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# Chemoenzymatic Synthesis of Sialic Acid Derivatives Using Immobilized *N*-Acetylneuraminate Lyase in a Continuous Flow Reactor


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Manuscript received: January 31, 2019; Revised manuscript received: April 1, 2019;  
Version of record online: May 8, 2019

 Supporting information for this article is available on the WWW under <https://doi.org/10.1002/adsc.201900146>

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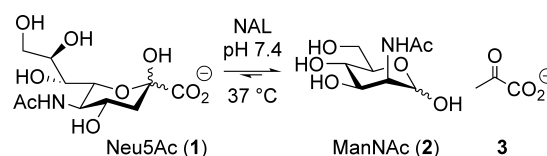
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**Abstract:** The synthesis of *N*-acetylneuraminic acid (Neu5Ac) derivatives is drawing more and more attention in glycobiology research because of the important role of sialic acids in e.g. cancer, bacterial, and healthy cells. Chemical preparation of these carbohydrates typically relies on multistep synthetic procedures leading to low overall yields. Herein we report a continuous flow process involving *N*-acetylneuraminate lyase (NAL) immobilized on Immobead 150P (Immobead-NAL) to prepare Neu5Ac derivatives. Batch experiments with Immobead-NAL showed equal activity as the native enzyme. Moreover, by using a fivefold excess of either *N*-acetyl-D-mannosamine (ManNAc) or pyruvate the conversion and isolated yield of Neu5Ac were significantly improved. To further increase the efficiency of the process, a flow setup was designed providing a chemoenzymatic entry into a series of *N*-functionalized Neu5Ac derivatives in conversions of 48–82%, and showing excellent stability over 1 week of continuous use.

**Keywords:** *N*-acetylneuraminic acid; *N*-acetylneuraminate lyase; flow chemistry; enzyme immobilization

due to the complexity of individual saccharide moieties and the numerous branching possibilities.<sup>[1]</sup> A unique carbohydrate that is generally encountered at the outer surface of glycans is the sialic acid *N*-acetylneuraminic acid (Neu5Ac, **1**). Neu5Ac has over 50 structurally related analogues, which can be recognized by specific receptors.<sup>[2]</sup> These receptors play a central role in health and disease and are potential targets for neuraminic acid-based drugs.<sup>[3]</sup>

Neu5Ac derivatives are most often prepared using multistep organic synthesis which is laborious, expensive, time consuming and typically low yielding. Alternatively, Neu5Ac derivatives can be obtained by using *N*-acetylneuraminate lyase (NAL), an enzyme which catalyzes the reversible aldol reaction of Neu5Ac (**1**) to form *N*-acetyl-D-mannosamine (**2**, ManNAc) and pyruvate (**3**, Scheme 1).<sup>[4]</sup> It has been shown that by using an excess of pyruvate or ManNAc derivative, the equilibrium is shifted toward the



**Scheme 1.** The reversible aldol reaction of Neu5Ac (**1**) to ManNAc (**2**) and pyruvate (**3**) catalyzed by NAL.

Carbohydrates constitute an important class of biomolecules with a wide array of biological functions

Neu5Ac product.<sup>[5]</sup> In contrast to chemically derived Neu5Ac substrates, ManNAc derivatives are synthetically readily accessible and due to the promiscuity of NAL give straightforward access to Neu5Ac derivatives.

The immobilization of NAL has been reported by several research groups and shows promise for large scale Neu5Ac production.<sup>[6]</sup> More recently, also cross-linked examples have been reported including CLEA, CLEC and CLIB which are notably stable, but challenging to prepare.<sup>[7]</sup> To the best of our knowledge, immobilized NAL has never been used in a continuous flow setup for the preparation of synthetic Neu5Ac derivatives. Based on the broad experience with chemoenzymatic reactions in our group, both in batch<sup>[8]</sup> and in flow systems,<sup>[9]</sup> and on our expertise in the chemistry and biology of sialic acids,<sup>[2,10]</sup> we herewith report a straightforward continuous flow system containing immobilized NAL providing a new and efficient chemoenzymatic entry into biologically relevant *N*-functionalized Neu5Ac derivatives.

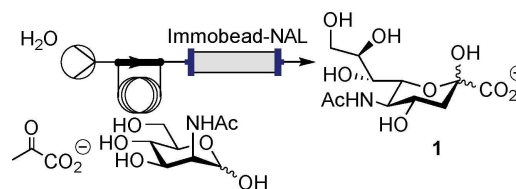
First, we investigated the immobilization of NAL to increase stability, shelf life, and ease of application in flow chemistry. Our initial attempt using an aqueous NAL solution in combination with oxirane-functionalized Immobead 150P (corresponding to 62 mg NAL per g Immobead 150P) was successful providing immobilized NAL (Immobead-NAL) with immobilization efficiencies of up to 74% (see: Supporting Information 1).<sup>[11]</sup>

In order to test the activity of Immobead-NAL, the chemoenzymatic synthesis of Neu5Ac (**1**) was investigated in batch experiments using ManNAc (**2**), pyruvate (**3**) and native NAL in a homogeneous

solution and Immobead-NAL in a heterogeneous formulation. In literature most often an excess of pyruvate is used, but this requires tedious purification in which residual buffer salts and pyruvate have to be removed.<sup>[3d,5d,12]</sup> In contrast, we showed that a fivefold molar excess of ManNAc in H<sub>2</sub>O led to conversions of up to 77% (Table 1), while the excess of ManNAc could be easily removed by anion exchange. All Immobead-NAL formulations were shown to be effective in the preparation of Neu5Ac with comparable conversions. Interestingly, three months old Immobead-NAL (stored at 0 °C) showed only a slight decrease in activity, indicating that the immobilized *N*-acetylneuraminate lyase can be stored for an extended period of time. Finally, increasing the molar excess of ManNAc did not lead to higher conversions and isolated yields, therefore a molar ratio of 5 of ManNAc and pyruvate was considered optimal in this setup.

We then applied Immobead-NAL in a flow system to efficiently screen reaction parameters and optimize conversions (Table 2). A flow reactor was designed using an HPLC cartridge, filled with freshly prepared Immobead-NAL, as a packed-bed reactor (see: Supporting Information 3). Prior to administering the substrates to the packed-bed reactor, it was preheated to 38 °C using a water bath. Perfluoroalkoxy (PFA) tubing (OD 1/16", ID 1/50") and flangeless fittings (1/16") were used to connect the linear flow setup.

**Table 2.** The effect of stoichiometry and concentration on the preparation of Neu5Ac (**1**) using Immobead-NAL in a continuous flow system.<sup>[a]</sup>



**Table 1.** The synthesis of Neu5Ac (**1**) in batch with various Immobead-NAL formulations and amounts of pyruvate.<sup>[a]</sup>

NAL formulation	ManNAc [mM]	pyruvate [mM]	conversion (%) <sup>[b]</sup>
aqueous NAL	250	50	67
		25	59
		12.5	60
crystalline NAL	250	50	77
		25	79
		12.5	74
Immobead-NAL (1 month)	250	50	62
		25	65
		12.5	63
Immobead-NAL (3 months)	250	50	58
		25	57
		12.5	63

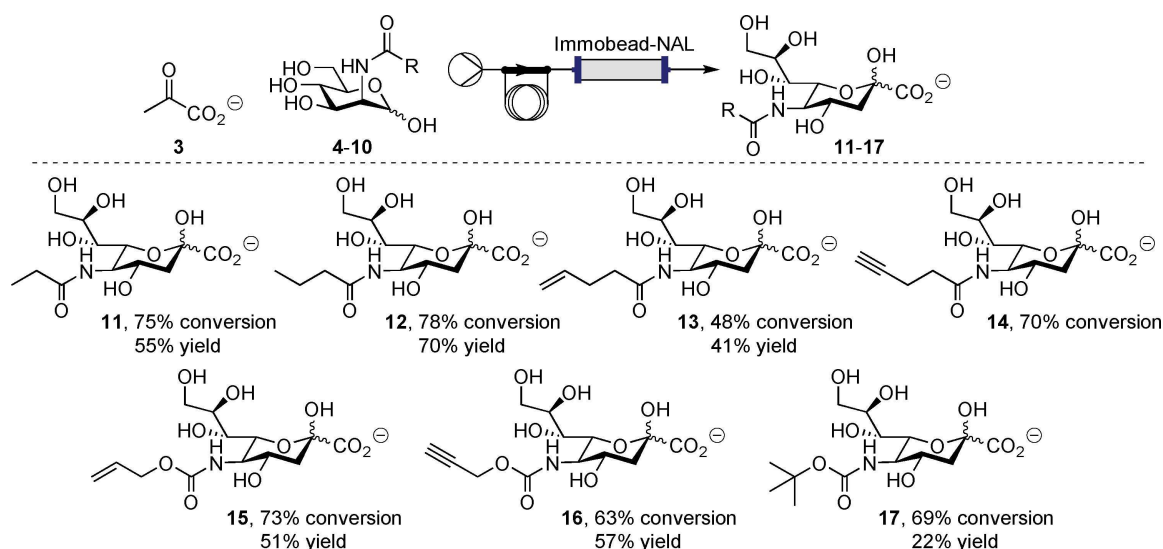
<sup>[a]</sup> Conditions: ManNAc (**2**, 450 μmol) and sodium pyruvate (90 or 45 or 23 μmol) in H<sub>2</sub>O (1.8 mL) at 38 °C for 24 h.

<sup>[b]</sup> Determined by <sup>1</sup>H-NMR analysis of the crude reaction mixture.

entry	pyruvate [mM]	ManNAc [mM]	conversion (%) <sup>[b]</sup>
<b>1</b>	1000	100	52
<b>2</b>	500	100	36
<b>3</b>	300	100	19
<b>4</b>	100	100	15
<b>5</b>	33	100	19
<b>6</b>	20	100	40
<b>7</b>	10	100	41
<b>8</b>	50	250	71
<b>9</b>	80	400	76
<b>10</b>	100	500	82

<sup>[a]</sup> Conditions: The solution (900 μL) was injected in a sample loop (1 mL) and pumped (0.05 mL·min<sup>-1</sup>) over the Immobead-NAL column at 38 °C and collected for 1.5 h.

<sup>[b]</sup> Determined by <sup>1</sup>H-NMR analysis of the crude reaction mixture.

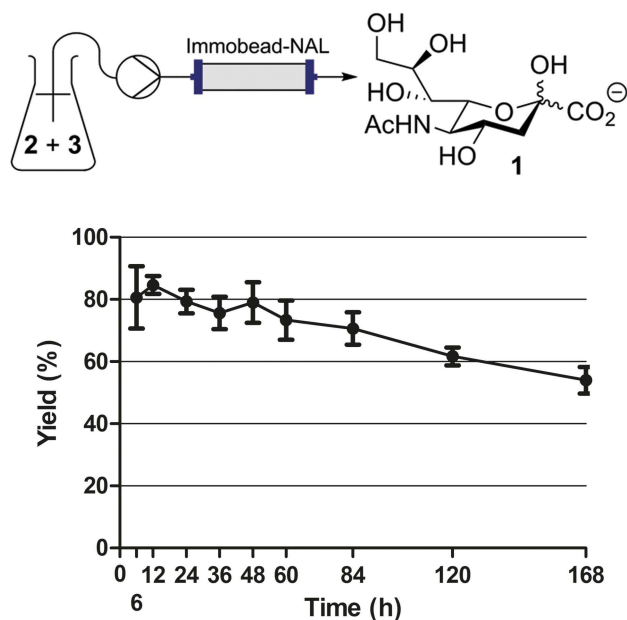


**Scheme 2.** The use of ManNAc derivatives 4–10 in the synthesis of Neu5Ac derivatives 11–17.

Initially we investigated stoichiometry and concentration as process parameters in the synthesis of Neu5Ac (**1**) in flow. At a fixed concentration of 100 mM ManNAc, neither excess of pyruvate nor ManNAc led to increase of the conversion beyond 50% (Table 2, entries 1–7). Attempts at lower flow speed did also not improve the conversion when using a fivefold excess of ManNAc (**2**) with respect to pyruvate (**3**, see: Supporting Information 2). Interestingly, use of higher concentrations of ManNAc (**2**) led to higher conversions of up to 82% (Table 2, entries 8–10). This result is supported by mechanistic insights of Wong et al.<sup>[4a]</sup> and by Groher and Hoelsch<sup>[13]</sup> showing a correlation in increased substrate concentration and its positive influence on Neu5Ac production. The upper limit for ManNAc concentrations was set to 500 mM due to its solubility in aqueous medium.

To broaden the scope of the chemoenzymatic process, synthetic ManNAc derivatives 4–10 were prepared in batch and subjected to the flow conditions (Scheme 2, see also: Supporting Information). In this reaction, the NAL appeared sufficiently promiscuous to successfully convert all synthetic ManNAc derivatives into the corresponding Neu5Ac analogues 11–17. Thus, the enzyme shows a relatively large substrate scope, which is not too much influenced by the fact that the water solubility of the ManNAc derivatives decreases with increasing size of the protecting group. In addition to monitoring conversions, the sialic acid products 11–17 were also obtained in fairly reasonable isolated yields confirming the synthetic feasibility of this system (Scheme 2). This includes the propargyloxycarbonyl (Poc)-functionalized Neu5Ac derivative **16** which has been extensively used in our group and was shown to be an effective substrate in bioorthogonal glycobiochemistry research.<sup>[2b,10]</sup>

Finally, we were interested in the robustness of the system and studied a seven-day continuous flow experiment. By pumping a stock solution of ManNAc (**2**, 55.7 g, 500 mM) and pyruvate (**3**, 5.5 g, 100 mM) over the Immobead-NAL reactor (approximately 35 mg NAL), more than 10 g of Neu5Ac (**1**) was produced. Even after 168 hours of continuous pumping at 0.05 mL·min<sup>-1</sup> marginal decrease in enzyme activity was observed (Figure 1, see also: Supporting



**Figure 1.** Preparative scale formation of Neu5Ac (**1**) in a continuous flow setup. Conditions: ManNAc (**2**, 500 mM), pyruvate (**3**, 100 mM), H<sub>2</sub>O, Immobead-NAL, flow 0.05 mL·min<sup>-1</sup>, 38 °C.

Information 4). The crude mixture was purified using anion exchange chromatography resulting in effective recovery of ManNAc (**2**) and production of Neu5Ac acid (**1**) in 58% isolated yield between time interval 0–120 h. These results clearly underline the stability of Immo-bead-NAL and the feasibility to scale up the flow process.

In summary, *N*-acetylneuraminase lyase (NAL) was immobilized and used to optimize the conversion of ManNAc and pyruvate into Neu5Ac. The oxirane-containing resin Immo-bead 150P was coupled to NAL with a binding efficiency of up to 74%. The resulting Immo-bead-NAL showed to be stable in aqueous solution over at least three months of storage. Optimal conversions were obtained using 500 mM ManNAc, 100 mM pyruvate and a flow rate of  $0.05 \text{ mL} \cdot \text{min}^{-1}$ , giving conversions of up to 82%.

We also demonstrated that the system was effective in the synthesis of a library of differently *N*-substituted Neu5Ac derivatives. Finally, the reactor was subjected to a durability test of 168 hours showing a relatively small decrease of *N*-acetylneuraminase lyase activity over a week time. Thus, using this immobilized lyase, an efficient and stable continuous flow process has been developed for the preparation of neuraminic acid derivatives.

## Experimental Section

### Chemicals and Enzymes

Chemicals used in this study were purchased from Fluorochem, Sigma Aldrich and Carbosynth with a purity grade of  $\geq 95\%$ . Demineralized water was used during all experiments. *N*-Acetylneuraminase lyase (NAL) was used as a crystalline powder ( $8.7 \text{ U} \cdot \text{mg}^{-1}$ ) or a suspension in aqueous solution ( $111 \text{ U} \cdot \text{mL}^{-1}$ ). The crystalline powder and aqueous solution were kept cool at  $4^\circ\text{C}$ , and the latter was shaken before use.

### Immobilization of NAL

The immobilization procedure comprised incubation of Immo-bead-150P (300 mg) and *N*-acetylneuraminase lyase (crystalline:  $50 \text{ mg}$ ,  $8 \text{ U} \cdot \text{mg}^{-1}$  or solution:  $900 \mu\text{L}$ ,  $111 \text{ U} \cdot \text{mL}^{-1}$ ) dissolved in aqueous  $\text{K}_2\text{PO}_4$  (1.25 M,  $\text{pH}=8.0$ ,  $45 \text{ mL}$ ) in a falcon tube. The mixture was shaken for 24 h at  $20^\circ\text{C}$ , and thereafter filtered using a cellulose paper. The obtained grain was washed twice with demineralized water ( $45 \text{ mL}$ ), and collected again. The beads were diluted in a glycine solution (2.0 M,  $\text{pH}=8.5$ ,  $45 \text{ mL}$ ) and shaken for 24 h at  $20^\circ\text{C}$ , and thereafter filtered using cellulose paper. The Immo-bead-150P was washed twice with demineralized water ( $30 \text{ mL}$ ), and twice with aqueous  $\text{K}_2\text{PO}_4$  (0.1 M,  $\text{pH}=7.4$ ,  $30 \text{ mL}$ ). The obtained immobilized lyase was stored at  $4^\circ\text{C}$  in a falcon tube, and used within three months of preparation.

### Determination of Binding Efficiency Using Lowry's Protein Determination

The binding of *N*-acetylneuraminase lyase to the beads was determined via the Lowry's protein assay of the washings during enzyme immobilization and measured in *triplo*.<sup>[11]</sup> The concentration of free protein was determined, effectively providing information about the total amount of protein coupled to the beads. The standard curve for protein determination was conducted using solutions of  $0.02 \text{ mg} \cdot \text{mL}^{-1}$  to  $0.1 \text{ mg} \cdot \text{mL}^{-1}$  of Bovine Serum Albumin (BSA) purchased from Sigma-Aldrich. Hereafter the protein concentration of aqueous *N*-acetylneuraminase lyase ( $100 \mu\text{L}$ ) was determined in *triplo*, and used to determine the protein content of the immobilization washings.

### Anion Exchange Purification of Neu5Ac (1)

A short cylindrical column ( $\pm 5 \times 1.5 \text{ cm}$ ;  $h \times d$ ) was charged with DEAE-Sephadex A-25  $\text{Cl}^-$  ( $\pm 4.5 \text{ g}$ ) and swollen using aqueous  $\text{NH}_4\text{HCO}_3$  (1.5 M,  $15 \text{ mL}$ ). The resin was filtered and washed thrice with water ( $3 \times 15 \text{ mL}$ ) before addition of the reaction mixture. The crude mixture was added to the resin and washed with water, followed by an increasing v/v% of formic acid in water, up to 20%. The obtained products were concentrated *in vacuo*.

### Flow Chemistry Setup

The Immo-bead-NAL resin (700 mg) was loaded directly in an HPLC cartridge ( $\pm 1.5 \text{ mL}$ ) using vacuum suction followed by washing with copious amounts of demineralized water. The ManNAc/pyruvate solution was prepared in demineralized water, and the pH adjusted to 7.0–7.5 using 1 M aqueous NaOH or HCl and a digital pH indicator. The HPLC cartridge was connected to the HPLC pump, and heated to  $38^\circ\text{C}$  in a water bath. After 30 min at  $38^\circ\text{C}$ , the pump was started at  $0.05 \text{ mL} \cdot \text{min}^{-1}$ . After the reaction mixture was pumped, additional demineralized water ( $\pm 5 \text{ mL}$ ) was used to remove remaining reactants. The product was concentrated *in vacuo* and the conversion determined using  $^1\text{H-NMR}$  in  $\text{D}_2\text{O}$ .

### Preparative Scale Experiment

The Immo-bead-NAL resin (700 mg) was loaded directly in an HPLC cartridge ( $\pm 1.5 \text{ mL}$ ) using vacuum suction followed by washing with copious amounts of demineralized water. ManNAc (55.7 g, 252 mmol) and sodium pyruvate (5.55 g, 50.4 mmol) were added to water (504 mL) and the pH was adjusted to 7.0–7.5 using 1 M aqueous NaOH or HCl using a digital pH indicator. The HPLC cartridge was connected directly to the HPLC pump without the sample loop, and heated to  $38^\circ\text{C}$  in a water bath. After 30 min at  $38^\circ\text{C}$ , the pump was set to  $0.05 \text{ mL} \cdot \text{min}^{-1}$ . After the indicated time intervals, the reaction mixture was separately collected for 10 min ( $3 \times$ ). The samples were concentrated *in vacuo* and the conversion determined using  $^1\text{H-NMR}$  in  $\text{D}_2\text{O}$ .



## Acknowledgements

We kindly acknowledge the funding by the H2020-FETOPEN-2016-2017 programme of the European Commission (Grant agreement number: 737266-ONE FLOW). This work was also supported by a European Research Council (ERC) Starting Grant (Grant agreement number: 758913) awarded to T. J. Boltje.

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