Chemoenzymatic Synthesis

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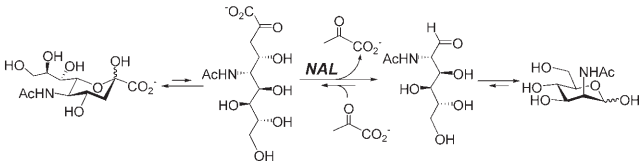
Disaccharides as Sialic Acid Aldolase Substrates: Synthesis of Disaccharides Containing a Sialic Acid at the Reducing End**

*Shengshu Huang, Hai Yu, and Xi Chen**

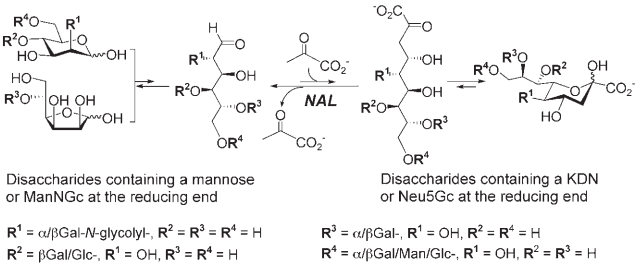
Sialic acid aldolases (EC 4.1.3.3), or *N*-acetylneuraminate lyases (Neu5Ac lyases or NALs), catalyze the reversible aldol cleavage of *N*-acetylneuraminic acid (Neu5Ac) to form pyruvate and *N*-acetyl-D-mannosamine (ManNAc) (Scheme 1).[1] They have been extensively studied and the reverse reaction has been widely applied in the enzymatic synthesis of sialic acids and their derivatives.[1a]



Scheme 1. Reaction catalyzed by sialic acid aldolases (NALs).

NALs are Schiff base forming type I aldolases. The NAL-catalyzed reaction proceeds through ring-opening of the α anomer of Neu5Ac[2] (Scheme 1) and a Schiff base intermediate is formed between a strictly conserved catalytic lysine residue in the enzyme and the ketone carbonyl group at the C2 position in the open ring form of Neu5Ac.[3,4] Although quite restricted in its donor substrate (for example, pyruvate), *Escherichia coli* aldolase is flexible about using a variety of hexoses, pentoses, and their derivatives as acceptor substrates for the synthesis of sialic acids and their analogues. C2-, C4-, C5-, and/or C6-substituted ManNAc or mannose are well tolerated.[5,6] The configurations at the C4 and C5 positions can also be different from those of ManNAc or mannose.[1a,4,5] Protein crystal-structure-based site-directed mutagenesis, structure-guided saturation mutagenesis, and directed evolution have been carried out to generate aldolase mutants with altered substrate specificity or broader substrate tolerance for the synthesis of novel products.[7]

A wild-type sialic acid aldolase has been cloned from *E. coli* K-12 (substrain MG1655) and used in the one-pot multiple-enzyme synthesis of cytidine 5′-monophosphate–sialic acid and sialoside derivatives in our laboratory.[6f–h] Our previous work has shown that a disaccharide, Galβ1,6Man, can also be used by the recombinant aldolase for the high-yield synthesis of the corresponding disaccharide, Galβ1,9KDN, containing a deaminoneuraminic acid (KDN) at the reducing end.[8] The resultant KDN-containing disaccharide is a part of the tetrasaccharide component found in the cell wall of *Streptomyces* sp. MB-8.[8] In this case, the disaccharide Galβ1,6Man can be considered as a mannose derivative in which the hydrogen atom on the C6 hydroxy group of the mannose is substituted with a galactose residue. In order to investigate whether disaccharides containing a mannose residue at the reducing end are generally tolerated by the sialic acid aldolase, we chemically synthesized a list of disaccharides with a galactose (Gal), a glucose (Glc), or a mannose (Man) residue α or β linked to the hydroxy group at the C2, C4, C5, or C6 positions of a mannose residue. Two disaccharides containing an *N*-glycolylmannosamine (ManNGc) at the reducing end, Galα1,2ManNGc (**23**) and Galβ1,2ManNGc (**24**, Table 1), were also synthesized. We demonstrate here that the two ManNGc-containing disaccharides and most of the disaccharides containing a mannose at the reducing end can be used as unusual substrates for the sialic acid aldolase (Scheme 2). This type of sialic acid



Scheme 2. Sialic acid aldolase catalyzed synthesis of disaccharides containing a sialic acid residue at the reducing end.

aldolase catalyzed chemoenzymatic reaction can thus be widely applied as a general and efficient method for producing disaccharides containing sialic acid at the reducing end. The resultant disaccharides can be used as precursors for the synthesis of naturally occurring carbohydrates containing nonterminal sialic acid residues, which have been found mainly as surface polysaccharides (for example, capsular polysaccharides or lipopolysaccharides) in some pathogenic

[*] S. Huang,[+] Dr. H. Yu,[+] Prof. Dr. X. Chen
Department of Chemistry
University of California, Davis
One Shields Avenue, Davis, CA 95616 (USA)
Fax: (+1) 530-752-8995
E-mail: chen@chem.ucdavis.edu
Homepage: http://chemgroups.ucdavis.edu/~chen/
[+] These authors contributed equally to this work.

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bacteria.[9] The synthesis of these molecules is essential to the investigation of their biological roles.

The chemical synthesis of disaccharides with a monosaccharide (selected from a galactose, a glucose, or a mannose) α or β linked to a mannose or a ManNGc residue at the reducing end was achieved by two general approaches. Both approaches used partially protected mannose derivatives **6**–**10** or partially protected ManNGc **11** as glycosyl acceptors (Table 1).

For the production of disaccharides with the glycosidic bond *trans* to the C2 group on the glycosyl donor, peracetylated D-galacto- (**2**), D-gluco- (**3**) and D-mannopyranosyl (**4**) trichloroacetimidates were synthesized and used as glycosyl donors.[10] The desired β-linked disaccharides Galβ1,2Man (**13**, 72%; Table 1, entry b), Galβ1,4Man (**15**, 59%; entry d), Glcβ1,4Man (**16**, 51%; entry e), Galβ1,5Man (**18**, 41%; entry g), Glcβ1,6Man (**22**, 68%; entry k), and Galβ1,2-ManNGc (**24**, 82%; entry m), as well as the α-linked disaccharide Manα1,6Man (**20**, 71%; entry i) were obtained by incubating the corresponding donor and acceptor in the presence of trimethylsilyl triflate (TMSOTf) promoter, followed by deprotection steps. The reactions proceeded with excellent stereoselectivity due to the assistance of the acetyl group at the C2 position of the glycosyl donors (neighboring-group participation).

Table 1: Chemical synthesis of disaccharides containing mannose or ManNGc at the reducing end and sialic acid aldolase catalyzed enzymatic synthesis of disaccharides containing KDN or Neu5Gc at the reducing end.

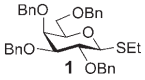
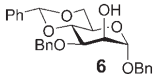
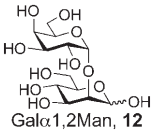
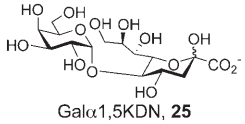
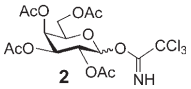
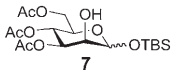
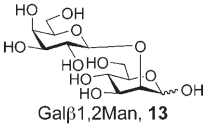
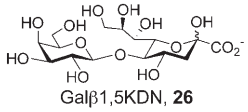
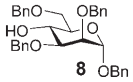
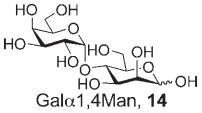
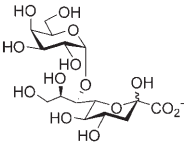
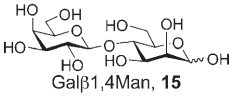
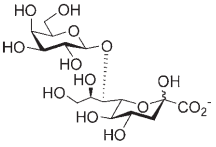
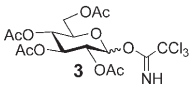
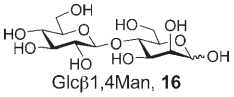
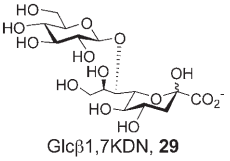
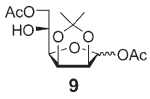
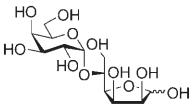
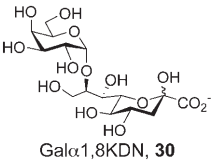
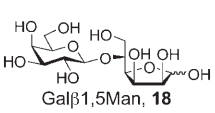
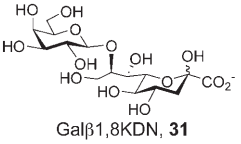
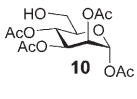
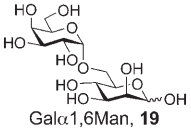
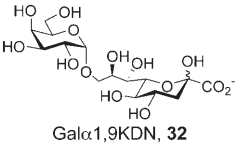
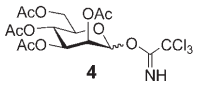
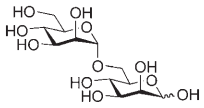
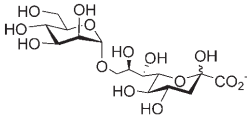
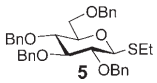
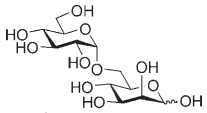
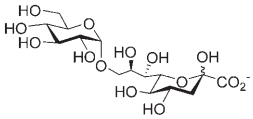
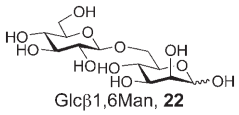
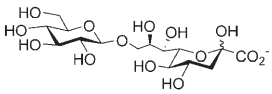
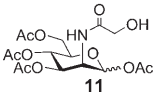
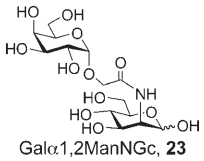
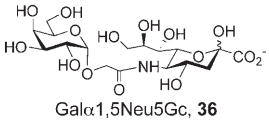
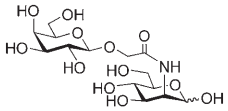
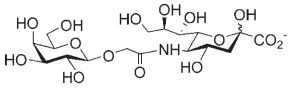
Entry	Chemical synthesis				Aldolase-catalyzed reaction	
	donor	acceptor	product[a]	yield [%][b]	product	yield [%]
a	 **1**	 **6**	 Galα1,2Man, **12**	50	 Galα1,5KDN, **25**	0
b	 **2**	 **7**	 Galβ1,2Man, **13**	72	 Galβ1,5KDN, **26**	0
c	**1**	 **8**	 Galα1,4Man, **14**	44	 Galα1,7KDN, **27**	ca. 5[c]
d	**2**	**8**	 Galβ1,4Man, **15**	59	 Galβ1,7KDN, **28**	38
e	 **3**	**8**	 Glcβ1,4Man, **16**	51	 Glcβ1,7KDN, **29**	35
f	**1**	 **9**	 Galα1,5Man, **17**	60	 Galα1,8KDN, **30**	62
g	**2**	**9**	 Galβ1,5Man, **18**	41	 Galβ1,8KDN, **31**	85
h	**1**	 **10**	 Galα1,6Man, **19**	62	 Galα1,9KDN, **32**	81

Table 1: (Continued)

Entry	Chemical synthesis				Aldolase-catalyzed reaction	
	donor	acceptor	product[a]	yield [%][b]	product	yield [%]
i	 **4**	**10**	 Manα1,6Man, **20**	71	 Manα1,9KDN, **33**	78
j	 **5**	**10**	 Glcα1,6Man, **21**	59	 Glcα1,9KDN, **34**	65
k	**3**	**10**	 Glcβ1,6Man, **22**	68	 Glcβ1,9KDN, **35**	83
l	**1**	 **11**	 Galα1,2ManNGc, **23**	65	 Galα1,5Neu5Gc, **36**	36
m	**2**	**11**	 Galβ1,2ManNGc, **24**	82	 Galβ1,5Neu5Gc, **37**	34

[a] The products of the chemical glycosylation are the potential substrates for the sialic acid aldolase catalyzed enzymatic reaction. [b] Yields for the reaction steps including glycosylation and deprotection. [c] Yield was estimated by thin-layer chromatography.

For the synthesis of disaccharides with the glycosidic bond *cis* to the C2 group on the glycosyl donor, thioethyl glycoside donors bearing a nonparticipating substituent at the C2 position were used.[11] Coupling of perbenzylated thiogalactoside donor **1** with acceptors **6** and **8**–**11** by using *N*-iodosuccinimide/trifluoromethanesulfonic acid (NIS/TfOH) as the catalyst, followed by appropriate deprotection steps, gave the expected α-linked disaccharides Galα1,2Man (**12**, 50%; Table 1, entry a), Galα1,4Man (**14**, 44%; entry c), Galα1,5Man (**17**, 60%; entry f), Galα1,6Man (**19**, 62%; entry h), and Galα1,2ManNGc (**23**, 65%; entry l), respectively. Similarly, glycosylation of thioglucoside donor **5** and acceptor **10** followed by deprotection afforded the disaccharide Glcα1,6Man (**21**) in 59% yield (Table 1, entry j).

All of the disaccharides obtained by chemical glycosylation were tested as potential substrates for the recombinant *E. coli* sialic acid aldolase. As shown in Table 1, except for (1→2)-linked disaccharides Galα1,2Man (**12**) and Galβ1,2-Man (**13**; Table 1, entries a and b) which were not acceptable to the aldolase, all of the disaccharides tested (Table 1, entries c–m) could be used as substrates for the sialic acid aldolase for the formation of disaccharides containing a sialic acid residue at the reducing end.

The yield of the aldolase-catalyzed disaccharide synthesis was closely related to the position of the glycosidic linkage in the disaccharide substrates (or the position of the monosaccharide substituent on the reducing mannose). In general, disaccharides with a monosaccharide linked to the C5 (Galα1,5Man (**17**) and Galβ1,5Man (**18**); Table 1, entries f and g) or C6 (Galα1,6Man (**19**), Manα1,6Man (**20**), Glcα1,6-Man (**21**), and Glcβ1,6Man (**22**); Table 1, entries h–k) hydroxy group of the mannose were good substrates for the aldolase. The corresponding KDN-containing disaccharides could be obtained with synthetic yields of 62–85%. Disaccharides with a monosaccharide linked to the C4 hydroxy group of the mannose (Galα1,4Man (**14**), Galβ1,4Man (**15**), and Glcβ1,4Man (**16**); Table 1, entries c–e) were acceptable substrates for the aldolase, with yields varying from 5–38%. Disaccharides containing a galactose residue directly linked to the C2 hydroxy group of the mannose (Galα1,2Man (**12**) and Galβ1,2Man (**13**); Table 1, entries a and b) were not acceptable substrates for the aldolase.

The length of the linkage between the two monosaccharides in the disaccharides could also be important to the aldolase activity. For example, although disaccharides with a galactose directly linked to the C2 position on the mannose were not aldolase substrates, disaccharides containing a galactose residue linked through an elongated *N*-glycolyl linkage to the C2 position of ManNGc (Galα1,2ManNGc (**23**) and Galβ1,2ManNGc (**24**); Table 1, entries l and m) were acceptable to the aldolase for the synthesis of the corresponding disaccharides Galα1,5Neu5Gc (**36**) and Galβ1,5-Neu5Gc (**37**) in 36 and 34% yields, respectively.

The aldolase activity was affected moderately by the α- or β-glycosidic linkage in the disaccharides. Overall, β-linked disaccharides were preferred by the aldolase. For example,

only about 5% of Galα1,4Man (**14**) could be processed by the aldolase for the formation of Galα1,7KDN (**27**; Table 1, entry c), while about 38% of the corresponding disaccharide with a β-linkage, Galβ1,4Man (**15**), was converted into the product Galβ1,7KDN (**28**) by the aldolase (Table 1, entry d). Similarly, a lower yield (62%) was observed for the aldolase-catalyzed formation of Galα1,8KDN (**30**) from Galα1,5Man (**17**; Table 1, entry f) compared to that (85%) for the formation of Galβ1,8KDN (**31**) from Galβ1,5Man (**18**; Table 1, entry 9). Another example is that the yield for the formation of Glcα1,9KDN (**34**) from Glcα1,6Man (**21**) was 65% (Table 1, entry j), while the yield for the formation of the corresponding β-linked disaccharide, Glcβ1,9KDN (**35**), from Glcβ1,6Man (**22**) was higher (83%; Table 1, entry k).

No significant effect on the aldolase activity was observed for disaccharides containing different monosaccharides at the nonreducing terminus. For example, the yields for the aldolase-catalyzed synthesis of Galβ1,7KDN (**28**, 38%; Table 1, entry d) and Glcβ1,7KDN (**29**, 35%; Table 1, entry e) were almost identical. Similarly, the yields for the aldolase-catalyzed synthesis of Galα1,9KDN (**32**, 81%; Table 1, entry h), Manα1,9KDN (**33**, 78%; Table 1, entry i), and Glcα1,9KDN (**34**, 65%; Table 1, entry j) were close.

Except for the formation of Galα1,7KDN (**27**) from Galα1,4Man (**14**) (Table 1, entry c) for which the yield was too low to obtain purified product, all of the other disaccharides, **15**–**24** (Table 1, entries d–m), were used with an excess amount of sodium pyruvate for the aldolase-catalyzed preparative-scale synthesis of disaccharides **28**–**37** containing KDN or Neu5Gc at the reducing terminus. These disaccharide products have been purified and characterized by NMR spectroscopy and high-resolution mass spectrometry (HRMS).

In conclusion, *E. coli* sialic acid aldolase has been found to be unusually flexible in accepting disaccharides containing a mannose or ManNGc at the reducing end as substrates for the synthesis of disaccharides containing a KDN or Neu5Gc at the reducing end. The position of the glycosidic linkage in the disaccharide substrates is critical for the activity of the sialic acid aldolase, but the effect of monosaccharide structure at the nonreducing end is insignificant. This chemoenzymatic approach is general and efficient for the synthesis of precursors for carbohydrates containing nonterminal sialic acid residues, which have been found as surface polysaccharides in some pathogenic bacteria.

Experimental Section

General procedures for aldolase-catalyzed reaction: A disaccharide (50–100 mg, 1 equiv) and sodium pyruvate (5 equiv) were dissolved in H_2O (5 mL). Tris(hydroxymethyl)aminomethane (Tris)/HCl buffer (1 mL, 1M, pH 7.5) was added, followed by addition of the *E. coli* aldolase (5 mg). The reaction solution was brought to 10 mL in volume by adding H_2O. The reaction mixture was incubated at 37 °C with agitation at 140 rpm. The reaction was monitored by thin-layer chromatographic analysis with developing solvent (EtOAc:MeOH:H_2O:HOAc 5:3:2:0.1, by volume) and stained with *p*-anisaldehyde sugar stain. When no additional products were observed (the general reaction time was 24 h), an equal volume (10 mL) of 95% EtOH was added to the reaction mixture. The precipitates were separated by centrifugation and discarded. The supernatant was concentrated by rotary evaporation and the product was purified on a Bio-Gel P-2 gel filtration column.

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