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

Lin Liu,

Complex Carbohydrate Research Center, The University of Georgia 315 Riverbend Road, Athens, GA 30602 (United States)

Jingying Zha,

Department of Infectious Diseases, MedImmune, LLC Gaithersburg, MD 20878 (United States)

Antonio DiGiandomenico,

Department of Infectious Diseases, MedImmune, LLC Gaithersburg, MD 20878 (United States)

Douglas McAllister,

Virostat Inc., Portland, ME 04104 (United States)

C. Kendall Stover,

Department of Infectious Diseases, MedImmune, LLC Gaithersburg, MD 20878 (United States)

Qun Wang, and

Department of Infectious Diseases, MedImmune, LLC Gaithersburg, MD 20878 (United States)

Geert-Jan Boons

Complex Carbohydrate Research Center, The University of Georgia 315 Riverbend Road, Athens, GA 30602 (United States)

Qun Wang: wangqu@medimmune.com; Geert-Jan Boons: gjboons@ccrc.uga.edu

Abstract

All *Enterobacteriaceae* express a polysaccharide known as Enterobacterial Common Antigen (ECA), which is an attractive target for the development of universal acting immunotherapies. We describe here the first chemical synthesis of ECA derived oligosaccharides for the development of such therapies. 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 protocols, appropriate timing for the introduction of carboxylic acids of ManpNAcA moieties and conditions for the reduction of multiple azido moieties. The synthetic compounds were employed to uncover

Correspondence to: Qun Wang, wangqu@medimmune.com; Geert-Jan Boons, gjboons@ccrc.uga.edu.

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immuno-dominant moieties of ECA. Furthermore, a mAb was developed that binds to ECA and can selectively recognize a wide range of intact *Enterobacteriaceae*.

Keywords

Oligosaccharides; Glycosylations; *Enterobacteriaceae*; Immunotherapies

Carbapenem-resistant *Enterobacteriaceae* (CRE) are gram-negative bacteria that are resistant to the carbapenem class of antibiotics, which are drugs of last resort. CRE cause infections with high mortality,^[1] and their prevalence is rapidly increasing through the transmission of carbapenem resistance via mobile genetic elements.^[2] Immuno-therapy with monoclonal antibodies (mAbs) has the potential of offering a new approach for the treatment of these infections.^[4]

All *Enterobacteriaceae* express a polysaccharide known as Enterobacterial Common Antigen (ECA) having the following structure: $\rightarrow 3)-\alpha\text{-D-Fucp}4\text{NAc}-(1\rightarrow 4)-\beta\text{-D-ManpNAcA}-(1\rightarrow 4)-\alpha\text{-D-GlcpNAc}-(1\rightarrow$.^[5] It is modified substoichiometrically (~70%) at C-6 of the *N*-acetyl-glucosamine 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 for example 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 relationships or used to determine minimal epitope for eliciting immune responses.^[13]

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

The synthesis of ECA represented a considerable synthetic challenge^[14] requiring the preparation of several unusual monosaccharides. Furthermore, all its glycosidic linkages

have 1,2-*cis*-configurations including a β -*N*-acetyl-D-mannosaminuronic acid (β -D-ManpNAcA), which are difficult to install in a stereoselective manner.^[15] Also, β -D-ManpNAcA 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 limiting the possibilities of 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 employing 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 the C-3 of the non-reducing terminal of the trisaccharide can selectively be removed allowing the preparation of a glycosyl donor and acceptor for oligosaccharide assembly. The levulinoyl (Lev) ester of the ManN moiety served as the surrogate for the uronic acids, which at an appropriate stage of synthesis can be removed 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 by the initial formation of a β -glucoside followed by inversion of configuration of the C-2 hydroxyl of the latter moiety.^[16] Thus, a *N*-iodosuccinimide (NIS)/ trimethylsilyl trifluoromethanesulfonate (TMSOTf) mediated glycosylation of **3** with **4** at $-20\text{ }^{\circ}\text{C}$ provided disaccharide **7** as only the β -anomer (91%) due to neighboring group participation of the allyloxycarbonyl (Alloc) group. The latter functionality was selectively removed by treatment with $\text{Pd}(\text{PPh}_3)_4$ and triflation of the resulting alcohol **8** with triflic anhydride followed by reaction with NaN_3 in DMF at $55\text{ }^{\circ}\text{C}$ gave the 2-azido-2-deoxy- β -mannoside **9** in an overall yield of 71%. A direct approach using 2-azido-2-deoxymannuronyl donor **2** and acceptor **4** gave a low yield of disaccharide probably due to low reactivity of uronyl donors (Scheme S2).^[17]

The benzylidene acetal of **9** was cleaved 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 to give acceptor **11**. A triflic acid mediated glycosylation of **1** with **11** in diethyl ether at $-20\text{ }^{\circ}\text{C}$ gave trisaccharide **5** as mainly the α -anomer (82%, $\alpha/\beta = 9/1$). The Lev ester of **5** was removed by hydrazine in pyridine and acetic acid to give alcohol **12**, which was oxidized using (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) and (diacetoxyiodo)benzene (BAIB) and the resulting carboxylic acid **13** was protected as a benzyl ester by 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). Probably, the carboxylic acid of the acceptor 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 a *N*-phenyl trifluoroacetimidate **16** 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 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**.

After extensive optimization (Table 1, see SI for details), the α -linked hexasaccharide **20** could be obtained in a yield of 43% by a 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). Thus, the various attempts to install the ManNA moieties highlight the importance of installing the carboxylic acids at an appropriate stage of synthesis, which in our strategy was possible due to the carefully selected orthogonal protecting groups.

The final challenge was the deprotection of the tri- and hexasaccharide. It is well known that D-mannosaminuronic esters have a propensity to form 2,6-lactams, and previously Birch reduction was employed for the deprotection of such oligosaccharides.^[15a] Such a strategy is, however, not feasible for ECA-derived compounds due to the presence of base sensitive acetyl esters. The need to simultaneously reduce six azido groups further complicated the deprotection. Multiple conditions were examined (Table 2, see SI for details) and it was found that **22** could be obtained in a yield of 71% using $\text{SnCl}_2/\text{PhSH}/\text{Et}_3\text{N}$ ^[22] as the reducing agent in a mixture of CH_3CN and THF for 45 min followed by *N*-acetylation using 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 multiple N-Sn coordinated intermediate might lead to substantial decomposition. Gratifyingly, the use of a mixture of solvents containing $\text{CH}_3\text{CN}/\text{CH}_3\text{OH}/\text{CH}_2\text{Cl}_2$, which was expected to break Sn-N bonds, gave after *N*-acetylation, hexasaccharide **23** in an acceptable yield of 51% (Table 2, entry 12).

Finally, hydrogenolysis of **23** using $\text{Pd}(\text{OH})_2$ 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 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 the synthetic oligosaccharides mimic natural occurring ECA, we examined the binding of the oligosaccharides to immune sera of mice exposed to various *K. pneumoniae* strains including multi-drug resistant clinical isolates such as *K. pneumoniae* carbapenemase producing strain (KPC3)^[23] and extended spectrum beta-lactamase (ESBL)-producing strains.^[24] The anti-sera exhibited moderate to strong binding to the hexasaccharide and trisaccharide conjugates **29–32** as determined by enzyme-linked immunosorbent assay (ELISA) confirming that naturally induced antibodies can recognize the synthetic oligosaccharides (Table 3 and Figure S6). 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 substantial 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 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 for all antigens were kept for further characterization. Importantly, the SM250-1A5 mAb was reactive to all members of the *Enterobacteriaceae* examined (e.g. *K. pneumoniae*, *S. sonnei*, *S. flexneri*, *C. freundii*, *E. coli*, *Y. enterocolitica*, *E. aerogenes*, *S. typhimurium*) but non-reactive to other gram-negative bacteria (*V. cholera*) and gram-positive bacteria (*L. monocytogenes*) as determined by whole cell ELISA (Table S3). The mAb did not recognize lipid A or OmpA, which is an integral membrane protein that is common to most members of *Enterobacteriaceae*. Based on these results, it was anticipated that the mAb recognizes ECA. Interestingly, it bound to trisaccharide **30** lacking the *O*-acetyl ester but did not bind to hexasaccharide **32** and acetylated derivatives **29** and **31** (Figure 2). These results demonstrate that ECA of intact bacteria is assessable to antibodies. Furthermore, the antibody recognizes a similar epitope as was observed for the polyclonal sera elicited against *K. pneumoniae* 9177 (O5:K57) and 43816 (O1:K2), and possibly monomeric ECA was responsible for this response. Bundle and coworkers have made a similar observation and have 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-ManpNAcA 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 of infections with *Enterobacteriaceae*. Current efforts are focused on using the synthetic oligosaccharides for mAb generation and for determining functional properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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

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

Scheme 1.

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

Scheme 2.
Deprotection and BSA conjugation.

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

Entry	Donor	Conditions	Yield, %	Side reaction
1	16	TfOH, 4Å MS, CH ₂ Cl ₂ , 0 °C	10	elimination
2	16	TfOH, AW-300, CH ₂ Cl ₂ , 0 °C	10	elimination, hydrolysis
3	19	TfOH, 4Å MS, CH ₂ Cl ₂ , -20 to 0 °C	<5	hydrolysis, rearrangement
4	19	TfOH, AW-300, CH ₂ Cl ₂ , -20 to 0 °C	<5	hydrolysis, rearrangement
5	19	AgOTf, 4Å MS, CH ₂ Cl ₂ , -40 to 0 °C	trace	hydrolysis
6	15	Ph ₂ SO, Tf ₂ O, TTBP, CH ₂ Cl ₂ , -78 to 0 °C	trace	donor recovered
7	16	TfOH, 4Å MS, Et ₂ O, -20 °C	0	hydrolysis
8	16	TfOH, AW-300, Et ₂ O, -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 ₂ O	<5	ND ^a
2	1,3-propanedithiol, Et ₃ N	trace	ND
3	PhSeH, Et ₃ N	10	ND
4	Mg/CH ₃ OH	0	ND
5	AcSH, 2,6-Lutidine, CHCl ₃	10	ND
6	AcSeH, THF	0	ND
7	AIBN/HBu ₃ Sn	trace	ND
8	Lindlar catalyst, H ₂ ,	trace	ND
9	Zn/Cu, HOAc, Ac ₂ O, THF	50	0
10	NaBH ₄ , NiCl ₂ , CH ₂ Cl ₂ , CH ₃ OH; Ac ₂ O	45	15
11	SnCl ₂ /PhSH/Et ₃ N, CH ₃ CN/THF; Ac ₂ O, CH ₃ OH	71	0
12	SnCl ₂ /PhSH/Et ₃ N, CH ₃ CN/CH ₂ Cl ₂ /CH ₃ OH; Ac ₂ O, CH ₃ OH	70	51

^a ND: Not determined.

Table 3Serum titers^a against synthetic ECA conjugates **29–32**

<i>K. pneumoniae</i> immune serum	Conjugate 29	Conjugate 30	Conjugate 31	Conjugate 32
5046 (O2a:K3)	2,800	1,600	73,000	38,700
8045 (O1:K1)	4,700	12,000	10,600	10,100
9135 (O4:K15)	8,200	9,700	23,500	15,400
9140 (O1:K20)	3,000	3,900	3,900	1,900
9177 (O5:K57)	1,300	9,300	200	500
9178 (O3:K58)	6,600	12,300	10,400	1,300
43816 (O1:K2)	1,300	8,300	300	600
3048513 (KPC3)	800	3,300	2,300	2,100
3048514 (KPC3)	3,400	3,200	6,500	4,100
3048561 (ESBL)	700	2,700	3,800	2,400

^aTiters are defined as the highest dilution yielding an optical density of 0.1.