

Efficient and Stereoselective Synthesis of $\alpha(2\rightarrow9)$ Oligosialic Acids: From Monomers to Dodecamers**

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N-acetyl neuraminic acid (Neu5Ac) is often present at the terminal end of glycoproteins or glycolipids.^[1] The linear homopolymers formed by Neu5Ac are called polysialic acids, three of which have been identified in nature (Figure 1). The

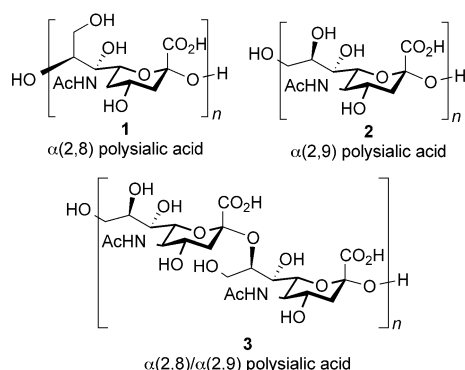


Figure 1. Structures of polysialic acids.

most common $\alpha(2\rightarrow8)$ polysialic acid (**1**)^[2] is found in mammalian tissues and bacteria (*Neisseria meningitidis* B, *Escherichia coli* K1, *Moraxella nonliquefaciens*, and *Mannheimia haemolytica* A2),^[2–4] and the less common $\alpha(2\rightarrow9)$ polysialic acid (**2**) and alternating $\alpha(2\rightarrow8)/\alpha(2\rightarrow9)$ polysialic acids (**3**) were discovered to form extracellular capsules of *N.*

meningitidis C and *E. coli* K92, respectively.^[3–5] Human pathogens encapsulated with polysialic acids cause invasive diseases such as meningitis and urinary tract infections.^[6] In pathogenic bacteria, these acidic polysaccharides serve as extracellular shields against the defense systems of their mammalian host. Therefore, polysialic acids are considered good targets for the development of bactericidal agents and antibacterial vaccines.^[7] For example, the current vaccines against meningococcal group C diseases are glycoconjugates of isolated $\alpha(2\rightarrow9)$ polysialic acids and a carrier protein such as diphtheria or tetanus toxoid.^[8] However, these kinds of vaccines are often heterogeneous or contaminated with other antigenic components because of the difficulty of purifying polysialic acids from natural sources.^[8b,9] An effective method to synthesize pure polysialic acids having a well-defined structure will not only simplify the complexities of vaccines but also provide a better understanding of the structure–activity relationships of polysialic acids in various biological events.^[10]

Chemical sialylation is complicated as a result of the intrinsic structural features of sialic acid, thus resulting in poor yields or stereoselectivities. Even though notable progress toward the development of sialic acid donors for efficient α sialylation have been reported in the last decade,^[11,12] the synthesis of poly/oligo sialic acid with satisfactory yields and excellent α selectivity is still very challenging.

The advancement of donor development led to many approaches for the synthesis of α -specific oligosialic acids, including the synthesis of $\alpha(2\rightarrow9)$ trisialic acid using C5-azido sialyl phosphite as donor,^[12a] the synthesis of $\alpha(2\rightarrow9)$ oligosialic acid using C5-TFA sialyl phosphite as a donor and C5-TFA thiosialoside as an acceptor,^[12b] and the synthesis of $\alpha(2\rightarrow8)$ tetrasialoside,^[12d] $\alpha(2\rightarrow9)$ trisialoside,^[12j] and $\alpha(2\rightarrow9)$ tetrasialoside^[13] using 5*N*,4*O*-carbonyl-protected thiosialosides as donors, the sequence of assembly starts from the reducing end to the nonreducing end, thus providing an opportunity to stereoselectively elongate the sugar chain one residue at a time. However, this approach has not successfully been used to synthesize an α -specific oligosialic acid polymer that is longer than a tetramer.

In principle, convergent block synthesis is an intrinsically better strategy for the preparation of oligomers or polymers and has been applied to the synthesis of some carbohydrate polymers.^[14] However, this strategy is hindered by the limited choice of leaving groups to ensure a proper reactivity and

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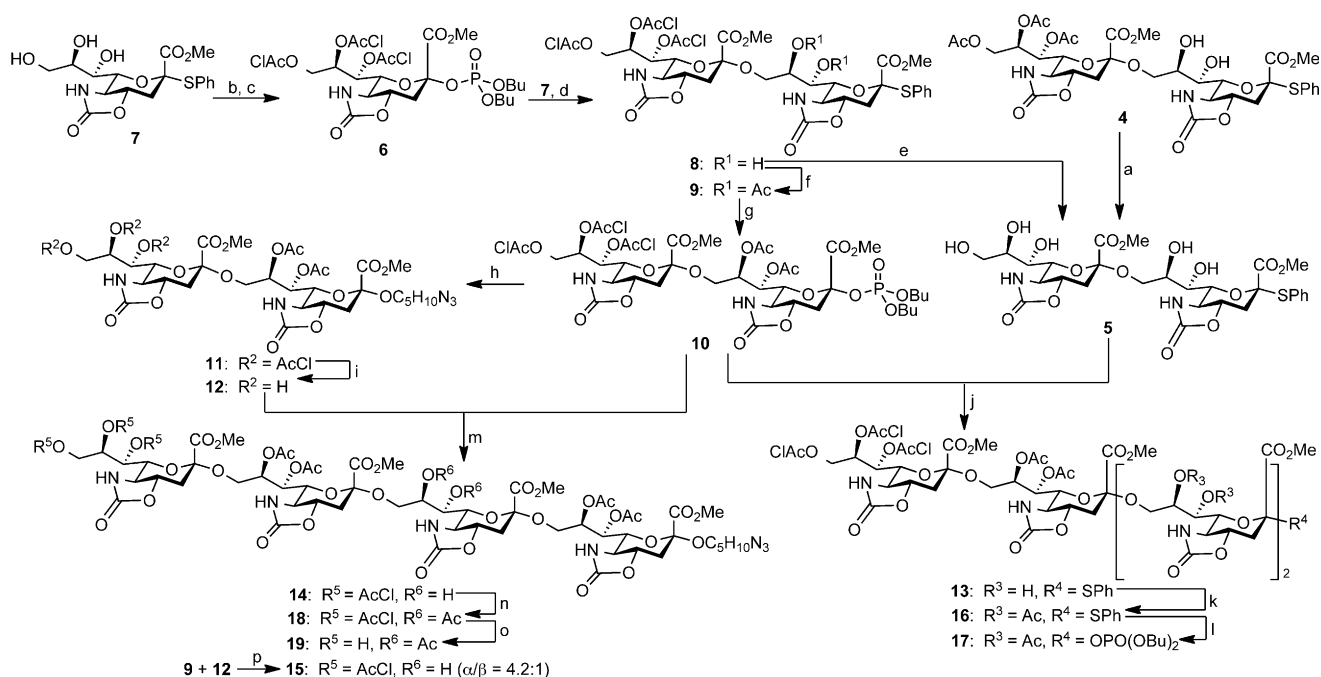
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Scheme 1. Synthesis of the $\alpha(2\rightarrow9)$ tetrasialyl donor **17** and acceptor **19**. Reagents and conditions: a) NaOMe, MeOH, RT, 37%; b) pyridine, ClAcCl, CH₂Cl₂, 0 °C, 90%; c) HOPO(OBu)₂, NIS, TFOH, CH₂Cl₂, 4 °C, 12 h, 96%; d) TMSOTf, CH₂Cl₂/CH₃CN (3:2), -60 °C, 80%; e) thiourea, 2,6-lutidine, DMF, 55 °C, 82%; f) pyridine, Ac₂O, DMAP, CH₂Cl₂, -50 °C to 0 °C, 80%; g) HOPO(OBu)₂, NIS, TFOH, CH₂Cl₂, 4 °C, 2 days, 80%; h) HOC₅H₁₀N₃, TMSOTf, CH₂Cl₂/CH₃CN (3:2), -50 °C, 96%; i) thiourea, 2,6-lutidine, DMF, 80 °C, 78%; j) TMSOTf, CH₂Cl₂/CH₃CN (3:2), -78 °C, 68%; k) pyridine, Ac₂O, DMAP, CH₂Cl₂, -50 °C to 0 °C, 70%; l) HOPO(OBu)₂, NIS, TFOH, CH₂Cl₂, 4 °C, 7 days, 80%; m) TMSOTf, CH₂Cl₂/CH₃CN (3:2), -78 °C, 70%; n) pyridine, Ac₂O, DMAP, CH₂Cl₂, -50 °C to 0 °C, 78%; o) thiourea, 2,6-lutidine, DMF, 80 °C, 45%; p) NIS, TFOH, CH₂Cl₂/CH₃CN (3:2), RT, 32%. DMAP = 4-dimethylaminopyridine; DMF = *N,N'*-dimethylformamide, NIS = *N*-iodosuccinimide, Tf = trifluoromethylsulfonyl, TMS = trimethylsilyl.

selectivity of an oligosaccharide donor. When a di/oligosialic acid unit was used as a glycosylation donor for the synthesis of longer oligosialic acids, it often resulted in poor α selectivity and yield. For example, two recent attempts using the 2+2 strategy to construct $\alpha(2\rightarrow9)$ tetrasialic acids led only to an inseparable mixture with moderate selectivity ($\alpha/\beta = 1.6:1$).^[13,15] Another observation was that the α selectivity decreased significantly when the length of sialic acid donor increased from monomer (α only) to tetramer ($\alpha/\beta = 1:1.3$).^[12b] Last year, we reported a new chemical sialylation approach that successfully constructed an $\alpha(2\rightarrow9)$ tetrasialoside derivative using a combination of 5*N*,4*O*-carbonyl protection and dibutyl phosphate as a reactive leaving group in a convergent 2+2 block synthesis that exclusively gave α selectivity and high yield.^[16] However, the intermediate disialyl pentanol acceptor **5** was obtained in only 37% yield because of the random opening of the 5*N*,4*O*-oxazolidine rings when **4** was exposed to the required strong basic conditions (Scheme 1). This low efficiency in deacetylation prevents further extension of sialic acid chain. Herein, we present an improved convergent block synthesis strategy with increased efficiency in the deacetylation steps to assemble a dodecasialic acid derivative in good yield with all α linkages.

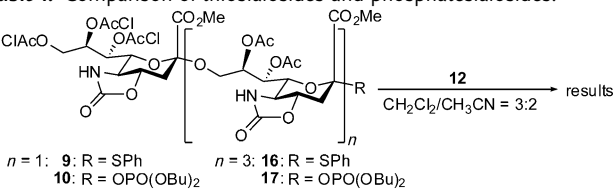
To improve the procedure for the preparation of the disaccharide acceptor **5**, three nonreducing terminal hydroxy groups were protected with a chloroacetyl group, which can be efficiently introduced and removed under milder reaction conditions without influencing the 5*N*,4*O*-oxazolidine rings

of the sialosides.^[12d,17] With this modification, the fully O-chloroacetylated sialyl phosphate product **6** was obtained in 86% yield after two steps from the thioglycoside **7** (Scheme 1).^[16] Notably, this reaction gave only the α -phosphate product **6** which was assigned by the $^3J(C_1-H_{3ax}) = 6.0$ Hz coupling constant.^[18] Glycosylation of the phosphate donor **6** and the triol acceptor **7** in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf) in CH₂Cl₂/CH₃CN (3:2) at -60 °C gave the 9'*O*,8'*O*,7'*O*-trichloroacetyl-protected $\alpha(2\rightarrow9)$ disialoside derivative **8** in 80% yield exclusively with α selectivity. The dechloroacetylation of **8** was carried out in the presence of thiourea and 2,6-lutidine in DMF at 55 °C to obtain **5** in 82% yield. In contrast, the disialyl phosphate donor **10** could be synthesized in 64% yield over two steps: acetylation of the thiosialoside **8** and phosphate formation under the standard reaction conditions. The α -only configurations were confirmed by the $^3J(C_1-H_{3ax})$ coupling constants of **10** (6.1 and 6.2 Hz). To test the reactivity and α selectivity of disialyl phosphate donor **10**, we used 5-azidopentan-1-ol as an acceptor to give the disialoside **11** in 96% yield with α -only configurations ($^3J_{C_1-H_{3ax}} = 5.4$ and 5.4 Hz). Dechloroacetylation of **11** provided triol **12** as an acceptor for further constructions of oligosialic acid.

With these encouraging results, the convergent 2+2 procedures were used in the sialylations of the pentanol **5** and triol **12** with the donor **10** to obtain tetrasialosides **13** and **14** ($^3J_{C_1-H_{3ax}} = 5.3, 4.0, 4.5$, and 5.4 Hz) in 68% and 70%, respectively, and exclusively with α -selectivity. To roughly

compare the influence of the leaving group, the disialyl thioglycoside **9** was also coupled with the disialyl acceptor **12** using NIS/TfOH as a promoter. The donor **9** could not be

Table 1: Comparison of thiosialosides and phosphatesialosides.

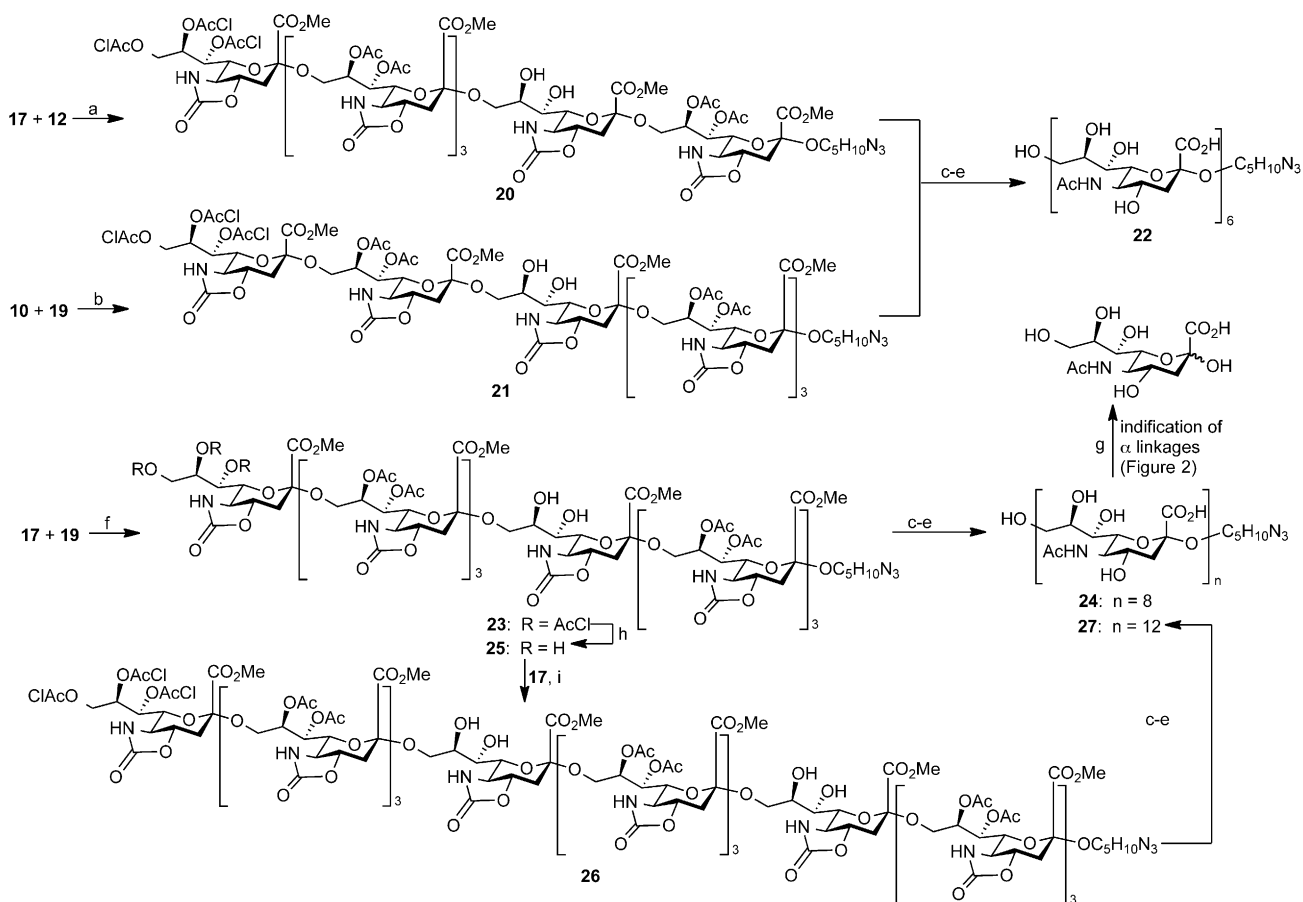


Entry	Donor	Conditions	Yield [%] ^[b]	α/β ^[c]
1	9	NIS/TfOH, RT, ^[a] 8 h	32	4.2:1
2	9	NIS/TfOH, RT, ^[a] 8 h ^[d]	45	4.2:1
3	10	TMSOTf, -78°C , 2 h	70	α only
4	16	NIS/TfOH, RT, ^[a] 16 h	5	1:3.2
5	17	TMSOTf, -78°C , 2 h	59	α only

[a] It was the lowest temperature when the donor could be activated smoothly. [b] The yield was calculated after purification. [c] The ratio was determined using the integral values of the corresponding peak in either the NMR spectrum or the HPLC trace. [d] Used dichloromethane as the solvent.

activated until the reaction temperature was raised to room temperature, and the product was an inseparable mixture of the tetrasialyl derivative **15** ($\alpha/\beta = 4.2:1$) in only 32 % or 45 % yield depending upon the solvent system (Table 1, entries 1 and 2). These results indicate that the phosphate leaving group of **10** in combination with the protecting groups provides an optimal reactivity and α selectivity for the convergent 2+2 glycosylation reaction. To investigate the convergent 4+4 strategy using a similar approach, tetrasialyl phosphate donor **17** ($^3J_{\text{C}_1-\text{H}_{3\text{ax}}} = 5.7 \text{ Hz}$, 5.7 Hz, 6.3 Hz, and 6.0 Hz) was synthesized after acetylation and phosphate formation from **13** in 56 % yield over two steps, and the tetrasialyl triol acceptor **19** was obtained after acetylation and dechloroacetylation from **14** in 35 % yield over two steps.

Prior to the construction of the octamer, the α selectivity of the tetrasialyl phosphate donor **17** was tested by coupling with the disialyl acceptor **12** in the presence of TMSOTf at -78°C in $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{CN}$ (3:2) for 2 hours. The hexamer **20** was obtained as a single stereoisomer in 59 % yield (Scheme 2). However, it was difficult to measure the $^3J(\text{C}_1-\text{H}_{3\text{ax}})$ coupling constants of all C1 carbon atoms on every monomer of the hexamer **20** because of the overlapping peaks in the NMR spectrum. Fortunately, we could ensure the



Scheme 2. Synthesis of the $\alpha(2\rightarrow9)$ hexasialic acid **22**, octasialic acid **24** and dodecasialic acid **27**. Reagents and conditions: a) TMSOTf, $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{CN}$ (3:2), -78°C , 59 %; b) TMSOTf, $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{CN}$ (3:2), -78°C , 55 %; c) LiOH, $\text{H}_2\text{O}/\text{MeOH}$ (1:1), 80°C ; d) Ac_2O , NaHCO_3 , H_2O ; e) NaOMe, MeOH, 40 % from **20** and **21**, 37 % from **23** and 33 % from **26** over three steps; f) TMSOTf, $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{CN}$ (3:2), -78°C , 58 %; g) hydrolysis by neuraminidase; h) thiourea, 2,6-lutidine, DMF, 80°C , 68 %; i) TMSOTf, $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{CN}$ (3:2), -78°C , 45 %.

configuration by a chemical protocol. First, we prepared the hexamer **21** in 55 % yield by the treatment of the anomerically pure disialyl donor **10** and tetrasialyl acceptor **19** under the same glycosylation conditions used to synthesize **20**. Then, after global deprotection and *N*-acetylation of the hexamers **20** and **21**, we confirmed that both strategies gave the same hexasialic acid **22** by comparing the ^1H and ^{13}C NMR spectra. Because the α -only linkages were confirmed for the disaccharides (**10** and **12**) and the tetrasaccharides (**17** and **19**), the anomeric configurations of **20** and **21** could also be confirmed to possess α -only linkages. Thus, the tetrasialyl phosphate **17** is demonstrated to be a useful α -selective donor for glycosylation. On the contrary, the tetrasialyl thioglycoside **16** gave only trace amounts of the hexasaccharide as a mixture of anomers ($\alpha/\beta = 1:3.2$; Table 1, entry 4) after reacting with the acceptor **12** by NIS/TfOH.

With the proper donor in hand, the octasialoside derivative **23** was obtained successfully in 58 % yield by the 4+4 coupling of tetrasialyl phosphate donor **17** and tetrasialyl acceptor **19** under the same glycosylation conditions (Scheme 2). As a result of the same problem of having a complex NMR spectrum, it is difficult to identify the configuration of the octamer **23** by NMR methods. Therefore, we have developed a combined enzymatic hydrolysis and high-performance capillary electrophoresis (HPCE) methods to determine the configuration of the octamer **23**. Octasialic acid **24** was obtained after global deprotection and *N*-acetylation of **23**, and **24** was then hydrolyzed by neuraminidase to release only α -linked sialic acid from the nonreducing terminal. The octamer **24** dissolved in ammonium acetate buffer and was treated with the neuraminidase from *Arthrobacter ureafaciens* at 37 °C for various time intervals. The progression of hydrolysis was monitored by HPCE analysis at each time interval (Figure 2).^[19] We observed that the octamer **24** was eventually degraded completely into its monomers. This stepwise digestive process could clearly confirm the α configurations of the octamer **24**. We also used this method to confirm the α linkages of the hexamer **22** (see the Supporting Information). To prove that the neuraminidase recognizes only α -linked sialic acid, an α/β mixture of tetrasialoside **15** was deprotected, *N*-acetylated, and treated with the neuraminidase. The results showed that a major portion of the tetramer was degraded completely to monomer forms but some tetramer, which was produced from the β coupling product of **9** and **12**, was degraded to the trimer forms (see the Supporting Information).

To our knowledge, this is the first report using a chemical method to create oligosialic acids containing more than five monomers with exclusively α configurations. To demonstrate that this powerful convergent block synthetic strategy can be used to assemble longer $\alpha(2\rightarrow9)$ oligosialic acids, the tetrasialyl phosphate donor **17** and octasialoside acceptor **25** were coupled to successfully obtain $\alpha(2\rightarrow9)$ dodecasialoside **26** in 45 % yield. The α -only configuration of dodecasialoside **26** was also confirmed by the combination of enzymatic hydrolysis and the HPCE method using dodecasialic acid **27** (obtained after deprotection and *N*-acetylation of **26**; see the Supporting Information). In using a palladium catalyst in hydrogenolysis, the terminal azide group of **22**, **24**, or **27** can

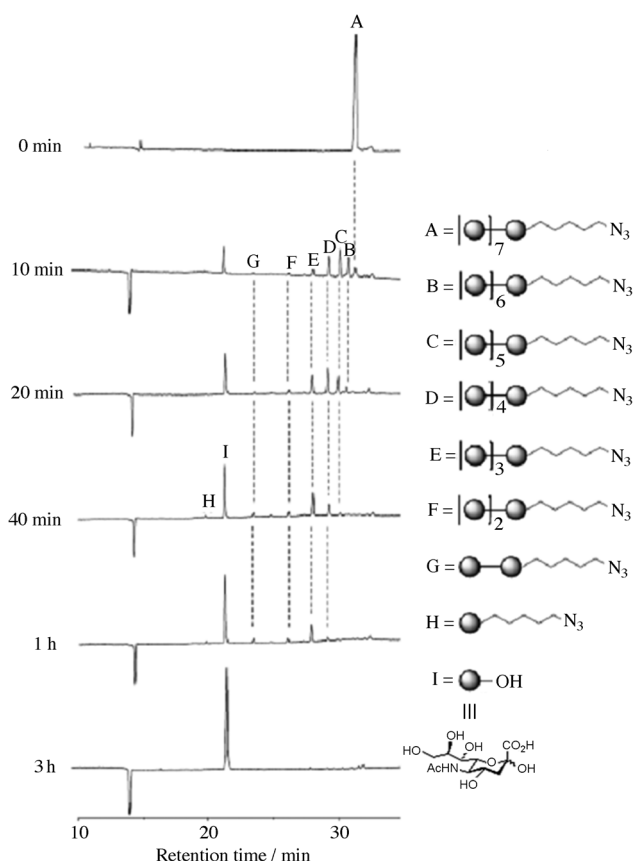


Figure 2. The hydrolysis of the octamer **24** by neuraminidase.

be converted into the amine group for bioconjugation in vaccine development and for glycan microarray assembly.

In conclusion, we have demonstrated that $\alpha(2\rightarrow9)$ oligosialic acids with a well-defined length can be synthesized efficiently by the use of 5*N*,4*O*-carbonyl-protected, phosphate-based donors using a convergent block synthesis strategy. The success of this convergent 4+8 strategy is significant because the α selectivity is retained even when the size of donor or acceptor increases. Moreover, our preliminary results showed that this method can be applied to the synthesis of $\alpha(2\rightarrow8)$ Neu5Ac tetrasialic acid and alternating $\alpha(2\rightarrow8)/\alpha(2\rightarrow9)$ tetrasialic acid. After systematic studies, we believe that this method can be applied to the synthesis of higher oligomers for not only the study of their biological functions but also the preparation of homogeneous polysialic acid–protein conjugates as vaccine candidates.

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[1] a) T. Angata, A. Varki, *Chem. Rev.* **2002**, *102*, 439–469; b) A. Varki, *Nature* **2007**, *446*, 1023–1029.

- [2] E. J. McGuire, S. B. Binkley, *Biochemistry* **1964**, *3*, 247–251.
- [3] A. K. Bhattacharjee, H. J. Jennings, C. P. Kenny, A. Martin, I. C. P. Smith, *J. Biol. Chem.* **1975**, *250*, 1926–1932.
- [4] a) T. Y. Liu, Gotschli, Ec, F. T. Dunne, E. K. Jonssen, *J. Biol. Chem.* **1971**, *246*, 4703–4712; b) T. Y. Liu, E. Gotschli, E. K. Jonssen, J. R. Wysocki, *J. Biol. Chem.* **1971**, *246*, 2849–2858; c) F. A. Troy, *Glycobiology* **1992**, *2*, 5–23.
- [5] W. Egan, T. Y. Liu, D. Dorow, J. S. Cohen, J. D. Robbins, E. C. Gotschlich, J. B. Robbins, *Biochemistry* **1977**, *16*, 3687–3692.
- [6] K. Jann, B. Jann, *Can. J. Microbiol.* **1992**, *38*, 705–710.
- [7] H. J. Jennings in *Carbohydrate-based Drug Discovery* (Ed.: C.-H. Wong), Wiley-VCH, Weinheim, **2003**, pp. 357–380.
- [8] a) P. Costantino, S. Viti, A. Podda, M. A. Velmonte, L. Nencioni, R. Rappuoli, *Vaccine* **1992**, *10*, 691–698; b) C.-Y. Wu, Y.-L. Huang, *Expert Rev. Vaccines* **2010**, *9*, 1257–1274.
- [9] a) S. Marburg, D. Jorn, R. L. Tolman, B. Arison, J. McCauley, P. J. Kniskern, A. Hagopian, P. P. Vella, *J. Am. Chem. Soc.* **1986**, *108*, 5282–5287; b) A. Bardotti, G. Averani, F. Berti, S. Berti, V. Carinci, S. D'Ascenzi, B. Fabbri, S. Glannini, A. Giannozzi, C. Magagnoli, D. Proietti, F. Norelli, R. Rappuoli, S. Ricci, P. Costantino, *Vaccine* **2008**, *26*, 2284–2296.
- [10] C. Sato, K. Kitajima, *Trends Glycosci. Glycotechnol.* **1999**, *11*, 371–390.
- [11] Recent reviews: a) G. J. Boons, A. V. Demchenko, *Chem. Rev.* **2000**, *100*, 4539–4566; b) D. K. Riss, R. J. Linhardt, *Curr. Org. Synth.* **2004**, *1*, 31–46; c) H. Ando, A. Imanura, *Trends Glycosci. Glycotechnol.* **2004**, *16*, 293–303; d) M. Kiso, H. Ishida, H. Ito in *Carbohydrates in Chemistry and Biology, Vol. 1* (Eds.: B. Ernst, G. W. Hart, P. Sinay), Wiley-VCH, Weinheim, **2000**, pp. 345–365; e) R. L. Halcomb, M. D. Chappel in *Glycochemistry: Principles, Synthesis and Applications* (Eds.: P. G. Wang, C. R. Bertozzi), Marcel Dekker, New York, **2001**, pp. 177–220; f) C. De Meo, U. Priyadarshani, *Carbohydr. Res.* **2008**, *343*, 1540–1552.
- [12] a) C. S. Yu, K. Niikura, C. C. Lin, C. H. Wong, *Angew. Chem.* **2001**, *113*, 2984–2987; *Angew. Chem. Int. Ed.* **2001**, *40*, 2900–2903; b) C.-C. Lin, K. T. Huang, C. C. Lin, *Org. Lett.* **2005**, *7*, 4169–4172; c) K. Tanaka, T. Goi, K. Fukase, *Synlett* **2005**, 2958–2962; d) H. Tanaka, Y. Nishiura, T. Takahashi, *J. Am. Chem. Soc.* **2006**, *128*, 7124–7125; e) D. Crich, W. J. Li, *J. Org. Chem.* **2007**, *72*, 2387–2391; f) D. Crich, W. J. Li, *J. Org. Chem.* **2007**, *72*, 7794–7797; g) M. D. Farris, C. De Meo, *Tetrahedron Lett.* **2007**, *48*, 1225–1227; h) D. Crich, B. Wu, *Org. Lett.* **2008**, *10*, 4033–4035; i) B. Sun, B. Srinivasan, X. F. Huang, *Chem. Eur. J.* **2008**, *14*, 7072–7081; j) H. Tanaka, Y. Tateno, Y. Nishiura, T. Takahashi, *Org. Lett.* **2008**, *10*, 5597–5600; k) F. F. Liang, L. Chen, G. W. Xing, *Synlett* **2009**, 425–428.
- [13] H. Tanaka, Y. Nishiura, T. Takahashi, *J. Org. Chem.* **2009**, *74*, 4383–4386.
- [14] a) S. Koto, T. Uchida, S. Zen, *Bull. Chem. Soc. Jpn.* **1973**, *46*, 2520–2523; b) H. Paulsen, *Angew. Chem.* **1982**, *94*, 184–201; *Angew. Chem. Int. Ed. Engl.* **1982**, *21*, 155–173; c) T. Ogawa, H. Yamamoto, T. Nukada, T. Kitajima, M. Sugimoto, *Pure Appl. Chem.* **1984**, *56*, 779–795; d) G. J. Boons, *Tetrahedron* **1996**, *52*, 1095–1121; e) R. Bommer, W. Kinzy, R. R. Schmidt, *Liebigs Ann. Chem.* **1991**, 425–433.
- [15] C. C. Lin, A. K. Adak, J. C. Horng, *Tetrahedron* **2009**, *65*, 4714–4725.
- [16] C. H. Hsu, K. C. Chu, Y. S. Lin, J. L. Han, Y. S. Peng, C. T. Ren, C. Y. Wu, C. H. Wong, *Chem. Eur. J.* **2010**, *16*, 1754–1760.
- [17] C. C. Lin, N. P. Lin, L. S. Sahabuddin, V. R. Reddy, L. D. Huang, K. C. Hwang, *J. Org. Chem.* **2010**, *75*, 4921–4928.
- [18] H. Hori, T. Nakajima, Y. Nishida, H. Ohnui, H. Meguro, *Tetrahedron Lett.* **1988**, *29*, 6317–6320.
- [19] a) M. C. Cheng, S. L. Lin, S. H. Wu, S. Inoue, Y. Inoue, *Anal. Biochem.* **1998**, *260*, 154–159; b) M. C. Cheng, K. T. Wang, S. Inoue, Y. Inoue, K. H. Khoo, S. H. Wu, *Anal. Biochem.* **1999**, *267*, 287–293.