Oligosaccharides

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Synthetic Enterobacterial Common Antigen (ECA) for the Development of a Universal Immunotherapy for Drug-Resistant *Enterobacteriaceae*

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Abstract: *All Enterobacteriaceae express a polysaccharide known as enterobacterial common antigen (ECA), which is an attractive target for the development of universally acting immunotherapies. The first chemical synthesis of ECA-derived oligosaccharides for the development of such therapies is described. A number of synthetic challenges had to be addressed, including the development of concise synthetic procedures for unusual monosaccharides, the selection of appropriate orthogonal protecting groups, the development of stereoselective glycosylation methods, appropriate timing for the introduction of the carboxylic acid groups on the ManpNAcA moieties, and the selection of appropriate conditions for the reduction of multiple azido moieties. The synthetic compounds were employed to uncover immunodominant moieties of ECA. Furthermore, a monoclonal antibody (mAb) was developed that binds to ECA and can selectively recognize a wide range of Enterobacteriaceae species.*

Carbapenem-resistant *Enterobacteriaceae* (CRE) are Gram-negative bacteria that are resistant to the carbapenem class of antibiotics, which are the drugs of last resort. CRE cause infections with high mortality[1] and their prevalence is rapidly increasing owing to the transmission of carbapenem resistance via mobile genetic elements.[2] New therapeutic strategies are urgently needed because of limited available therapeutic options.[3] Immunotherapy with monoclonal antibodies (mAbs) could potentially offer a new approach for the treatment of these infections.[4]

All *Enterobacteriaceae* express a polysaccharide known as enterobacterial common antigen (ECA), which has the following structure: →3)-α-D-Fuc*p*4NAc-(1→4)-β-D-Man*p*NAcA-(1→4)-α-D-Glc*p*NAc-(1→.[5] It is modified substoichiometrically (ca. 70%) at C-6 of the *N*-acetylglucosamine moieties with acetyl esters.[6] ECA is important for cell envelope integrity,[7] flagellum expression,[8] and resistance to acetic acid and bile salts.[9] Evidence is also emerging that ECA is a virulence factor, and mutant bacteria that cannot produce ECA exhibit attenuated infection in oral and intraperitoneal mouse models.[10] These properties and the universal occurrence of ECA among *Enterobacteriaceae* make it an attractive target for the development of cross-protective vaccines or mAbs.

The development of immunotherapeutics based on ECA requires a detailed knowledge of the saccharide structures that can be recognized by naturally induced antibodies.[11] Such efforts need well-defined oligosaccharides conjugated to carrier proteins to establish structural motifs that can provide protection and for raising monoclonal antibodies. Although ECA can be isolated from natural sources, chemical synthesis offers a much more attractive approach for obtaining such compounds.[12] It allows the installation of reactive linkers for controlled conjugation of oligosaccharides to carrier proteins. This issue is particularly relevant for ECA, which is a highly functionalized compound containing sensitive functional groups such as acetyl esters that complicate conjugation chemistry. Furthermore, chemical synthesis can provide substructures required for establishing structure–activity

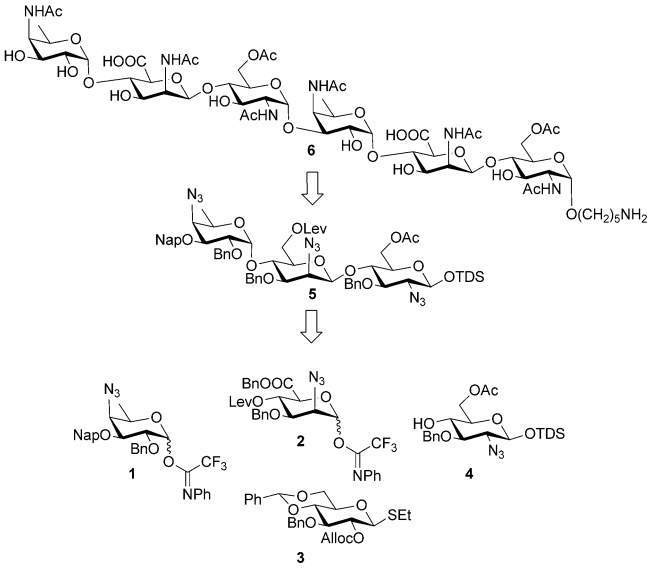

Figure 1. The ECA repeating unit and building blocks for chemical synthesis.

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relationships or determining a minimal epitope for eliciting immune responses.[13]

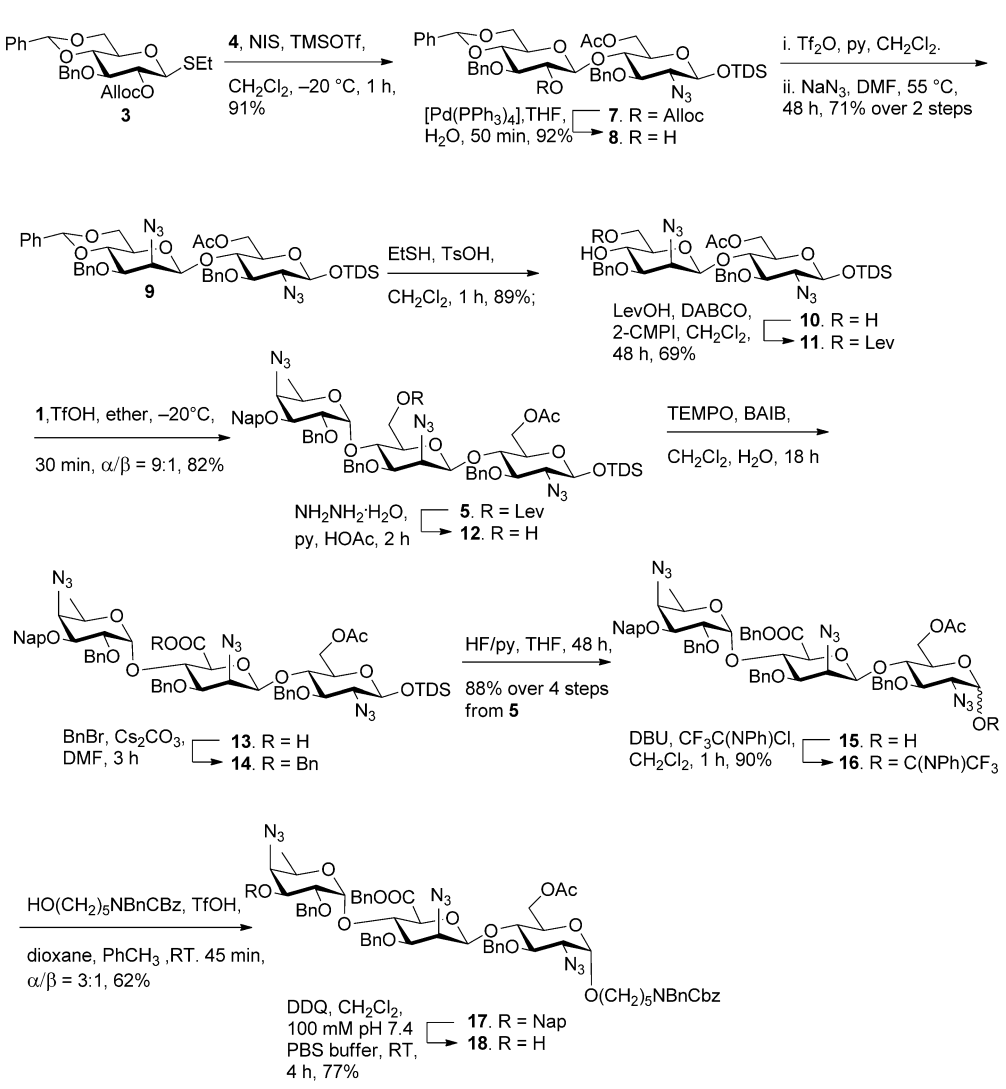
We report herein the preparation of a panel of tri- and hexasaccharides derived from ECA that are modified with an anomeric aminopentyl linker, which allows controlled conjugation to bovine serum albumin (BSA). The conjugates were employed for determining immunodominant epitopes and for characterizing a monoclonal antibody that exhibits broad recognition of *Enterobacteriaceae*.

The synthesis of ECA represented a considerable synthetic challenge[14] and required the preparation of several unusual monosaccharides. Furthermore, all of its glycosidic linkages, including a β-*N*-acetyl-D-mannosaminuronic acid (β-D-Man*p*NAcA), have 1,2-*cis*-configurations, which are difficult to install in a stereoselective manner.[15] Additionally, β-D-Man*p*NAcA has poor glycosyl donor and acceptor properties and readily forms a lactam. The latter problem was compounded by the fact that an anomeric aminopentyl linker needed to be installed, thus limiting the possibilities for amino-group manipulations. Finally, the target compounds contain acetyl esters and therefore base-sensitive protecting groups had to be avoided.

A synthetic strategy was designed that employs a repeating trisaccharide (**5**, Figure 1) modified by a set of carefully chosen orthogonal protecting groups. The thexyldimethylsilyl (TDS) group at the anomeric center and a 2-naphthylmethyl (Nap) ether at C-3 of the nonreducing terminal of the trisaccharide can selectively be removed, thereby allowing the preparation of a glycosyl donor and acceptor for oligosaccharide assembly. The levulinoyl (Lev) ester of the ManN moiety served as a surrogate for the uronic acids, which can be removed at an appropriate stage of the synthesis to liberate an alcohol for oxidation to a carboxylic acid.

The trisaccharide was assembled from monosaccharide building blocks **1**, **3**, and **4**. The β-mannosidic linkage was installed through the initial formation of a β-glucoside followed by inversion of configuration of the C-2 hydroxy group of the latter moiety.[16] A *N*-iodosuccinimide (NIS)/trimethylsilyl trifluoromethanesulfonate (TMSOTf)-mediated glycosylation of **3** with **4** at −20 °C thus provided disaccharide **7** solely as the β anomer (91 %) owing to neighboring-group participation from the allyloxycarbonyl (Alloc) group. The latter functionality was selectively removed through treatment with [Pd(PPh$_3$)$_4$], and triflation of the resulting alcohol **8** with triflic anhydride followed by reaction with NaN_3 in DMF at 55 °C gave the 2-azido-2-deoxy-β-mannoside **9** in an overall yield of 71 %. A direct approach with 2-azido-2-deoxymannuronyl donor **2** and acceptor **4** gave a low yield of disaccharide, probably owing to the low reactivity of uronyl donors (Scheme S2 in the Supporting Information).[17]

The benzylidene acetal of **9** was cleaved by using ethane thiol and a catalytic amount of *p*-toluene sulfonic acid,[18] and the primary hydroxyl of the resulting compound **10** was selectively protected as a Lev ester[19] to give acceptor **11**. A triflic acid mediated glycosylation of **1** with **11** in diethyl ether at −20 °C gave trisaccharide **5** mainly as the α anomer (82 %, α/β = 9:1). The Lev ester of **5** was removed with hydrazine in pyridine and acetic acid to give alcohol **12**, which was oxidized



Scheme 1. Synthesis of the trisaccharide acceptor and donor through a post-glycosylation oxidation approach.

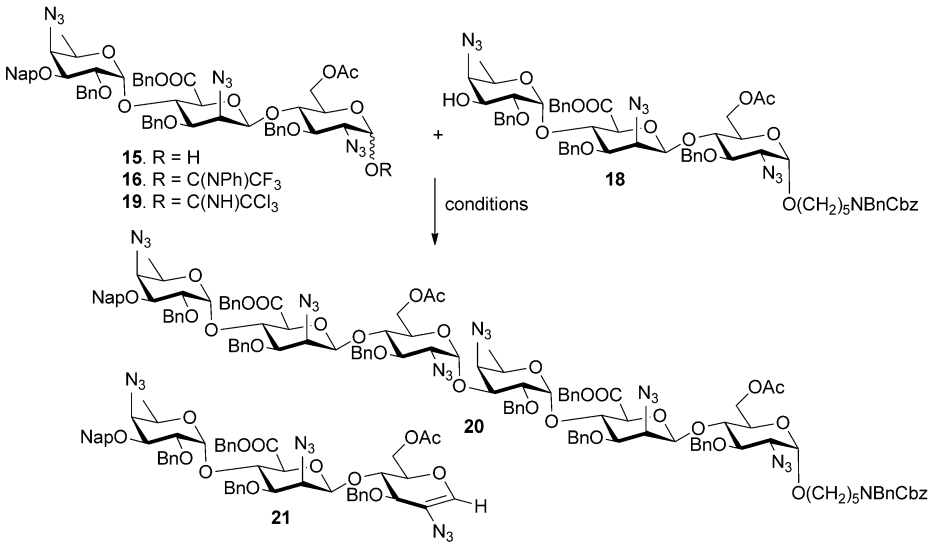
with (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) and (diacetoxyiodo)benzene (BAIB). The resulting carboxylic acid **13** was protected as a benzyl ester through reaction with benzyl bromide in the presence of Cs_2CO_3[20] to afford **14**. A strategy in which the primary alcohol of **10** was oxidized and protected as a benzyl ester and then used as an acceptor in a glycosylation with **1** resulted in a trace amount of trisaccharide **14** (Scheme S3). The carboxylic acid of the acceptor probably reduced the reactivity of the neighboring C-4 alcohol, thereby complicating the glycosylation.[17a]

The anomeric TDS of **14** was removed and the resulting lactol **15** was converted into the *N*-phenyl trifluoroacetimidate **16** by using standard conditions (88%, 4 steps). A triflic acid promoted glycosylation of **16** with benzyl carboxybenzyl (Cbz) protected aminopentanol in a mixture of toluene and dioxane[21] gave the spacer-linked trisaccharide **17** as a separable mixture of α/β anomers (66%, $\alpha/\beta = 3{:}1$). The Nap ether of **17** was removed by using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in a mixture of DCM and phosphate buffered saline (PBS) to provide glycosyl acceptor **18** (Scheme 1).

After extensive optimization (Table 1, see the Supporting Information for details), the α-linked hexasaccharide **20** could be obtained in a yield of 43% through triflic acid mediated glycosylation of **16** with **18** in diethyl ether in the presence of acid-washed molecular sieves. Surprisingly, a post-glycosylation oxidation strategy in which trisaccharide **5** was converted into a glycosyl donor and acceptor for oligosaccharide assembly gave the unwanted β-glycoside (Scheme S4). Our various attempts to install the ManNA moieties highlight the importance of installing the carboxylic acids at an appropriate stage of the synthesis, which in our strategy was made possible by the carefully selected orthogonal protecting groups.

The final challenge was deprotection of the tri- and hexasaccharide. It is well know that D-mannosaminuronic esters have a propensity to form 2,6-lactams, and Birch reduction was previously employed for the deprotection of such oligosaccharides.[15a] However, such a strategy is not feasible for ECA-derived compounds owing to the presence of base-sensitive acetyl esters. The need to simultaneously reduce six azido groups further complicated the deprotection. Various conditions were examined (Table 2, see the Supporting Information for details) and it was found that **22** could be obtained in a yield of 71% by using $SnCl_2/PhSH/Et_3N$[22] as the reducing agent in a mixture of CH_3CN and THF for 45 min followed by N-acetylation with acetic anhydride (Table 2, entry 11). Disappointingly, the application of these reaction conditions to hexasaccharide **20** led mainly to decomposition. It was anticipated that the formation of a multiple N–Sn coordinated intermediate might lead to substantial decomposition. Gratifyingly, the use of a mixture of solvents comprising $CH_3CN/CH_3OH/CH_2Cl_2$, which was expected to break the Sn–N bonds, gave hexasaccharide **23** in an acceptable yield of 51% after N-acetylation (Table 2, entry 12).

Table 1: Establishing optimal glycosylation conditions for the formation of hexasaccharide **20**.

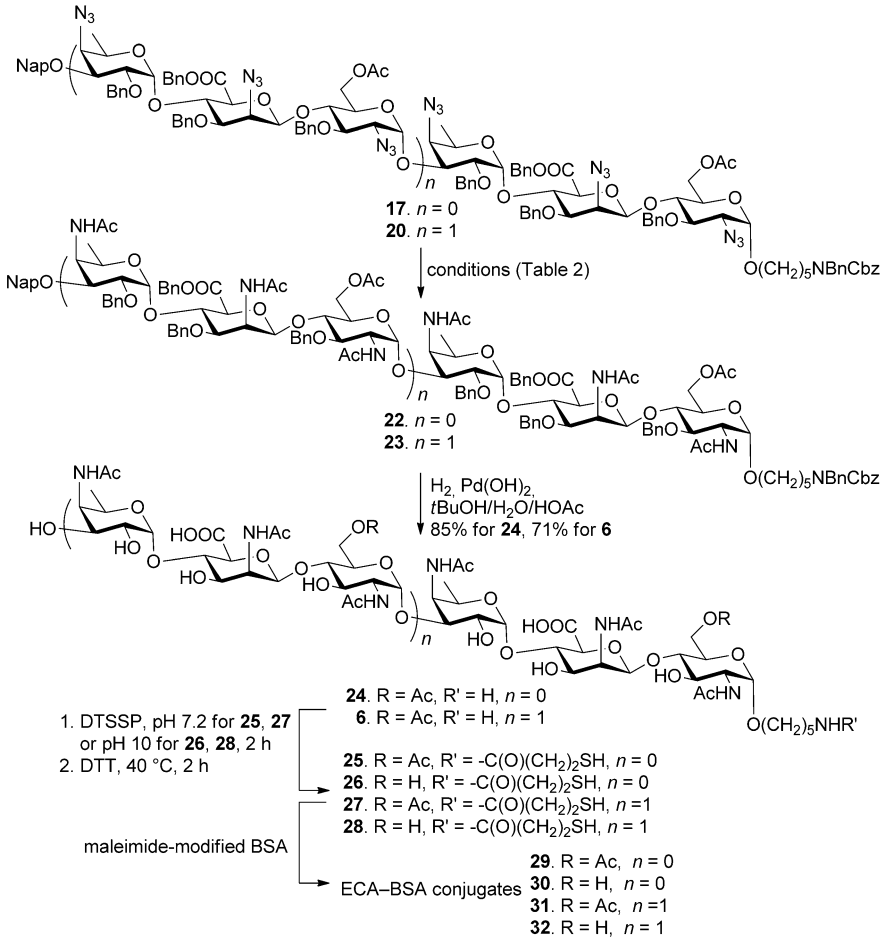


Entry	Donor	Conditions	Yield [%]	Side reaction
1	**16**	TfOH, 4 Å MS, CH_2Cl_2, 0°C	10	elimination
2	**16**	TfOH, AW-300, CH_2Cl_2, 0°C	10	elimination, hydrolysis
3	**19**	TfOH, 4 Å MS, CH_2Cl_2, −20 to 0°C	<5	hydrolysis, rearrangement
4	**19**	TfOH, AW-300, CH_2Cl_2, −20 to 0°C	<5	hydrolysis, rearrangement
5	**19**	AgOTf, 4 Å MS, CH_2Cl_2, −40 to 0°C	trace	hydrolysis
6	**15**	Ph_2SO, Tf_2O, TTBP, CH_2Cl_2, −78 to 0°C	trace	donor recovered
7	**16**	TfOH, 4 Å MS, Et_2O, −20°C	0	hydrolysis
8	**16**	TfOH, AW-300, Et_2O, −20 to 0°C	43	hydrolysis, elimination

Table 2: Establishing optimal conditions for azide reduction.

Entry	Conditions	Yield of **22** [%]	Yield of **23** [%]
1	PMe_3, THF, H_2O	<5	ND[a]
2	1,3-propanedithiol, Et_3N	trace	ND[a]
3	PhSeH, Et_3N	10	ND[a]
4	Mg/CH_3OH	0	ND[a]
5	AcSH, 2,6-Lutidine, $CHCl_3$	10	ND[a]
6	AcSeH, THF	0	ND[a]
7	$AIBN/HBu_3Sn$	trace	ND[a]
8	Lindlar catalyst, H_2	trace	ND[a]
9	Zn/Cu, HOAc, Ac_2O, THF	50	0
10	$NaBH_4$, $NiCl_2$, CH_2Cl_2, CH_3OH; Ac_2O	45	15
11	$SnCl_2/PhSH/Et_3N$, CH_3CN/THF; Ac_2O, CH_3OH	71	0
12	$SnCl_2/PhSH/Et_3N$, CH_3CN/CH_2Cl_2/CH_3OH; Ac_2O, CH_3OH	70	51

[a] ND: Not determined.



Scheme 2. Deprotection and BSA conjugation.

Finally, hydrogenolysis of **23** by using $Pd(OH)_2$ in a mixture of *t*BuOH/H_2O/HOAc afforded ECA hexasaccharide **6**. Compound **24** was prepared in a similar manner (Scheme 2.) Oligosaccharides **25**–**28** were conjugated to BSA by using thiol–maleimide coupling chemistry to provide conjugates **29**, **30**, **31**, and **32**, respectively. The saccharide content of the conjugates was determined by MALDI-TOF mass spectrometry.

To confirm that the synthetic oligosaccharides mimic natural occurring ECA, we examined the binding of the oligosaccharides to immune sera from mice exposed to various *K. pneumoniae* strains, including multidrug-resistant clinical isolates, such as *K. pneumoniae* carbapenemase-producing strain (KPC3)[23] and extended-spectrum beta-lactamase (ESBL)-producing strains.[24] The antisera exhibited moderate to strong binding to the hexasaccharide and trisaccharide conjugates **29**–**32**, as determined by enzyme-linked immunosorbent assay (ELISA), thus confirming that naturally induced antibodies can recognize the synthetic oligosaccharides (Table 3 and Figure S6 in the Supporting Information). In most cases, the presence of the O-acetyl esters had only a moderate effect on serum titers. In the case of 9177 (O5:K57) and 43816 (O1:K2), the trisaccharide conjugates **29** and **30** gave substantially higher titers, which can best be explained by monomeric ECA being an immunodominant epitope.

We explored a strategy to generate ECA-recognizing mAbs. Mice were immunized with a mixture of six *Shigella* strains that had been washed and boiled for 2 h to denature the proteins. The spleens of the immunized mice were harvested and employed for hybridoma generation. The resulting clones were screened by ELISA for binding to whole cells of *S. flexneri*, *S. sonnei*, and *S. dysenteriae*, and clones that were positive against all of the tested strains were kept for further characterization. Importantly, the SM250-1 A5 mAb was reactive to all of the *Enterobacteriaceae* examined (*K. pneumoniae*, *S. sonnei*, *S. flexneri*, *C. freundii*, *E. coli*, *Y. enterocolitica*, *E. aerogenes*, *S. typimurium*) but nonreactive to other Gram-negative bacteria (*V. cholera*) and Gram-positive bacteria (*L. monocytogenes*), as determined by whole-cell ELISA (Table S3 in the Supporting Information). The mAb did not recognize either lipid A or OmpA, which is an integral membrane protein that is common to many Gram-negative bacteria. Based on these results, it was anticipated that the mAb recognizes ECA. Interestingly, it bound to trisaccharide **30**, which lacks the O-acetyl ester, but it did not bind to hexasaccharide **32** or acetylated derivatives **29** and **31** (Figure 2). These results demonstrate that ECA from intact bacteria is accessible to antibodies. Furthermore, the antibody recognizes a similar epitope to that observed for the polyclonal sera elicited against *K. pneumoniae* 9177 (O5:K57) and 43816 (O1:K2), and monomeric ECA is possibly responsible for this response. Bundle and co-workers have made a similar observation and have

Table 3: Serum titers[a] against synthetic ECA conjugates **29**–**32**.

K. pneumoniae immune serum	Conjugate **29**	Conjugate **30**	Conjugate **31**	Conjugate **32**
5046 (O2a:K3)	2800	1600	73 000	38 700
8045 (O1:K1)	4700	12 000	10 600	10 100
9135 (O4:K15)	8200	9700	23 500	15 400
9140 (O1:K20)	3000	3900	3900	1900
9177 (O5:K57)	1300	9300	200	500
9178 (O3:K58)	6600	12 300	10 400	1300
43 816 (O1:K2)	1300	8300	300	600
30 48 513 (KPC3)	800	3300	2300	2100
30 48 514 (KPC3)	3400	3200	6500	4100
30 48 561 (ESBL)	700	2700	3800	2400

[a] Titers are defined as the highest dilution yielding an optical density of 0.1.

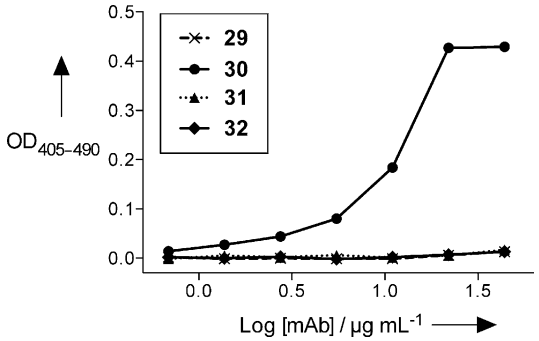


Figure 2. Immunoreactivity of ECA–BSA conjugates **29**–**32** with mAb SM250-1A5.

shown that an antibody recognizing *C. albicans* binds optimally to di- and trisaccharide epitopes, whereas larger oligosaccharides were bound with markedly lower affinities.[25]

In summary, a synthetic strategy has been developed that addresses the challenges associated with the synthesis of oligosaccharides containing β-D-Man*p*NAcA moieties and offers opportunities for the preparation of many other biologically important oligosaccharides containing this type of monosaccharide. The synthetic ECA oligosaccharides offer promising reagents for the development of universal immunotherapies for infections caused by *Enterobacteriaceae*. Current efforts are focused on using the synthetic oligosaccharides for mAb generation and for determining functional properties.

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[1] M. McKenna, *Nature* **2013**, *499*, 394–396.
[2] a) N. Gupta, B. M. Limbago, J. B. Patel, A. J. Kallen, *Clin. Infect. Dis.* **2011**, *53*, 60–67; b) A. J. Mathers, H. L. Cox, B. Kitchel, H. Bonatti, A. K. C. Brassinga, J. Carroll, W. M. Scheld, K. C. Hazen, C. D. Sifri, *mBio* **2011**, *2*, e00204-11.
[3] P. Savard, T. M. Perl, *Curr. Opin. Infect. Dis.* **2012**, *25*, 371–377.
[4] a) O. Leavy, *Nat. Rev. Immunol.* **2010**, *10*, 297–297; b) J. ter Meulen, *Infect. Dis. Clin. N. Am.* **2011**, *25*, 789–802.
[5] a) A. Dell, J. Oates, C. Lugowski, E. Romanowska, L. Kenne, B. Lindberg, *Carbohydr. Res.* **1984**, *133*, 95–104; b) C. Lugowski, E. Romanowska, L. Kenne, B. Lindberg, *Carbohydr. Res.* **1983**, *118*, 173–181; c) D. Männel, H. Mayer, *Eur. J. Biochem.* **1978**, *86*, 361–370.
[6] M. Bruix, J. Jiménez-Barbero, P. Cronet, *Carbohydr. Res.* **1995**, *273*, 157–170.
[7] M. E. Castelli, E. G. Véscovi, *J. Bacteriol.* **2011**, *193*, 63–74.
[8] M. E. Castelli, G. V. Fedrigo, A. L. Clementín, M. V. Ielmini, M. F. Feldman, E. G. Véscovi, *J. Bacteriol.* **2008**, *190*, 213–220.
[9] F. Ramos-Morales, A. I. Prieto, C. R. Beuzón, D. W. Holden, J. Casadesús, *J. Bacteriol.* **2003**, *185*, 5328–5332.
[10] J. J. Gilbreath, J. Colvocoresses Dodds, P. D. Rick, M. J. Soloski, D. S. Merrell, E. S. Metcalf, *Infect. Immun.* **2012**, *80*, 441–450.
[11] M. B. Oleksiewicz, G. Nagy, E. Nagy, *Arch. Biochem. Biophys.* **2012**, *526*, 124–131.
[12] T. J. Boltje, T. Buskas, G.-J. Boons, *Nat. Chem.* **2009**, *1*, 611–622.
[13] R. D. Astronomo, D. R. Burton, *Nat. Rev. Drug Discovery* **2010**, *9*, 308–324.
[14] A previous effort to prepare ECA focused on an unnatural trisaccharide: H. Paulsen, J. P. Lorentzen, *Angew. Chem. Int. Ed. Engl.* **1985**, *24*, 773–775; *Angew. Chem.* **1985**, *97*, 791–792.
[15] a) M. T. C. Walvoort, G. Lodder, H. S. Overkleeft, J. D. C. Codée, G. A. van der Marel, *J. Org. Chem.* **2010**, *75*, 7990–8002; b) L. J. van den Bos, B. A. Duivenvoorden, M. C. de Koning, D. V. Filippov, H. S. Overkleeft, G. A. van der Marel, *Eur. J. Org. Chem.* **2007**, 116–124.
[16] K.-i. Sato, A. Yoshitomo, *Chem. Lett.* **1995**, 39–40.
[17] a) J. D. Codée, A. E. Christina, M. T. Walvoort, H. S. Overkleeft, G. A. van der Marel, *Top. Curr. Chem.* **2011**, *301*, 253–289; b) A. Wadouachi, J. Kovensky, *Molecules* **2011**, *16*, 3933–3968.
[18] K. C. Nicolaou, C. A. Veale, C. K. Hwang, J. Hutchinson, C. V. C. Prasad, W. W. Ogilvie, *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 299–303; *Angew. Chem.* **1991**, *103*, 304–308.
[19] T. Mukaiyama, *Angew. Chem. Int. Ed. Engl.* **1979**, *18*, 707–721; *Angew. Chem.* **1979**, *91*, 798–812.
[20] E. M. Scanlan, M. M. Mackeen, M. R. Wormald, B. G. Davis, *J. Am. Chem. Soc.* **2010**, *132*, 7238–7239.
[21] A. Demchenko, T. Stauch, G.-J. Boons, *Synlett* **1997**, 818–820.
[22] a) M. Bartra, P. Romea, F. Urpí, J. Vilarrasa, *Tetrahedron* **1990**, *46*, 587–594; b) J. W. Lee, P. L. Fuchs, *Org. Lett.* **1999**, *1*, 179–182.
[23] A. Leavitt, S. Navon-Venezia, I. Chmelnitsky, M. J. Schwaber, Y. Carmeli, *Antimicrob. Agents Chemother.* **2007**, *51*, 3026–3029.
[24] J. D. D. Pitout, K. B. Laupland, *Lancet Infect. Dis.* **2008**, *8*, 159–166.
[25] a) M. A. Johnson, J. Cartmell, N. E. Weisser, R. J. Woods, D. R. Bundle, *J. Biol. Chem.* **2012**, *287*, 18078–18090; b) D. R. Bundle, C. Nycholat, C. Costello, R. Rennie, T. Lipinski, *ACS Chem. Biol.* **2012**, *7*, 1754–1763.

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