an An-60 Ti rotor. BC2L-A and compounds **12** and **14** were diluted with the experimental buffer (20 mM Tris/HCl, 150 mM NaCl, 0.5 mM $CaCl_2$, pH 7.5) and several mixtures with different lectin to ligand ratios were prepared. In all cases, the concentration of BC2L-A was 72 μM and lectin:ligand molar ratios of 1:0.1, 1:0.5, 1:1, 1:2 and 1:10 were tested. BC2L-A without the ligand added was also measured. .Sedimentation velocity experiments were conducted in standard double-sector centerpiece cells loaded with 425 ul of sample and 425 ul of reference solution (20 mM Tris/HCl, 150 mM NaCl, 0.5 mM $CaCl_2$, pH 7.5). Data were collected using absorbance optics at 20° C at a rotor speed of 48,000 rpm. Scans were collected at 280 nm at 5-min intervals and 0.003 cm spatial resolution in continuous scan mode. The partial specific volume of protein and the solvent density and viscosity were calculated from the amino acid sequence and buffer composition, respectively, using the software Sednterp (http://bitcwiki.sr.unh.edu). The data were analyzed with the continuous c(s) distribution model implemented in the program Sedfit 14.6.[20] For the regularization procedure, a confidence level of 0.95 was used. High-quality plots of continuous distributions were created in GUSSI 1.10.[17]

4.7. Synthesis

4.7.1. General method A for azide-alkyne click reaction

(5 mL) Et_3N (1 equiv. /alkyne) and Cu(I)I (0.1 equiv. /alkyne) were added to a stirred solution of alkyne (0.2 mmol) and azide (1.25 equiv./alkyne) in DMF or CH_3CN under an argon atmosphere and stirred overnight at room temperature. The reaction mixture was evaporated, and the crude product was purified by flash column chromatography to give the desired compound.

4.7.1. General method B for Zemplén-deacetylation

The catalytic amount of NaOMe (pH ~ 9) was added to a stirred solution of ester (0.2 mmol) in dry MeOH (5 mL) and stirred overnight at room temperature. The reaction mixture was neutralized with Amberlite IR-120 ion-exchange resin, filtered and evaporated, then the crude product was purified by flash column chromatography and gel filtration to give the desired compound.

*4.7.2. Compound **7***

Azide compound **1** (152 mg, 0.2 mmol) and alkyne **3** (30 mg, 0.04 mmol) were reacted in CH_3CN according to the general method A. The crude product was purified by flash column chromatography (95:5 CH_2Cl_2:MeOH) to give compound **7** (16 mg, 11%) as a colourless syrup. $[\alpha]^{24}_D$ +88.30 (*c* 0.49, $CHCl_3$); R_f 0.30 (95:5 CH_2Cl_2: MeOH); ^{1}H NMR ($CDCl_3$, 500.76 MHz): δ (ppm) 7.75 (s, 4H, 4x H-Tr), 6.65 (s, 8H, 8x H-Ar), 5.38- 5.35 (overlapping signals, 8H, 4x H-1', 4x H-2'), 5.30 (t, 4H, 4x H-4', *J*= 9.9 Hz), 5.24 (dd, 4H, 4x H-3', J_1= 3.1 Hz, J_2= 9.9 Hz), 5.03 (m, 4H, 4x H-4), 4.85 (s, 4H, 4x H-1), 4.97 (s, 8H, 4x OCH_2Ar), 4.67 (m, 8H, 4x NCH_2), 4.38 (m, 4H, 4x H-5'), 4.28 (dd, 4H, J_1= 5.3 Hz, J_2= 12.3 Hz, 4x H-6'a), 4.20 (dd, 4H, J_1= 2.4 Hz, J_2= 12.3 Hz, 4x H-6a), 4.15 (dd, 4H, J_1= 5.3 Hz, J_2= 12.3 Hz, 4x H-6b), 4.11 (dd, 4H, J_1= 5.3 Hz, J_2= 12.3 Hz, 4x H-6'b), 3.97 (m, 4H, 4x H-5), 3.93 (m, 4H, 2x ArCH_{2a}Ar), 3.80- 3.50 (overlapping signals, 56H, OCH_2), 3.24 (m, 4H, 4x H-2), 2.87 (m, 4H, 2x ArCH_{2b}Ar), 2.23 (m, 8H, 4x H-3), 2.17, 2.11, 2.09, 2.06, 2.04, 1.99, (s, 72H, 24x COCH_3),1.07 (s, 36H, C(CH_3)$_3$). ^{13}C NMR ($CDCl_3$, 125.76 MHz): δ (ppm) 170.8, 170.5, 169.9, 169.8, 169.7 (*C*O), 152.5 (C_q-Ar), 143.9 (C_q-Tr), 133.9 (C_q-Ar), 133.7 (C_q-Ar), 129.7 (C_q-Ar), 125.2 (CH-Tr), 125.1 (CH-Ar), 98.8 (C-1), 82.4 (C-1'), 70.9, 70.5, 70.4, 70.1, 69.3, 68.8, 68.7, 66.2, 64.7 (skeleton carbons and O*C*H$_2$), 62.9 (C-6), 62.3 (C-6'), 50.5 (N*C*H$_2$), 44.9 (C-2), 33.9 (*C*(CH$_3$)$_3$), 31.7 (Ar*C*H$_2$Ar), 31.3 (C(*C*H$_3$)$_3$), 29.1 (C-3), 21.0, 20.9, 20.7, 20.6 (CO*C*H$_3$). MALDI-TOF (positive ion): *m/z* calcd for $C_{184}H_{260}N_{12}NaO_{76}S_4$: 4004.56 $[M+Na]^+$ Found: 4004.60.

*4.7.3. Compound **8***

Azide compound **2** (106 mg, 0.2 mmol) and alkyne **3 (**30 mg, 0.04 mmol) were reacted in DMF according to the general method. The crude product was purified by flash column chromatography (8:5:1 CH_2Cl_2:MeOH:H_2O) to give compound **8** (13 mg, 12%) as a colourless syrup. $[\alpha]^{24}_D$ +88.30 (*c* 0.49, MeOH); R_f 0.56 (16:10:3 CH_2Cl_2:MeOH:water) ^{1}H NMR (DMSO, 500.76 MHz): δ (ppm) 8.04 (s, 4H, 4x H-Tr), 6.70 (s, 8H, 8x H-Ar), 5.20 (s, 4H, 4x H-1'), 4.76 (s, 4H, 4x H-1), 4.56 (m, 8H, 4x NCH_2), 4.15- 3.15 (overlapping signals, 136H, skeleton hydrogens, ArCH_2Ar and OCH_2), 3.09 (s, 4H, 4x H-2), 2.05 (m, 8H, 4x H-3), 1.07 (s, 36 H, C(CH_3)$_3$), ^{13}C NMR (DMSO, 125.76 MHz): δ (ppm) 143.7 (C_{qvat}-Tr), 133.6 (C_q-Ar), 124.5 (CH-Tr), 123.7 (CH-Ar), 98.4 (C-1), 86.1 (C-1'), 74.6, 74.4, 71.7, 71.6, 69.7, 69.6, 69.5, 69.4, 68.8, 67.3, 65.7, 61.9 (skeleton carbons and O*C*H$_2$), 61.1 (C-6), 60.7 (C-6'), 49.2 (N*C*H$_2$), 45.4 (C-2), 33.5 (C-3 and *C*(CH$_3$)$_3$), 31.1 C(*C*H$_3$)$_3$, 29.7 (Ar*C*H$_2$Ar). MALDI-TOF (positive ion): *m/z* calcd for $C_{136}H_{212}N_{12}NaO_{52}S_4$: 2996.31 $[M+Na]^+$ Found: 2996.30

*4.7.4. Compound **9***

Azide compound **1** (168 mg, 0.2 mmol) and alkyne **4** (60 mg, 0.08 mmol) were reacted in CH_3CN according to the general method A. The crude product was purified by flash chromatography (95:5 CH_2Cl_2: MeOH) to give compound **9** (164 mg, 88%) as a colourless syrup. $[\alpha]^{24}_D$ +72.07 (*c* 0.37, $CHCl_3$); R_f 0.27 (95:5 CH_2Cl_2: MeOH); ^{1}H NMR ($CDCl_3$, 400.28 MHz): δ (ppm) 8.13 (s, 2H, 2x H-Tr), 7.03 (s, 4H, 4x H-Ar), 6.84 (s, 4H, 4x H-Ar), 5.38- 5.37 (overlapping signals, 4H, 2x H-1', 2x H-2'), 5.30 (t, 2H, 2x H-4' *J*= 9.9 Hz), 5.23 (dd, 2H, 2x H-3', J_1= 3.1 Hz, J_2= 9.9 Hz), 5.17 (s, 6H, 3x OCH_2Ar), 5.03 (m, 2H, 2x H-4), 4.84 (s, 2H, 2x H-1), 4.57 (m, 4H, 2x NCH_2), 4.37 (m, 2H, 2x H-5'), 4.30 (dd, 2H, 2x H-6'a), 4.24 (d, 4H, ArCH_{2a}Ar), 4.18- 4.05 (overlapping signals, 6H, 2x H-6, 2x H-6'b), 3.96 (m, 2H, 2x H-5), 3.86 (m, 6H, 3x OCH_2), 3.79 (m, 2H, OCH_2), 3.70- 3.46 (overlapping signals, 20H, OCH_2), 3.29 (d, 4H, ArCH_{2b}Ar, *J*= 12.8 Hz), 3.23 (m, 2H, 2x H-2), 2.20 (m, 4H, 2x H-3), 2.17, 2.10, 2.09, 2.05, 2.04, 1.99 (s, 36H, 12 x COCH_3), 1.27 (s, 18H, C(CH_3)$_3$), 0.98 (s, 18H, C(CH_3)$_3$). ^{13}C NMR ($CDCl_3$, 100.28 MHz): δ (ppm) 170.8, 170.5, 169.7, 169.9 (*C*O), 150.3, 149.5 (C_q-Ar), 147.3, (C_q-Tr), 143.9, 141.7 (C_q-Ar), 132.5 (C_q-Ar), 127.6 (C_q-Ar), 125.6 (CH-Ar), 125.0 (CH-Ar), 124.5 (CH-Tr), 98.7 (C-1), 82.3 (C-1'), 70.8, 70.4, 70.3, 69.9, 69.3, 69.2, 68.7, 66.8, 66.1, 64.6 (skeleton carbons and O*C*H$_2$), 62.8 (C-6'), 62.2 (C-6), 50.1 (N*C*H$_2$), 44.6 (C-2), 33.9 (*C*(CH$_3$)$_3$), 33.7 (*C*(CH$_3$)$_3$), (31.7 (ArCH_2Ar), 31.6 (C(*C*H$_3$)$_3$), 30.9 (C(*C*H$_3$)$_3$), 29.1 (C-3), 20.9, 20.8, 20.6, 20.5 (CO*C*H$_3$). MALDI-TOF (positive ion): *m/z* calcd for $C_{114}H_{158}N_6NaO_{40}S_2$: 2337.99 $[M+Na]^+$ Found: 2337.99

*4.7.5. Compound **10***

Compound **9** (164 mg, 0.07 mmol) was deprotected according to general method B to result in compound **10** (47 mg, 73%). $[\alpha]^{24}_D$ +117.0 (*c* 0.05, MeOH); R_f 0.61 (8:5:1 CH_2Cl_2: MeOH:water); ^{1}H NMR (DMSO, 500.76 MHz): δ (ppm): 8.24 (s, 2H, H-Tr),7.40- 6.90 (s, 8H, H-Ar), 5.19 (s, 2H, 2x H-1'), 5.15 (s, 2H, OCH_2), 4.75 (s, 2H, 2x H-1), 4.80 (m, 4H, NCH_2), 4.75- 3.15 (overlapping signals, 72H, skeleton hydrogens, ArCH_2Ar and OCH_2), 3.09 (m, 2H, 2x H-2), 2.05, 2.03 (m, 4H, 2x H-3), 1.15, 1.09 (s, 36 H, 4 x C(CH_3)$_3$), ^{13}C NMR (DMSO, 125.76 MHz): δ (ppm) 151.9, 142.6 (C_q-Tr), 134.1, 133.1, 129.8, 128.9, 127.5 (C_q-Ar), 125.5 (CH-Ar), 125.1 (CH-Tr), 98.4 (C-1), 86.1 (C-1'), 74.6, 74.4, 71.7, 71.6, 69.7, 69.6, 68.9, 67.3, 65.7, 61.9 (skeleton carbons and O*C*H$_2$), 61.06 (C-6), 61.04 (C-6'), 49.5 (N*C*H$_2$), 45.5 (C-2), 33.5 (2C, C-3 and *C*(CH$_3$)$_3$), 32.5 (Ar*C*H$_2$Ar), 31.4 (C(*C*H$_3$)$_3$), 31.1 (C(*C*H$_3$)$_3$). MALDI-TOF (positive ion): *m/z* calcd for $C_{94}H_{134}N_6NaO_{28}S_2$ [M+Na]: 1833.86. Found: 1833.87.

*4.7.6. Compound **11***

Azide compound **1** (256 mg, 0.3 mmol) and alkyne **5** (25 mg, 0.08 mmol) were reacted in CH_3CN according to the general method A. The crude product was purified by flash chromatography (95:5 CH_2Cl_2: MeOH) to give compound **11** (110 mg, 54%) as a colourless syrup. $[\alpha]^{24}_D$ +3.32 (*c* 0.09, $CHCl_3$); R_f 0.26 (95:5 CH_2Cl_2: MeOH); ^{1}H NMR ($CDCl_3$, 500.76 MHz): δ (ppm) 7.94 (s, 3H, 3x H-Tr), 7.46 (s, 2H, 2x H-Ar), 5.38-5.35 (overlapping signals, 6H, 3x H-1', 3x H-2'), 5.31 (t, 3H, 3x H-4', *J*= 9.9 Hz), 5.28- 5.20 (overlapping signals, 5H, 3x H-3', OCH_2), 5.04 (m, 3H, 3x H-4), 4.85 (s, 3H, 3x H-1), 4.58 (m, 6H, 3x NCH_2), 4.38 (m, 3H, 3x H-5'), 4.28 (dd, 3H, J_1= 5.3 Hz, J_2= 12.3 Hz, 3x H-6'a), 4.19 (dd, 3H, J_1= 2.4 Hz, J_2= 12.3 Hz, 3x H-6a), 4.15 (dd, 3H, J_1= 5.3 Hz, J_2= 12.3 Hz, 3x H-6b), 4.10 (dd, 3H, J_1= 5.3 Hz, J_2= 12.3 Hz, 3x H-6'b), 3.97 (m, 3H, 3x H-5), 3.95- 3.50 (overlapping signals, 5H, OCH_2, OCH_3), 3.86 (m, 2H, OCH_2), 3.80 (m, 2H, OCH_2), 3.70- 3.50 (overlapping signals, 40H, OCH_2), 3.24 (m, 3H, 3x H-2), 2.23 (m, 6H, 3x H-3), 2.17, 2.11, 2.09, 2.06, 2.04, 1.99, (s, 54H, 18x COCH_3). ^{13}C NMR ($CDCl_3$, 125.76 MHz): δ (ppm) 170.5, 169.8, 169.9, 169.8, 169.7, 169.6 (*C*O), 152.0 (C_q-Ar), 144.0 (C_q-Ar), 143.7 (C_q-Tr), 141.8 (C_q-Ar), 124.9 (CH-Tr), 124.3 (CH-Ar), 109.1 (CH-Ar), 98.8 (C-1), 82.4 (C-1'), 70.9, 70.5, 70.4, 70.1, 69.4, 69.3, 69.1, 68.7, 66.8, 66.2, 64.8 (skeleton carbons and O*C*H$_2$), 62.8 (C-6), 62.3 (C-6'), 52.3 (O*C*H$_3$), 50.0 (N*C*H$_2$), 44.7 (C-2), 29.2 (C-3), 21.0, 20.9, 20.7, 20.6 (CO*C*H$_3$). MALDI-TOF (positive ion): *m/z* calcd for $C_{113}H_{161}N_9NaO_{59}S_3$: 2706.86 $[M+Na]^+$ Found: 2706.89.

*4.7.7. Compound **12***

Azide compound **2** (356 mg, 0.64 mmol) and alkyne **5** (50 mg, 0.17 mmol) were reacted in DMF according to the general

method A. The crude product was purified by flash chromatography (8:5:1 CH_2Cl_2: MeOH:water) to give compound **12** (240 mg, 63%) as a colourless syrup.

Compound **12** was also prepared by deacetylation of compound **11** (110 mg, 0.04 mmol) according to general method B to result in compound **12** (55 mg, 70%). $[\alpha]^{24}_D$ +86.43 (*c* 0.17, MeOH); R_f 0.32 (8:5:1 CH_2Cl_2: MeOH:water); ^{1}H NMR (DMSO, 500.76 MHz): δ (ppm) 8.18 (s, 3H, H-Tr), 7.44 (s, 2H, H-Ar), 5.20- 5.19 (overlapping signals, 9H, 3x H-1', 3x OCH_2), 4.76 (s, 3H, 3x H-1), 4.56 (m, 6H, 3x NCH_2,), 3.85 (s, 3H, OCH_3), 3.84 (m, 6H, 3x OCH_2), 3.81- 3. 46 (overlapping signals, 84H, skeleton hydrogens and OCH_2,), 3.40 (m, 3H, 3x H-4), 3.10 (m, 3H, 3x H-2), 1.99, 1.93 (m, 6H, 3x H-3), ^{13}C NMR (DMSO, 125.76 MHz): δ (ppm) 165.8 (*C*O), 151.8 (C_q-Ar), 143.0 (C_q-Tr), 142.3 (C_q-Ar), 141.1 (C_q-Ar), 124.8 (CH-Tr), 108.7 (CH-Ar), 98.3 (C-1), 86.1 (C-1'), 74.6, 74.4, 71.6, 71.5, 69.8, 69.6, 69.5 , 68.7, 67.3, 65.5 , 62.3, 61.9 (skeleton carbons and OCH_2), 61.1 (C-6'), 61.0 (C-6), 52.3 (OCH_3), 49.4 (NCH_2), 45.4 (C-2), 33.5 (C-3). MALDI-TOF (positive ion): *m/z* calcd for $C_{77}H_{125}N_9NaO_{41}S_3$ $[M+Na]^+$ 1950.70. Found: 1950.75.

*4.7.9. Compound **13***

Azide compound **1** (204 mg, 0.25 mmol) and alkyne **6** (15 mg, 0.05 mmol) were reacted in CH_3CN according to the general method A. The crude product was purified by flash chromatography (98:2 CH_2Cl_2: MeOH) to give compound **13** (91 mg, 50%) as a colourless syrup. $[\alpha]^{24}_D$ +73.85 (*c* 0.08, $CHCl_3$); R_f 0.31 (95:5 CH_2Cl_2: MeOH); ^{1}H NMR ($CDCl_3$, 500.76 MHz): δ (ppm) 7.72 (s, 4H, 4x H-Tr), 5.38- 5.37 (overlapping signals, 8H, 4x H-1', 4x H-2'), 5.30 (t, 4H, 4x H-4', *J*= 9.9 Hz), 5.23 (dd, 4H, 4x H-3', J_1= 3.1 Hz, J_2= 9.9 Hz), 5.03 (m, 4H, 4x H-4), 4.85 (s, 4H, 4x H-1), 4.55 (t, 8H, 4x NCH_2), 4.53 (m, 8H, 4x OCH_2,) 4.38 (m, 4H, 4x H-5'), 4.28 (dd, 4H, J_1= 5.3 Hz, J_2= 12.3 Hz, 4x H-6'a), 4.19 (dd,, 4H, J_1= 2.4 Hz, J_2= 12.3 Hz, 4x H-6a), 4.14 (dd, 4H, J_1= 5.3 Hz, J_2= 12.3 Hz, 4x H-6b), 4.11 (dd, 4H, J_1= 5.3 Hz, J_2= 12.3 Hz, 4x H-6'b), 3.97 (m, 4H, 4x H-5), 3.89 (m, 8H, 4x OCH_2,), 3.80 (m, 4H, 2x OCH_2), 3.85- 3.56 (overlapping signals, 56H, 28x OCH_2), 3.48 (s, 8H, 4x C(CH_2), 3.24 (m, 4H, 4x H-2), 2.22 (m, 4H, 4x H-3), 2.17, 2.10, 2.09, 2.05, 2.04, 1.99 (s, 54H, 18 x COCH_3), ^{13}C NMR ($CDCl_3$, 125.76 MHz): δ (ppm) 170.7, 170.4, 169.8, 169.7, 159.5, 168.5 (6C, 6 x *C*O), 144.9, (C_q-Tr), 123.6 (CH-Tr), 98.7 (C-1), 82.3 (C-1'), 70.8, 70.4, 70.3, 69.9, 69.3, 69.2, 69.1, 68.7, 66.8, 66.1, 64.6, 64.5 (73 C, skeleton carbons and OCH_2), 62.8 (C-6), 62.1 (C-6'), 51.0 (NCH_2), 45.1 (C_q), 44.6 (C-2), 29.5 (C-3), 20.9, 20.7, 20.6 (COCH_3). MALDI-TOF (positive ion): *m/z* calcd for $C_{145}H_{216}N_{12}NaO_{76}S_4$: 3492.22 $[M+Na]^+$ Found: 3492.32.

*4.7.10. Compound **14***

Azide compound **2** (297 mg, 0.5 mmol) and alkyne **6** (30 mg, 0.1 mmol) were reacted in DMF according to the general method A. The crude product was purified by flash chromatography (24:8:1 CH_2Cl_2: MeOH:water) to give compound **14** (165 mg, 65%) as a colourless syrup.

Compound **14** was was also prepared by deacetylation of compound **13** (75 mg, 0.02 mmol)) according to general method B to result in to result in compound **14** (43 mg, 84%). $[\alpha]^{24}_D$ +89.72 (*c* 0.27, MeOH); R_f 0.20 (24:8:1 methanol:toluene:water); ^{1}H NMR (DMSO, 500.76MHz): δ (ppm) 8.04 (s, 4H, 4x H-Tr), 5.21 (s, 4H, 4x H-1'), 4.79 (s, 4H, 4x H-1), 4.53 (m, 8H, 4x NCH_2), 4.48 (m, 8H, 4xOCH_2), 3.90- 3.20 (overlapping signals, 128H, skeleton hydrogens and OCH_2), 3.12 (s, 4H, 4x H-2), 2.09 (m, 4H, 4x H-3a), 1.96 (m, 4H, 4x H-3b), ^{13}C NMR (DMSO, 125.76 MHz): δ (ppm) 144.1 (C_q-Tr), 124.0 (CH-Tr), 98.4 (C-1), 86.4 (C-1'), 74.6, 74.4, 71.7, 71.6, 69.8, 69.7, 69.6, 69.5, 68.7, 67.3, 65.7, 64.1, 61.9 (skeleton carbons and OCH_2), 61.1 (C-6'), 61.0 (C-6), 49.3 (NCH_2), 45.4 (C-2), 44.8 (C_q), 33.5 (C-3). MALDI-TOF (positive ion): *m/z* calcd for $C_{97}H_{168}N_{12}NaO_{52}S_4$ $[(M+Na)^+]$: 2483.97. Found: 2483.94.

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- Four glycoclusters with terminal 1,2-thio-linked pseudomannobiosides were prepared.
- Two glycoclusters were studied in lectin-binding assays with the BC2L-A lectin.
- ITC, SPR, yeast agglutination test and analytical ultracentrifugation were applied.
- The mannoclusters cross-link and aggregates BC2L-A of *Burkholderia cenocepacia.*