Published in final edited form as:

Angew Chem Int Ed Engl. 2017 November 06; 56(45): 13973–13978. doi:10.1002/anie.201700964.

A Semi-Synthetic Glycoconjugate Vaccine Candidate for Carbapenem-Resistant *Klebsiella pneumoniae*

Prof. Dr. Peter H. Seeberger^{*},

Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces, Am Mühlenberg 1, 14424 Potsdam (Germany)

Institute of Chemistry and Biochemistry, Freie Universität Berlin, Arnimallee 22, 14195 Berlin (Germany)

Dr. Claney L. Pereira^{*},

Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces, Am Mühlenberg 1, 14424 Potsdam (Germany)

Institute of Chemistry and Biochemistry, Freie Universität Berlin, Arnimallee 22, 14195 Berlin (Germany)

Dr. Naeem Khan⁺,

Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces, Am Mühlenberg 1, 14424 Potsdam (Germany)

Institute of Chemistry and Biochemistry, Freie Universität Berlin, Arnimallee 22, 14195 Berlin (Germany)

Dr. Guozhi Xiao⁺,

Department of Biomolecular Systems, Max Planck Institute of Colloids and Interfaces, Am Mühlenberg 1, 14424 Potsdam (Germany)

Institute of Chemistry and Biochemistry, Freie Universität Berlin, Arnimallee 22, 14195 Berlin (Germany)

Dr. Elizabeth Diago-Navarro,

Department of Medicine, Division of Infectious Diseases, Stony Brook University, 101 Nicolls Road, Stony Brook, NY 11794 (USA)

Dr. Katrin Reppe,

Charité—Universitätsmedizin Berlin, Department of Infectious Diseases and Pulmonary Medicine, Charitéplatz 1, 10117 Berlin (Germany)

Prof. Dr. Bastian Opitz,

^{*}Prof. Dr. P. H. Seeberger, Dr. C. L. Pereira: peter.seeberger@mpikg.mpg.de; claney.pereira@vaxxilon.com.

⁺These authors contributed equally to this work.

Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under: <https://doi.org/10.1002/anie.201700964>.

Homepage: <http://www.mpihg.mpg.de/en/bs>

Conflict of interest

The authors declare no conflict of interest.

Charité—Universitätsmedizin Berlin, Department of Infectious Diseases and Pulmonary Medicine, Charitéplatz 1, 10117 Berlin (Germany)

Prof. Dr. Bettina C. Fries, and

Department of Medicine, Division of Infectious Diseases, Stony Brook University, 101 Nicolls Road, Stony Brook, NY 11794 (USA)

Prof. Dr. Martin Witzernath

Charité—Universitätsmedizin Berlin, Department of Infectious Diseases and Pulmonary Medicine, Charitéplatz 1, 10117 Berlin (Germany)

Abstract

Hospital-acquired infections are an increasingly serious health concern. Infections caused by carbapenem-resistant *Klebsiella pneumoniae* (CR-*Kp*) are especially problematic, with a 50% average survival rate. CR-*Kp* are isolated from patients with ever greater frequency, 7% within the EU but 62% in Greece. At a time when antibiotics are becoming less effective, no vaccines to protect from this severe bacterial infection exist. Herein, we describe the convergent [3+3] synthesis of the hexasaccharide repeating unit from its capsular polysaccharide and related sequences. Immunization with the synthetic hexasaccharide **1** glycoconjugate resulted in high titers of cross-reactive antibodies against CR-*Kp* CPS in mice and rabbits. Whole-cell ELISA was used to establish the surface staining of CR-*Kp* strains. The antibodies raised were found to promote phagocytosis. Thus, this semi-synthetic glycoconjugate is a lead for the development of a vaccine against a rapidly progressing, deadly bacterium.

Keywords

antibiotic resistance; hexasaccharide total synthesis; *Klebsiella pneumoniae*; vaccines

The bacterium *Klebsiella pneumoniae* (*Kp*) is the leading cause of nosocomial respiratory and urinary tract infections as well as bacteremia, primarily among newborns and immuno-compromised patients.^[1] Carbapenem-resistant *Kp* (CR-*Kp*) are now commonly encountered in hospitals worldwide. Outbreaks occur with increasing frequency,^[2] and these strains are highly contagious^[3] and cause high morbidity and mortality.^[4] Isolates belonging to the sequence type 258 (ST258) that expresses *Kp* carbapenemase are largely responsible for the global spread of CR-*Kp*.^[5] Efficacious vaccination of risk groups is direly needed as treatment options for CR-*Kp* are diminishing.

Successful vaccines against streptococci^[6] and meningococci^[7] are based on conjugates of capsular polysaccharides (CPS) with carrier proteins. Currently, no vaccines for prophylactic or therapeutic use against *Kp* are available. These Gram-negative pathogens are surrounded by CPS and lipopolysaccharides (LPS). Active immunization with LPS-containing vaccines holds severe risks associated with endotoxic components such as Lipid A.^[8] CPS, on the other hand, are highly immunogenic and nontoxic.^[9] A 24-valent *Klebsiella* vaccine manufactured by the Swiss Serum and Vaccine Institute, composed of CPS antigens from *Kp* and *K. oxytoca* that are expressed by about 70% of *Klebsiella* strains associated with bacteremia, proved safe and immunogenic in Phase I clinical trials.^[10] Challenges

associated with glycan isolation rendered the vaccine too costly and development ended following Phase I clinical trials.^[11]

The production of well-defined glycans by chemical synthesis is attractive as it is a reliable and scalable method. Long lasting, T-cell-dependent protection from bacterial infections can be provided by oligosaccharide antigens that are linked to a carrier protein,^[12] as demonstrated by a semi-synthetic glycoconjugate vaccine against *Haemophilus influenzae* type b marketed in Cuba.^[13] Semi-synthetic oligosaccharide vaccine candidates against *Shigella*,^[14] meningococci,^[15] and streptococci^[16] are currently under preclinical evaluation.

Clinical CR-*Kp* isolates responsible for a serious 2011 outbreak killing six of the 18 infected patients were found to have identical CPS comprising a hexasaccharide repeating unit (RU) (Figure 1a).^[17] This RU resembles CPS of other isolated serotypes even though it is not identical. A vaccine based on hexasaccharide antigen **1** may provide a much needed defense against these devastating pathogens (Figure 1b).

Herein, we report the synthesis of hexasaccharide **1** as well as related substructures **2–6** (Figure 1b) and their immunological evaluation in vivo. Glycan microarray studies revealed that only hexasaccharide **1** is recognized by monoclonal antibody (MAb) 1C9 that cross-reacts with CR-*Kp* CPS.^[20] The glycoconjugate hexasaccharide CRM197-**1** vaccine candidate is immunogenic in mice, and is promising for further preclinical development to protect against CR-*Kp* outbreaks.

To prepare the glycans, a convergent [3+3] approach based on three monosaccharide building blocks, Rha **7**,^[18] GalA **8**, and Gal **9**,^[19] was selected (Scheme 1). Differentially protected GalA **8** carrying benzyl ether (Bn), allyl ether (All) and levulinyl ester (Lev) groups at C2, C3, and C4 was to be synthesized by cyclization of thioacetal **10**, which is derived from aldehyde **11** and auxiliary **12** via aldol addition. For glycan immobilization on array surfaces and subsequent immunization studies an aminopentanol linker is included at the reducing end. The synthesis of differentially protected GalA building block **8** via a de novo approach^[21–23] commenced with the treatment of auxiliary **12** with LDA at -78°C , followed by addition of aldehyde **11** (Supporting Information) to furnish aldol adduct **13** (d.r. = 10.4:1; Scheme 2).^[24] Levulation afforded fully protected thioacetal **10**; treatment with trifluoroacetic acid and anisole led to cleavage of *p*-methoxybenzyl ether, and concomitant cyclization produced the desired thioglycoside **14** as a mixture of anomers (α/β = 3.9/1). Isolation of the α -product followed by methanolysis of the chiral auxiliary under mild acidic conditions provided GalA **8**.^[25]

The assembly of trisaccharide donor **18** commenced by the selective removal of the C4-Lev ester of **8** upon exposure to hydrazine, providing alcohol **15** (Scheme 2). Glycosylation of **15** with rhamnosyl building block **7** afforded disaccharide **16** as the α -anomer. A two-step removal of the allyl group via the enol ether provided disaccharide **17**.^[26] Glycosylation of **17** with galactosyl trichloroacetimidate **9** proceeded smoothly at -30°C to give trisaccharide thioglycoside **18** as a single β -anomer, which was converted to glycosyl *N*-phenyltrifluoroacetimidate **19**.^[27]

An iterative glycosylation and deprotection strategy was employed to construct trisaccharide acceptor **22**, using rhamnosyl trichloroacetimidate building block **7** (Scheme 3). Coupling of Rha **7** with the protected aminopentanol linker afforded α -linked rhamnoside; subsequent removal of the C2-acetate by a treatment with sodium methoxide provided acceptor **20**. Repeating this glycosylation–deprotection sequence twice with Rha **7** produced trisaccharide acceptor **22**.

With donors **18** and **19** as well as acceptor **22** in hand, the key [3+3] glycosylation was investigated. The identification of effective reaction conditions proved challenging owing to the poor reactivity and sterics of the uronic acid donor **18** and the inert C-2 hydroxyl of the trisaccharide acceptor **22**.^[28,29] Ultimately, thioglycoside **18** failed to react with acceptor **22**. However, *N*-phenyltrifluoroacetimidate **19**, after some optimization, reacted smoothly in a mixture of toluene and diethyl ether at -70°C to provide the hexasaccharide **23** (Scheme 3). The desired configuration of the α -isomer of **23** was confirmed by the coupling constant between galacturonic acid C-1 and H-1 ($^1J_{\text{C-H}} = 172\text{ Hz}$; Supporting Information).^[30]

The initial plan for the global deprotection of **23**, relying on the initial cleavage of acetate and methyl esters followed by hydrogenation of the benzyl groups, did not meet with success as only the pentasaccharide β -elimination product was obtained (data not shown). In contrast, when hydrogenolysis was carried out first, followed by saponification, the target hexasaccharide **1** was obtained in 76% yield (Scheme 3). NMR spectra of synthetic hexasaccharide **1** were in good agreement with those reported for the CPS from the CR-*Kp* outbreak^[17] (Supporting Information). The substructures **2–6** (Figure 1b) were also synthesized for epitope mapping studies (Supporting Information).

The synthetic glycans along with the native CR-*Kp* CPS were printed on microarray slides and incubated with the partially protective mouse IgM monoclonal antibody (MAb) 1C9 raised against CR-*Kp* CPS.^[20] MAb 1C9 specifically recognizes RU hexasaccharide **1** as no binding was observed for the other synthetic glycans (Figure 2A). Immunogenic structures that induce cross-reactive immune responses to native CPS can be determined by CPS-based inhibition studies.^[15] Accordingly, the MAb 1C9 was first incubated with CR-*Kp* CPS, and *S. pneumoniae* type 3 (ST3) CPS was used as a negative control (Figure 2C). MAb binding was abolished after pre-incubation with CR-*Kp* CPS but without other synthetic glycans (Figure 2B,E), suggesting that hexasaccharide **1** effectively mimics the natural CPS epitope. Therefore, **1** was further immunologically characterized.

Carbohydrate-based, type 2 T-independent antigens are poorly immunogenic,^[31] and small synthetic glycans alone are ineffective in eliciting an immune response. Attachment of synthetic glycan antigens to carrier proteins that induce a T-cell response enhances glycan immunogenicity and triggers specific memory responses. We used CRM197, a nontoxic mutant of diphtheria toxin, as a carrier because it is a component of several marketed conjugate vaccines.^[32] The *p*-nitrophenyladipate ester^[33] derivative of hexasaccharide **1** was covalently coupled to CRM197 to yield the CRM197-**1** conjugate with an average of 7.5 hexasaccharide **1** molecules per CRM197 (Supporting Information, Figure S2).

The immunogenicity of the CRM197-**1** conjugate formulated with either Freund's adjuvant (FA) or human approved aluminum hydroxide was assessed. Mice were immunized subcutaneously and received two booster shots, one on day 14 and one on day 35 (Figure 3A). High antibody titers were detected using glycan microarrays one week after the final immunization (Figure 3B,C). The antibody response was adjuvant-dependent, as the conjugate formulated with FA induced more cross-reactive antibodies than the alum formulation. Antibodies against FA-formulated conjugate recognize both trisaccharides **2** and **3**, whereas those elicited in response to the alum formulation cross-react only with trisaccharide **3** (Figure 3B,C). Antibody binding to hexasaccharide **1** and trisaccharide **3** was similar for both formulations (Figure 3C). Interestingly, the FA-adjuvanted conjugate also induces CPS cross-reactive antibodies whereas the alum formulation does not (Figure 3D). Synthetic glycan antigens elicit a significantly stronger antibody response in rabbits when compared to mice.^[34] Rabbits were immunized with CRM197-**1** conjugate formulated with alum, an adjuvant approved for use in humans.^[35] The serological analysis using glycan microarrays and ELISA revealed that CRM197-**1** conjugate elicits a robust immune responses against native CR-*Kp* CPS (Supporting Information, Figure S3). Collectively, these results demonstrate that the CRM197-**1** conjugate is immunogenic in mice and rabbits and produces antibodies that are cross-reactive with native CR-*Kp* CPS.

In order for CRM197-**1** to qualify as a vaccine lead, immunological memory and anti-CPS antibody-mediated protection through challenge has to be demonstrated. CR-*Kp* strains are avirulent in mice; therefore, there is currently no established animal challenge model available to test CRM197-**1**.^[20] The production of different types of antibodies is being used to assess the immunological response in vivo. The majority of antibodies produced by mice in response to immunization with CRM197-**1** are IgG1 as determined by glycan array analyses (Figure 4A,C), whereas less IgG2a and IgG3 are observed (Figure 4D,E). The CRM197-**1**-specific total serum antibody level (IgG and IgM) is significantly higher than the PBS group (Figure 4F,G). The strong immunological response and antibody-class switch underscores the potential of hexasaccharide **1** as a vaccine lead against CR-*Kp*.

In order for an antibody to be protective in vivo, it has to be able to bind to *Klebsiella* and induce antibody-dependent in vitro phagocytosis that typically correlates with in vivo protection.^[36] Therefore, we assessed the surface binding of CRM197-**1**-specific antibodies to intact *Klebsiella* strains (both carbapenem-resistant and carbapenem-sensitive). The whole-cell ELISA assay suggests that anti-CRM197-**1** antibodies bind broadly to CR-*Kp* strains, whereas there was no binding observed to other capsular antigen-expressing strains (Figure 5A).

The functional relevance of the antibodies induced in response to immunization with CRM197-**1** conjugate was assessed by a opsonophagocytic killing assay (OPKA). Differentiated HL-60 cells were incubated with CR-*Kp* strains 85/12 and NRZ-00319 preopsonized with anti-CRM197-**1** antibodies. The phagocytic activity of *Klebsiella* was assessed by plating on blood agar plates and was expressed as percent killing. CRM197-**1** conjugate immunization elicits opsonic antibodies that exhibit phagocytic activity against CR-*Kp* strains tested (Figure 5B–E). Antibodies promoting bacterial killing of 50% or more were considered biologically significant. The antibody titer values for 50% killing for 85/12

and NRZ-00319 are 153.6 and 134.2, respectively (Figure 5C,E). CRM197-1 conjugate produce functional antibodies that promote uptake and killing of *Klebsiella* by phagocytic cells in mice as well as in rabbits (Supporting Information, Figure S4).

In summary, synthetic oligosaccharides help to identify and define glycan cell-surface epitopes as a basis for vaccine development against bacterial infections. The synthesis of CR-*Kp* CPS hexasaccharide RU 1 and related glycans 2–6 enabled glycan microarray analyses that revealed that hexasaccharide 1 is selectively recognized by the monoclonal antibody 1C9, which cross-reacts with CR-*Kp* CPS.^[17] The CRM197-1 conjugate elicits high antibody titers in mice and rabbits and shows immunological memory in mice, cross-reacting with native CPS. Immunization with CRM197-1 conjugate elicits CR-*Kp* CPS specific antibodies that bind to the surface of intact bacteria and result in vitro phagocytic killing of CR-*Kp* *Klebsiella* strains. The full potential of CRM197-1 as a vaccine candidate can be evaluated only once a suitable animal challenge model, currently being established for CR-*Kp* strains, is available.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the Max Planck Society and the Körber Foundation (Award to P.H.S.) for generous financial support. The authors thank Dr. Allison Berger for editing the manuscript and Dr. Andreas Geissner for help in microarray printing. This work was supported in part by the German Research Foundation (SFB-TR 84, C3 and C6 to M.W., C8 to P.H.S., and A1 and A5 to B.O.), and by the German Ministry for Education and Research (e:Med and CAPSyS to M.W.). The authors gratefully thank Ivonne Pfeifer (Robert-Koch-Institute, Wernigerode, Germany) and Martin Kaase (National Reference Laboratory for Multidrug-Resistant GRAM-Negative Bacteria, Bochum, Germany) for kindly providing *K. pneumoniae* strains 85/12, NRZ-00319, NRZ-02497, and NRZ-01099. We are grateful to Catherine Chaput for the *K. pneumoniae* strain Kp52145.

References

1. Podschun R, Ullmann U. Clin Microbiol Rev. 1998; 11:589–603. [PubMed: 9767057]
2. a) Kaur J, Sheemar S, Chand K, Chopra S, Mahajan G. Int J Curr Microbiol Appl Sci. 2016; 5:727–733. b) Yu J, Tan K, Rong Z, Wang Y, Chen Z, Zhu X, Wu L, Tan L, Xiong W, Sun Z, Chen L. BMC Infect Dis. 2016; 16:563. [PubMed: 27733128] c) Kola A, Piening B, Pape UF, Schlieker WV, Kaase M, Geffers C, Wiedenmann B, Gastmeier P. Antimicrob Resist Infect Control. 2015; 4:8. [PubMed: 25815166] d) Stillwell T, Green M, Barbadora K, Ferrelli JG, Roberts TL, Weissman SJ, Nowalk A. J Pediatr Infect Dis. 2014; 4:330–338. e) Pereira GH, Garcia DO, Mostardeiro M, Fanti KS, Levin AS. Mem Inst Oswaldo Cruz. 2013; 108:113–115. [PubMed: 23440125]
3. a) Yigit H, Queenan AM, Anderson GJ, Domenech-Sanchez A, Biddle JW, Steward CD, Alberti S, Bush K, Tenover FC. Antimicrob Agents Chemother. 2001; 45:1151–1161. [PubMed: 11257029] b) Nordmann P, Cuzon G, Naas T. Lancet Infect Dis. 2009; 9:228–236. [PubMed: 19324295] c) Gupta N, Limbago BM, Patel JB, Kallen AJ. Clin Infect Dis. 2011; 53:60–67. [PubMed: 21653305]
4. a) Patel G, Huprikar S, Factor SH, Jenkins SG, Calfee DP. Infect Control Hosp Epidemiol. 2008; 29:1099–1106. [PubMed: 18973455] b) Hirsch EB, Tam VH. J Antimicrob Chemother. 2010; 65:1119–1125. [PubMed: 20378670] c) Livermore DM, Warner M, Mushtaq S, Doumith M, Zhang J, Woodford N. Int J Antimicrob Agents. 2011; 37:415–419. [PubMed: 21429716] d) Ben-David D, Kordevani R, Keller N, Tal I, Marzel A, Gal-Mor O, Maor Y, Rahav G. Clin Microbiol Infect. 2012; 18:54–60.
5. Kitchel B, Rasheed JK, Patel JB, Srinivasan A, Venezia SN, Carmeli Y, Brolund A, Giske CG. Antimicrob Agents Chemother. 2009; 53:3365–3370. [PubMed: 19506063]

6. Galiza EP, Heath PT. *Minerva Med.* 2007; 98:131–143. [PubMed: 17519855]
7. Poland GA. *Clin Infect Dis.* 2010; 50:S45–S53. [PubMed: 20144016]
8. a) Cross AS. *Virulence.* 2014; 5:219–225. [PubMed: 23974910] b) Ahmad TA, Sayed LHE, Haroun M, Hussein AA, Ashry ESHE. *Vaccine.* 2012; 30:2411–2420. [PubMed: 22100884]
9. Cryz SJ, Fürer E, Germanier R. *Infect Immun.* 1985; 150:225–230.
10. a) Edelman R, Taylor DN, Wasserman SS, McClain JB, Cross AS, Sadoff JC, Que JU, Cryz SJ. *Vaccine.* 1994; 12:1288–1294. [PubMed: 7856293] b) Campbell WN, Hendrix E, Cryz SJ, Cross AS. *Clin Infect Dis.* 1996; 23:179–181. [PubMed: 8816151]
11. Follador R, Heinz E, Wyres KL, Ellington MJ, Kowarik M, Holt KE, Thomson NR. *Microb Genomics.* 2016; 2:e000073.
12. a) Seeberger PH, Werz DB. *Nature.* 2007; 446:1046–1051. [PubMed: 17460666] b) Boltje TJ, Kim JH, Park J, Boons GJ. *Nat Chem.* 2009; 1:611–622. [PubMed: 20161474] c) Adamo R, Nilo A, Castagner B, Boutureira O, Berti F, Bernardes GJL. *Chem Sci.* 2013; 4:2995–3008. [PubMed: 25893089]
13. Verez-Bencomo V, Fernández-Santana V, Hardy E, Toledo ME, Rodríguez MC, Heynngnezz L, Rodríguez A, Balz A, Herrera L, Izquierdo M, Villar A, Valdés Y, Cosme K, Deler ML, Montane M, Garcia E, Ramos A, Aguilar A, Medina E, Toraño G, Sosa I, Hernandez I, Martinez R, Muzachio A, Carmentales A, Costa L, Cardoso F, Campa C, Diaz M, Roy R. *Science.* 2004; 305:522–525. [PubMed: 15273395]
14. a) Put RMF, Kim TH, Guerreiro C, Thouron F, Hoogerhout P, Sansonetti PJ, Westdijk J, Stork M, Phalipon A, Mulard LA. *Bioconjugate Chem.* 2016; 27:883–892. b) Phalipon A, Tanguy M, Grandjean C, Guerreiro C, Belot F, Cohen D, Sansonetti PJ, Mulard LA. *J Immunol.* 2009; 182:2241–2247. [PubMed: 19201878] c) Pozsgay V, Kielb JK, Schneerson R, Robbins JB. *Proc Natl Acad Sci USA.* 2007; 104:14478–14482. [PubMed: 17726093]
15. Wang CH, Li ST, Lin TL, Cheng YY, Sun TH, Wang JT, Cheng TJR, Mong KKT, Wong CH, Wu CY. *Angew Chem Int Ed.* 2013; 52:9157–9161. *Angew Chem.* 2013; 125:9327–9331.
16. a) Pereira CL, Geissner A, Anish C, Seeberger PH. *Angew Chem Int Ed.* 2015; 54:10016–10019. *Angew Chem.* 2015; 127:10154–10157. b) Geissner A, Pereira CL, Leddermann M, Anish C, Seeberger PH. *ACS Chem Biol.* 2016; 11:335–344. [PubMed: 26674834]
17. a) Kubler-Kielb J, Vinogradov E, Ng WI, Maczynska B, Junka A, Bartoszewicz M, Zelazny A, Bennett J, Schneerson R. *Carbohydr Res.* 2013; 369:6–9. [PubMed: 23360863] b) Sarkar V, Mukhopadhyay B. *RSC Adv.* 2016; 6:40147–40154. c) Pozsgay V, Brisson JR, Jennings HJ. *Can J Chem.* 1987; 65:2764–2769.
18. Barroso S, Geerdink D, ter Horst B, Casas-Arce E, Minnaard AJ. *Eur J Org Chem.* 2013:4642–4654.
19. Shi L, Kim YJ, Gin DY. *J Am Chem Soc.* 2001; 123:6939–6940. [PubMed: 11448206]
20. Diago-Navarro E, Chen L, Passet V, Burack S, Hernando AU, Kodiyanplakkal RP, Levi MH, Brisse S, Kreiswirth BN, Fries BC. *J Infect Dis.* 2014; 210:803–813. [PubMed: 24634498]
21. For selected reviews on de novo carbohydrate synthesis, see: a) Schmidt RR. *Pure Appl Chem.* 1987; 59:415–424. b) Kirschning A, Jesberger M, Schöning KU. *Synthesis.* 2001:507–540. c) Hemeon I, Bennet AJ. *Synthesis.* 2007:1899–1926. For selected examples of de novo carbohydrate synthesis, see: d) Northrup AB, MacMillan DWC. *Science.* 2004; 305:1752–1755. [PubMed: 15308765] e) Babu RS, Zhou M, O'Doherty GA. *J Am Chem Soc.* 2004; 126:3428–3429. [PubMed: 15025462] f) Enders D, Grondal C. *Angew Chem Int Ed.* 2005; 44:1210–1212. *Angew Chem.* 2005; 117:1235–1238. g) Crich D, Navuluri C. *Org Lett.* 2011; 13:6288–6291. [PubMed: 22070804] h) Lorpitthaya R, Suryawanshi SB, Wang S, Pasunooti KK, Cai S, Ma J, Liu XW. *Angew Chem Int Ed.* 2011; 50:12054–12057. *Angew Chem.* 2011; 123:12260–12263. i) Voigt B, Scheffler U, Mahrwald R. *Chem Commun.* 2012; 48:5304–5306. j) Peifer M, Berger R, Shurtleff VW, Conrad JC, MacMillan DWC. *J Am Chem Soc.* 2014; 136:5900–5903. [PubMed: 24670208]
22. Stallforth P, Adibekian A, Seeberger PH. *Org Lett.* 2008; 10:1573–1576. [PubMed: 18363404]
23. For recent de novo carbohydrate syntheses from our laboratory, see: a) Pragani R, Seeberger PH. *J Am Chem Soc.* 2011; 133:102–107. [PubMed: 21142035] b) Yang Y, Martin CE, Seeberger PH. *Chem Sci.* 2012; 3:896–899. c) Calin O, Eller S, Hahm HS, Seeberger PH. *Chem Eur J.* 2013; 19:3995–4002. [PubMed: 23447496] d) Yang Y, Oishi S, Martin CE, Seeberger PH. *J Am Chem*

- Soc. 2013; 135:6262–6271. [PubMed: 23521711] e) Schumann B, Pragani R, Anish C, Pereira CL, Seeberger PH. Chem Sci. 2014; 5:1992–2002.f) Matthies S, Stallforth P, Seeberger PH. J Am Chem Soc. 2015; 137:2848–2851. [PubMed: 25668389]
24. The diastereoselectivity of the Evans aldol reaction was improved greatly, from 4.9:1 to 10.4:1, by replacing the benzyl group with an allyl group at the C2 of aldehyde **11**.
25. Characteristic coupling constants between H-4 and H-5 ($J = 1.6$ Hz) as well as H-3 and H-4 ($J = 3.6$ Hz) unambiguously revealed the desired galacto configuration of building block 8. NOE correlations between H-5 and H-4, as well as between H-4 and H-3, confirmed the assignment.
26. a) Baudry D, Ephritikhine M, Felkin H. J Chem Soc Chem Commun. 1978:694–695.b) Boonyarattanakalin S, Liu X, Michieletti M, Lepenies B, Seeberger PH. J Am Chem Soc. 2008; 130:16791–16799. [PubMed: 19049470]
27. a) Yu B, Tao H. Tetrahedron Lett. 2001; 42:2405–2407.b) Yu B, Tao H. J Org Chem. 2002; 67:9099–9102. [PubMed: 12467439] c) Yu B, Sun J. Chem Commun. 2010; 46:4668–4679.
28. For a review on uronic acid derivatives in oligosaccharide synthesis, see:a) van den Bos LJ, Codée JDC, Litjens REJN, Dinkelaar J, Overkleeft HS, van der Marel GA. Eur J Org Chem. 2007:3963–3976.b) Codée JD, Christina AE, Walvoort MT, Overkleeft HS, van der Marel GA. Top Curr Chem. 2011; 301:253–289. [PubMed: 21222193] c) Wadouachi A, Kovensky J. Molecules. 2011; 16:3933–3968.
29. For recent selected examples of the direct use of uronic acid as glycosyl donor in oligosaccharide synthesis, see:a) Ma Y, Cao X, Yu B. Carbohydr Chem. 2013; 377:63–74.b) Dhamale OP, Zong C, Al-Mafraji K, Boons GJ. Org Biomol Chem. 2014; 12:2087–2098. [PubMed: 24549353]
30. Bock K, Pedersen C. J Chem Soc Perkin Trans. 1974; 2:293–297.
31. Weintraub A. Carbohydr Res. 2003; 338:2539–2547. [PubMed: 14670715]
32. Shinefield HR. Vaccine. 2010; 28:4335–4339. [PubMed: 20452430]
33. Wu X, Ling CC, Bundle DR. Org Lett. 2004; 6:4407–4410. [PubMed: 15548037]
34. Schumann B, Hahm HS, Parameswarappa SG, Reppe K, Wahlbrink A, Govindan S, Kaplonek P, Pirofski L-A, Witzenrath M, Anish C, Pereira CL, Seeberger PH. Sci Transl Med. 2017; 9:eaaf5347. [PubMed: 28275152]
35. Astronomo RD, Burton DR. Nat Rev Drug Discovery. 2010; 9:308–324. [PubMed: 20357803]
36. Cohen TS, Pelletier M, Cheng L, Pennini ME, Bonnell J, Cvitkovic R, Chang C, Xiao X, Cameroni E, Corti D, Semenova E, Warrener P, Sellman BR, Suzich J, Wang Q, Stover CK. JCI Insight. 2017; 2:e92774.

Figure 1.

A) Hexasaccharide RU from two CR-*Kp* clones isolated during a 2011 hospital outbreak. B) Synthetic hexasaccharide **1** and substructures **2–6** used for immunological studies.

Figure 2.

CR-*Kp* CPS inhibits binding of MAb 1C9 to hexasaccharide **1**. A) Glycan microarray was incubated with different amounts of MAb 1C9. Bound antibodies were detected using goat anti-mouse IgM Alexa-fluor 594 secondary antibodies. B) Pre-adsorption of MAb 1C9 with CR-*Kp* CPS (10 $\mu\text{g mL}^{-1}$) followed by incubation with the printed array inhibits binding to **1**. C) ST3 CPS was used as a negative control. D) Glycan microarray printing pattern. E) Mean fluorescence intensities (MFI) measured for the inhibition assay. Data are represented as mean $\pm$ SD (in triplicate) following subtraction of background values.

Figure 3.

Mice immunized with CRM197-1 produce serum antibodies that recognize CPS. A) Schematic immunization schedule. Mice (three individuals per group) were immunized on day 0 with CRM197-1 conjugate (3 μ g sugar per dose) formulated in aluminum hydroxide (alum) or FA and boosted on days 14 and 35 with the same amount of antigen subcutaneously in 100 μ L volume. Control mice received PBS with alum. B) A representative microarray scan following incubation of pooled sera from each group. C,D) The MFI of serum antibody cross-reactivity with synthetic structures and native CPS were expressed as mean $\pm$ SD of three microarray spots.

Figure 4.

Hexasaccharide **1**-specific serum antibody isotyping. Immunization with CRM197-**1** conjugate induces high antibody titers in mice. Mouse sera obtained at different time points were qualitatively analyzed using glycan microarrays for multiple antibody isotypes. The MFI of hexasaccharide **1**-specific serum A) IgG, B) IgM antibodies at different time points. The IgG isotype antibodies as expressed in MFI values of ($n = 3$) are shown in C) IgG1, D) IgG2a, and E) IgG3. Total serum antibody titers at day 49 F) IgG and G) IgM. MFIs for antibody titers was plotted as mean $\pm$ SD (triplicate) and analyzed by unpaired t -test using GraphPad Prism. p values of <0.05 were considered statistically significant. * P 0.1, ** P 0.05, *** P 0.01 and **** P 0.001.

Figure 5.

A) Surface binding of CRM197-1 antibodies was analyzed by whole-cell ELISA. Antibody binding was assessed for CR-*Kp* strains 85/12, NRZ-00319, NRZ-02497, NRZ-01099, carbapenem-sensitive strains ATCC43816 (serotype K1), Kp52145 (serotype 2), and other capsular antigen expressing strains *Salmonella typhi*, *Legionella pneumophila*, *Clostridium difficile*, and *Streptococcus pneumoniae*. Surface staining was performed with murine anti-CRM197-1 antibodies ($n = 3$) raised with alum and FA formulation. PBS with alum was served as control. Anti-CRM197-1 antibodies promote the phagocytosis of CR-*Kp*. The differentiated HL-60 cells were incubated with B,C) 85/12 and D,E) NRZ-00319 strains pretreated with anti-CRM197-1 antibodies and control sera, and *Klebsiella* survival was assessed after 90 min. Percent killing of *Klebsiella* were calculated based on viable *Klebsiella* colonies obtained relative to the no sera control. Values represent an independent experiment done in duplicates. Values represent mean $\pm$ SD. ND = not detected.

Scheme 1.
Retrosynthetic analysis of hexasaccharide **1**.

Scheme 2.

Synthesis of trisaccharide building blocks **18** and **19**.

Scheme 3.
[3+3] Glycosylation to complete hexasaccharide **1**.