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## Sialidase-catalyzed one-pot multienzyme (OPME) synthesis of sialidase transition-state analogue inhibitors

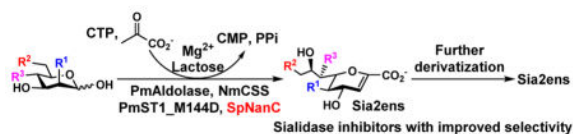
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### Abstract

Sialidase transition state analog inhibitor 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (Neu5Ac2en, DANA) has played a leading role in developing clinically used anti-influenza virus drugs. Taking advantage of the Neu5Ac2en-forming catalytic property of *Streptococcus pneumoniae* sialidase SpNanC, an effective one-pot multienzyme (OPME) strategy has been developed to directly access Neu5Ac2en and its C-5, C-9, and C-7-analogs from *N*-acetylmannosamine (ManNAc) and analogs. The obtained Neu5Ac2en analogs can be further derivatized at various positions to generate a larger inhibitor library. Inhibition studies demonstrated improved selectivity of several C-5- or C-9-modified Neu5Ac2en derivatives against several bacterial sialidases. The study provides an efficient enzymatic method to access sialidase inhibitors with improved selectivity.

### Graphical Abstract



### Keywords

biocatalysis; enzymatic synthesis; Neu5Ac2en; sialidase; sialidase inhibitor

Sialic acids are common terminal monosaccharides on the carbohydrate moieties of mammalian cell surface glycoconjugates and play important biological roles. Sialidases or neuraminidases, the exoglycosidases that catalyze the cleavage of the terminal sialic acids, are widely spread in vertebrates and microbes residing in or infecting vertebrates.<sup>1–6</sup> Viral

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#### Notes

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Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.xxxx

Cloning, expression, purification, and characterization of SpNanC; detailed synthetic procedures, nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (HRMS) data, and NMR spectra for products (PDF)

and bacterial sialidases are attractive targets for designing inhibitors as potent antimicrobial therapeutics.<sup>7–11</sup> The sialidase transition state analog, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (Neu5Ac2en or DANA), has been used as an important probe for structural and mechanistic studies of sialidases.<sup>12–19</sup> It was also a lead compound for rational design of clinical anti-influenza virus drugs Relenza (Zanamivir or 4-deoxy-4-guanidino-Neu5Ac2en)<sup>20–22</sup> and Tamiflu (Oseltamivir).<sup>23–24</sup> Recently, designing and synthesizing 2,3-dehydro-2-deoxy-sialic acids (Sia2ens) and their derivatives have been actively pursued for developing anti-viral and anti-bacterial therapeutics as well as tools for functional studies of human sialidase isoforms.<sup>25–27</sup> Except for a few examples of *de novo* synthesis of Zanamivir aiming at reducing the cost,<sup>28–29</sup> chemical modification of selectively protected sialic acids has been a common strategy to obtain Sia2ens.<sup>30–34</sup> For synthesizing Neu5Ac2en analogs, derivatization can be introduced before<sup>34</sup> or after<sup>33, 35</sup> the formation of the C2–C3 double bond. These methods require multiple protection and deprotection steps. In comparison, biocatalysis has advantages of high efficiency, environmental friendliness, and excellent regio- and stereo-selectivity without protecting group manipulation and has been increasingly used for synthesizing carbohydrates and derivatives. In this work, we aim to develop an effective enzymatic method that allows the formation of Sia2ens directly from the corresponding sialic acids or six-carbon precursors in one pot.

*Streptococcus pneumoniae* sialidase SpNanC, one of the three sialidases (SpNanA, SpNanB, and SpNanC) identified from hundreds of *Streptococcus pneumoniae* strains,<sup>36</sup> was chosen for synthesizing Sia2en and analogs. SpNanC specifically recognizes  $\alpha$ 2–3-linked sialosides and catalyzes the formation of the general sialic acid transition state analog inhibitor Neu5Ac2en, which can be further hydrated by SpNanC to form *N*-acetylneuraminic acid (Neu5Ac).<sup>37–38</sup> Combining the Neu5Ac2en-forming property of SpNanC and the highly efficient one-pot multienzyme (OPME) sialoside formation strategy<sup>39</sup> that we developed previously, a new OPME system has been developed that can be used to produce a diverse array of Sia2en derivatives directly from sialic acid derivatives or their six-carbon precursors.

As shown in Scheme 1, four enzymes are used in the OPME system to produce Sia2ens from the corresponding six-carbon precursors of sialic acids. Sialic acid aldolase from *Pasteurella multocida* (PmAldolase)<sup>40</sup> catalyzes the formation of sialic acid from its six-carbon monosaccharide precursor and pyruvate. CMP-sialic acid synthetase from *Neisseria meningitidis* (NmCSS)<sup>41</sup> catalyzes the conversion of cytidine 5'-triphosphate (CTP) and sialic acid for the formation CMP-sialic acid, which is used together with an acceptor such as lactose by *Pasteurella multocida* sialyltransferase 1 M144D mutant (PmST1\_M144D)<sup>42–43</sup> for the synthesis of the corresponding  $\alpha$ 2–3-linked sialosides, providing the substrates for SpNanC for the production of target Sia2ens.

To obtain SpNanC as a key biocatalyst for the OPME synthesis of Sia2ens, full length SpNanC from *Streptococcus pneumoniae* TIGR4 was cloned in pMAL-c4X vector and expressed as an N-terminal maltose-binding protein (MBP)-fused and C-terminal His<sub>6</sub>-tagged recombinant enzyme in *Escherichia coli* BL21(DE3) cells. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that Ni<sup>2+</sup>-affinity column-

purified MBP-SpNanC-His<sub>6</sub> was presented as two bands with a major band at about 80 kDa corresponding to the protein without the MBP-tag and a faint band at around 120 kDa corresponding to the expected size of the full-length fusion protein. N-Terminal protein sequencing of the major 80 kDa-band indicated that the MBP-tag and the first twenty-six amino acids (26 aa) of SpNanC were cleaved during the expression and purification. As the resulting protein retained activity, the N-terminal 26 aa-truncated protein was re-cloned in pET22b(+) vector as a C-His<sub>6</sub>-tagged recombinant enzyme (26SpNanC-His<sub>6</sub>). Approximately 108 mg of Ni<sup>2+</sup>-column purified 26SpNanC-His<sub>6</sub> (abbreviated as SpNanC, Figure S1) could be routinely obtained from one liter *Escherichia coli* culture.

SpNanC was active in a pH range of 5.5–9.0 with an optimal activity at pH 6.5 (Figure S2). It did not require a metal ion for activity and the addition of a reducing reagent dithiothreitol (DTT) did not affect its activity (Figure S3). Kinetics studies using Neu5Ac $\alpha$ 2–3Gal $\beta$ pNP as the substrate indicated that SpNanC was a highly active sialidase ( $k_{cat}/K_M = 150 \text{ s}^{-1} \text{ mM}^{-1}$  with  $K_M = 2.7 \pm 0.5 \text{ mM}$  and  $k_{cat} = 406.8 \pm 39.0 \text{ s}^{-1}$ ) compared to a previously reported recombinant SpNanC using  $\alpha$ 2–3-sialyllactose as the substrate ( $k_{cat}/K_M = 0.29 \text{ s}^{-1} \text{ mM}^{-1}$  with  $K_M = 1.08 \pm 0.35 \text{ mM}$ ,  $k_{cat} = 0.313 \pm 0.052 \text{ s}^{-1}$ ).<sup>37</sup>

Substrate specificity studies of SpNanC using a library of *para*-nitrophenol (pNP)-tagged  $\alpha$ 2–3-linked sialosides<sup>44–47</sup> indicated that the enzyme could tolerate various modifications at C-5, C-9, and C-7 of Neu5Ac (Table 1). Different from what was described in a previous report,<sup>48</sup>  $\alpha$ 2–3-linked sialosides containing *N*-glycolylneuraminic acid (Neu5Gc), a non-human sialic acid form,<sup>49</sup> or its C-9 derivatives were also suitable substrates for the enzyme.  $\alpha$ 2–3-Linked sialosides containing 2-keto-3-deoxynonulsonic acid (Kdn) or its derivatives with modifications at C-5 or C-9 were not effective substrates of SpNanC. Among compounds tested, two of the sialosides containing C7-modified Kdn (Kdn7OMe $\alpha$ 2–3Gal $\beta$ pNP and Kdn7deoxy $\alpha$ 2–3Gal $\beta$ pNP) could be cleaved off by SpNanC with low yields (23% and 10% respectively in reactions using 3  $\mu$ g of SpNanC).

The substrate promiscuities of SpNanC (Table 1) and the enzymes involved in the one-pot multienzyme (OPME) synthesis of sialosides<sup>40–43</sup> that we reported previously<sup>39</sup> provide an opportunity for synthesizing Sia2ens directly from the six-carbon precursors of sialic acids. As SpNanC has a dual function of forming Neu5Ac2en and further hydrating it to form Neu5Ac,<sup>37–38</sup> the amount of SpNanC used in the one-pot four-enzyme reaction needs to be controlled. Small-scale reactions using ManNAc (1) as the starting material followed by thin layer chromatography (TLC) and mass spectroscopy (MS) analyses suggested that a concentration of SpNanC in the range of 0.004–0.04 mg/mL was optimal for reactions using 10 mM of ManNAc. Due to its low cost, commercially available lactose was chosen as the sialyltransferase acceptor. As shown in Scheme 1, lactose was regenerated in the SpNanC-catalyzed Sia2en-production reaction and was needed only for a catalytic amount. An optimal concentration of lactose was found to be 0.25 equivalent (2.5 mM) of ManNAc where it provided a good balance of reaction rate and the consumption of reagents. A Tris-HCl buffer with pH 7.5 was chosen for the Sia2en-production OPME reaction to balance the activities of enzymes involved including PmAldolase (good activity in pH 6.0–9.0),<sup>40</sup> NmCSS (good activity in pH 7.0–10.0),<sup>50</sup> PmST1\_M144D (good  $\alpha$ 2–3-sialyltransferase activity in pH 6.0–10.0 similar to PmST1<sup>42</sup>), and SpNanC (good activity in pH 6.0–7.5)

(Figure S2). Under the optimal conditions determined by small-scale reactions, preparative-scale reactions were carried out and the amounts of the purified products were used to calculate the synthetic yields. Neu5Ac2en (**6**) was synthesized from ManNAc (**1**) in 72% yield (Table 2). Similar reaction conditions were used to synthesize 2,3-dehydro-2-deoxy-*N*-glycolylneuraminic acid (Neu5Gc2en, **7**)<sup>34</sup> from Neu5Gc precursor, *N*-glycolylmannosamine (ManNGc, **2**).<sup>41</sup> To our surprise, only a trace amount of the desired product was obtained. The slower formation of Neu5Gc-containing sialosides was identified to be the cause for the low yield. To improve the yield, an alternative two-step process was developed. In this case, a one-pot three-enzyme sialylation reaction<sup>39</sup> was carried out for overnight using equal molar amounts of lactose and ManNGc before the addition of SpNanC. Using this two-step process, the yield for Neu5Gc2en (**7**) was improved to 60% (Table 2). A similar two-step process applied for Neu5Ac2en (**6**) production led to the improvement of the yield to 82% from 72% using the one-step process.

Other than Neu5Gc2en which is a C-5 derivative of Neu5Ac2en, two C-9 derivatives of Neu5Ac2en were successfully synthesized. Neu5Ac9N<sub>3</sub>2en (**8**)<sup>33, 35</sup> containing a 9-azido group was synthesized from ManNAc6N<sub>3</sub> (**3**)<sup>42</sup> with a yield of 61% using the one-step process. The two-step process improved the yield to 79%. On the other hand, Neu5Ac9NAc2en (**9**)<sup>33</sup> containing a 9-acetamido group was synthesized from ManNAc6NAc (**4**)<sup>51</sup> in a yield of 63% using the one-step process and a yield of 71% was obtained using the two-step process. Neu5Ac9NAc2en (**9**) is a more stable analog of Neu5Ac9OAc2en<sup>51</sup> and is a promising inhibitor against sialidases that prefer to cleave 9-*O*-acetyl Neu5Ac.

The OPME system was also used for synthesizing Neu5Ac7deoxy2en (**10**), a C-7 derivative of Neu5Ac2en.<sup>52-53</sup> From ManNAc4deoxy (**5**),<sup>47</sup> Neu5Ac7deoxy2en (**10**) was obtained in a low 11% yield using the one-step process and the two-step process improved the yield to 25%. The lower yield was due to the lower efficiency in sialoside formation.

In general, the one-pot process was time efficient and the reactions were usually completed in 2–6 h. In comparison, the two-step process required a longer 12–20 h duration but usually led to improved yields, especially for sialic acid modifications causing the slowing down of the sialoside formation process. The enzymatic approach compared favorably to the traditional chemical methods which used organic solvents and involved multiple protection and deprotection steps. For example, chemical synthesis of Neu5Ac9NAc2en (**9**) from Neu5Ac involved nine steps and multiple purification processes with an overall yield of 9–14%.<sup>33</sup> For the chemoenzymatic approach described here, ManNAc6NAc (**4**) was chemically synthesized from ManNAc via ManNAc6N<sub>3</sub> (**3**)<sup>42</sup> intermediate in three steps with a 48% overall yield.<sup>51</sup> Considering the 63% or 71% yield for the one-step or two-step OPME synthetic process (Table 2), the overall yield for Neu5Ac9NAc2en (**9**) was 30% or 34% starting from ManNAc without the need of purifying the intermediates of enzymatic reactions.

The C9-azido group of Neu5Ac9N<sub>3</sub>2en (**8**) allowed easy derivatization via Neu5Ac9NH<sub>2</sub>2en (**11**) which was obtained by reduction of the azido group to an amino group using 1,3-propanedithiol (Scheme 2). Installation of various acyl groups was achieved conveniently to

form Neu5Ac9NPent2en (**12**)<sup>33</sup> with an improved selectivity against human NEU1<sup>33</sup> and Neu5Ac9NCyclopro2en (**13**)<sup>33</sup> with a higher selectivity against viral sialidases.<sup>54</sup> The C-5 position of Neu5Ac2en (**6**) was also easily derivatized by removal of the *N*-acetyl group using an aqueous sodium hydroxide solution (2 M) to produce Neu2en (**14**) with a free amino group at C-5 which allowed facile acylation (Scheme 3). Neu5Gc2en (**7**) and Neu5PhAc2en (**15**)<sup>55</sup> were conveniently obtained by this approach.

The obtained Sia2ens except for Neu5Ac9N<sub>3</sub>2en (**8**)<sup>35</sup> were tested as inhibitors against several glycoside hydrolase GH33 family sialidases and a glycosyltransferase GT80 sialyltransferase with  $\alpha$ 2–3-sialidase activity categorized in the Carbohydrate Active Enzyme (CAZy) database ([www.cazy.org](http://www.cazy.org)).<sup>56–58</sup> These include human cytosolic sialidase hNEU2<sup>34</sup> and several bacterial sialidases such as recombinant sialidases cloned from *Streptococcus pneumoniae* (SpNanA,<sup>59</sup> SpNanB,<sup>59</sup> and SpNanC<sup>60</sup>), *Pasteurella multocida* (PmST1, a bacterial sialyltransferase which also has  $\alpha$ 2–3-sialidase activity),<sup>42</sup> *Bifidobacterium infantis* (BiNanH2),<sup>61</sup> as well as commercially available sialidases from *Arthrobacter ureafaciens* (AuSialidase), *Clostridium perfringens* (CpNanH and CpNanI), and *Vibrio cholerae* (VcSialidase). Inhibition studies using Neu5Ac $\alpha$ 2–3Gal $\beta$ pNP as the sialidase substrate<sup>44, 62–63</sup> and 0.1 mM of each inhibitor showed (Table S1) that Neu5Ac2en (**6**) and its derivatives at C-5 (**7**, **15**), C-9 (**9**, **12**, **13**), and C-7 (**10**) were not effective inhibitors against SpNanB, SpNanC, CpNanH,<sup>64–65</sup> or PmST1. On the other hand, among all compounds tested, Neu5Ac2en (**6**) was the least selective one. At 0.1 mM, it had more than 50% inhibition against SpNanA, AuSialidase, CpNanI, VcSialidase, BiNanH2, and hNEU2. C9-Derivatization of Neu5Ac2en (compounds **9**, **12**, **13**) lowered the inhibitory activity against AuSialidase, VcSialidase, and hNEU2. In comparison, C5-derivatization of Neu5Ac2en also improved the inhibitory selectivity. For example, at 0.1 mM, Neu5Gc2en (**7**) retained more than 50% inhibition against only VcSialidase and hNEU2, similar to that reported previously.<sup>34</sup> Neu5PhAc2en (**15**) retained inhibitory activity against hNEU2 only, but not any bacterial sialidases tested. The previously identified selectivity of bacterial sialidases toward C7-deoxy Neu5Ac based on substrate specificity studies<sup>47</sup> was confirmed by the selective inhibitory activity of Neu5Ac7deoxy2en (**10**) against bacterial sialidases including SpNanA, AuSialidase, and CpNanI, but not hNEU2. Nevertheless, it was not an effective inhibitor against VcSialidase or BiNanH2.

For inhibitors with more than 65% inhibitory activity against certain sialidases at 0.1 mM concentration, IC<sub>50</sub> values were obtained. As shown in Table 3, derivatization at C-9 (compounds **9**, **12**, **13**) did not significantly alter the inhibitory activity of Neu5Ac2en (**6**) against SpNanA or BiNanH2 and the IC<sub>50</sub> values retained in the ranges of 6.8–11.0  $\mu$ M and 29–40  $\mu$ M, respectively. The C7-deoxy modification in Neu5Ac7deoxy2en (**10**) did not alter the inhibition activity against SpNanA, but decreased inhibitory activity against AuSialidase resulting in an IC<sub>50</sub> value of 17  $\mu$ M (increased from 8.1  $\mu$ M).

In conclusion, using the special property of SpNanC-catalyzed reaction, a new one-pot multienzyme strategy has been successfully developed for the synthesis of sialidase transition state analog inhibitors, 2,3-dehydro-2-deoxy-sialic acids (Sia2ens), and analogs. Such compounds can be further derivatized at various positions. Inhibition studies

demonstrated improved selectivity of several Neu5Ac2en (**6**) derivatives with modifications at C-5, C-9, or C-7 against several bacterial sialidases.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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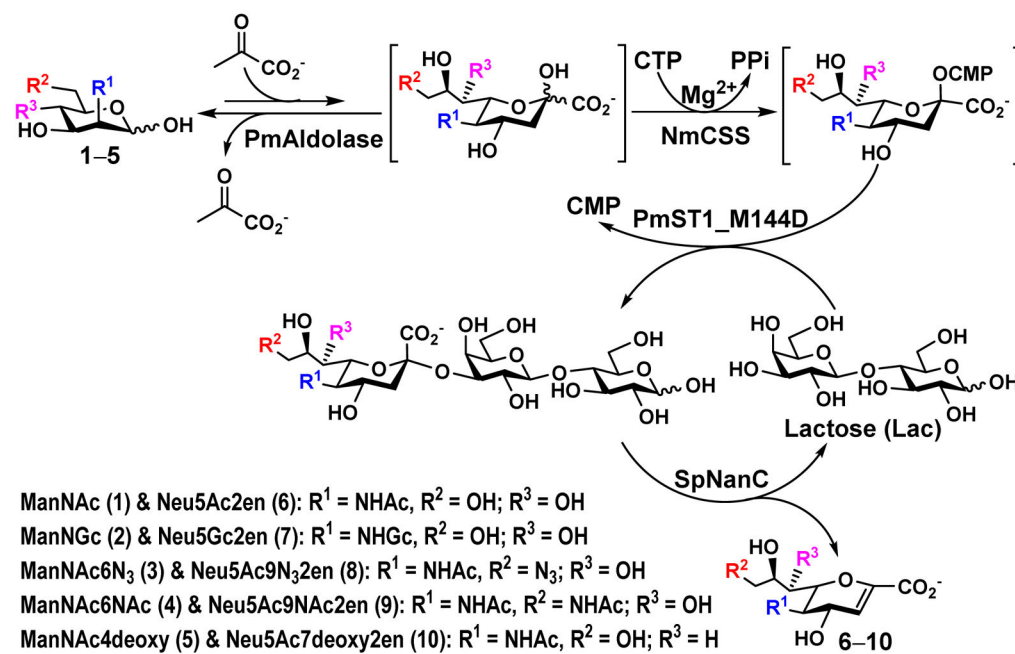
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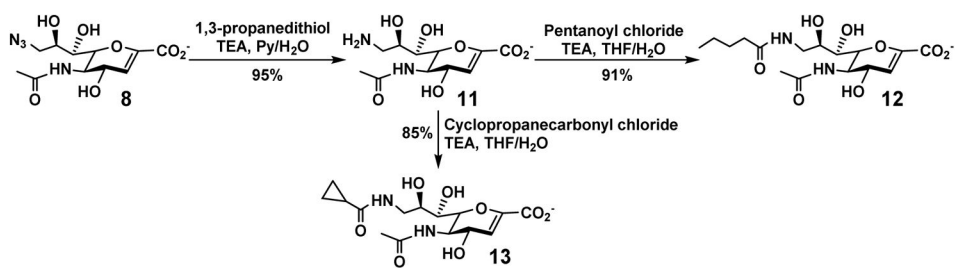
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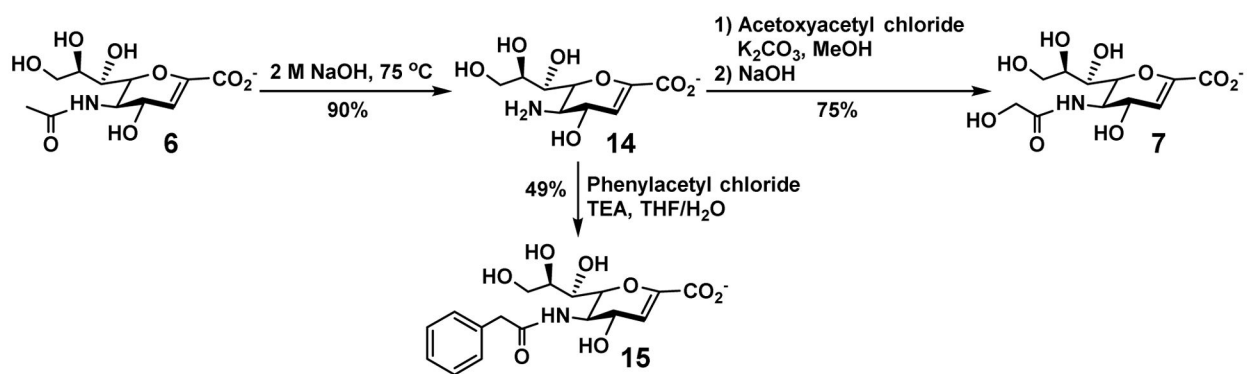




**Scheme 1.**  
One-pot Multienzyme (OPME) Synthesis of Sia2ens



**Scheme 2.**  
Derivatization at C-9 of Neu5Ac9N<sub>3</sub>2en (8)



**Scheme 3.**  
Derivatization at C-5 of Neu5Ac2en (6)

Table 1

## Substrate Specificity of SpNanC

| Substrate                                 | Percentage conversion (%) |
|---|---------------------------|
| Neu5Ac $\alpha$ 2-3Gal $\beta$ pNP        | 85.1 $\pm$ 2.4            |
| Neu5AcFa2-3Gal $\beta$ pNP                | 74.8 $\pm$ 3.2            |
| Neu5AcOMe $\alpha$ 2-3Gal $\beta$ pNP     | 81.8 $\pm$ 1.7            |
| Neu5AcN $_3$ $\alpha$ 2-3Gal $\beta$ pNP  | 87.9 $\pm$ 1.6            |
| Neu5Ac9Fa2-3Gal $\beta$ pNP               | 75.6 $\pm$ 3.9            |
| Neu5Ac9OMe $\alpha$ 2-3Gal $\beta$ pNP    | 88.5 $\pm$ 5.1            |
| Neu5Ac9deoxy $\alpha$ 2-3Gal $\beta$ pNP  | 93.5 $\pm$ 3.1            |
| Neu5Ac7Fa2-3Gal $\beta$ pNP               | 91.0 $\pm$ 4.3            |
| Neu5Ac7OMe $\alpha$ 2-3Gal $\beta$ pNP    | 96.5 $\pm$ 1.7            |
| Neu5Ac7deoxy $\alpha$ 2-3Gal $\beta$ pNP  | 91.2 $\pm$ 1.8            |
| Neu5Ac7N $_3$ $\alpha$ 2-3Gal $\beta$ pNP | 65.9 $\pm$ 1.1            |
| Neu5Gc $\alpha$ 2-3Gal $\beta$ pNP        | 80.6 $\pm$ 6.7            |
| Neu5Gc9Fa2-3Gal $\beta$ pNP               | 72.3 $\pm$ 2.6            |
| Neu5Gc9OMe $\alpha$ 2-3Gal $\beta$ pNP    | 75.0 $\pm$ 1.9            |
| Neu5Gc9deoxy $\alpha$ 2-3Gal $\beta$ pNP  | 85.5 $\pm$ 1.4            |
| Neu5Gc9N $_3$ $\alpha$ 2-3Gal $\beta$ pNP | 89.5 $\pm$ 3.5            |
| Kdn $\alpha$ 2-3Gal $\beta$ pNP           | 4.50 $\pm$ 0.45           |
| Kdn5Fa2-3Gal $\beta$ pNP                  | 0.48 $\pm$ 0.00           |
| Kdn5N $_3$ $\alpha$ 2-3Gal $\beta$ pNP    | 0.32 $\pm$ 0.45           |
| Kdn5deoxy $\alpha$ 2-3Gal $\beta$ pNP     | 5.31 $\pm$ 0.23           |
| Kdn5OMe $\alpha$ 2-3Gal $\beta$ pNP       | 1.53 $\pm$ 1.02           |
| Kdn9Fa2-3Gal $\beta$ pNP                  | 3.70 $\pm$ 1.14           |
| Kdn9OMe $\alpha$ 2-3Gal $\beta$ pNP       | 4.02 $\pm$ 1.36           |
| Kdn7Fa2-3Gal $\beta$ pNP                  | 0.56 $\pm$ 0.11           |
| Kdn7OMe $\alpha$ 2-3Gal $\beta$ pNP       | 23.4 $\pm$ 3.3            |
| Kdn7deoxy $\alpha$ 2-3Gal $\beta$ pNP     | 10.1 $\pm$ 0.0            |
| Kdn7N $_3$ $\alpha$ 2-3Gal $\beta$ pNP    | 4.50 $\pm$ 0.45           |

Table 2

## Sia2ens Obtained by OPME Synthesis

| Substrate                     | Product                           | Yield <sup>a</sup> | Yield <sup>b</sup> |
|-------------------------------|-----------------------------------|--------------------|--------------------|
| ManNAc (1)<br>                | Neu5Ac2en (6)<br>                 | 72%                | 82%                |
| ManNGc (2)<br>                | Neu5Gc2en (7)<br>                 | <5% <sup>c</sup>   | 60%                |
| ManNAc6N <sub>3</sub> (3)<br> | Neu5Ac9N <sub>3</sub> 2en (8)<br> | 61%                | 79%                |
| ManNAc6NAc (4)<br>            | Neu5Ac9NAc2en (9)<br>             | 63%                | 71%                |
| ManNAc4deoxy (5)<br>          | Neu5Ac7deoxy2en (10)<br>          | 11%                | 25%                |

<sup>a</sup>One-step process.<sup>b</sup>Two-step process.<sup>c</sup>Estimated by TLC analysis.

**Table 3**  $IC_{50}$  Values of Sia2ens Against Bacterial Sialidases SpNanA, AuSialidase, and BiNanH2

| Sialidases  | $IC_{50}$ values of different inhibitors ( $\mu$ M) |                |                |                |               |
|-------------|---|----------------|----------------|----------------|---------------|
|             | 6   | 9              | 10             | 12             | 13            |
| SpNanA      | 10.0 $\pm$ 0.2                                      | 11.0 $\pm$ 0.2 | 10.7 $\pm$ 0.4 | 10.5 $\pm$ 0.5 | 6.8 $\pm$ 0.2 |
| AuSialidase | 8.1 $\pm$ 0.3                                       | -              | 17 $\pm$ 1     | -              | -             |
| BiNanH2     | 40 $\pm$ 2  | 33 $\pm$ 1     | -              | 34 $\pm$ 2     | 29 $\pm$ 2    |