RSC Advances

RSC Publishing

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *RSC Adv.*, 2013, **3**, 21335

Received 30th August 2013
Accepted 11th September 2013

DOI: 10.1039/c3ra44791j

www.rsc.org/advances

Enzymatic synthesis of colorimetric substrates to determine α-2,3- and α-2,6-specific neuraminidase activity†

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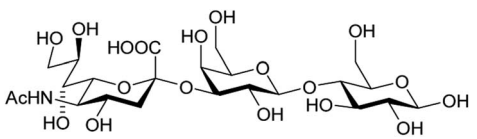
Glycoconjugates containing either terminal α-2,3- or α-2,6-Neu5Ac-Gal disaccharides are found on cell surfaces of many animal glycans. Each linkage can be specifically recognized by lectins and enzymes such as neuraminidases. Here we describe a one-step enzymatic synthesis of two colorimetric substrates that allow for fast distinction of specific neuraminidase activity.

Sialic acids (*e.g.* Neu5Ac) are a family of monosaccharides which are commonly found on the terminus of glycans present on animal cell surfaces and are responsible for regulating a number of biological recognition processes.[1,2] Influenza virus hemaglutinin[3] and numerous lectins such as Siglecs,[4,5] which are involved in immune responses have been shown to bind sialic acids present on cell surfaces.[6] In animal cell surface glycans, sialic acids are often linked to galactose with either α-2,3- or α-2,6 glycosidic bonds depending on the tissue and organism (as in **1** and **2**; Scheme 1).[7] Linkage specificity of lectins and enzymes can have significant biological impact. For example, influenza viral infection depends on the host sialic acid containing ligands:[3,8] whilst bird flu viruses recognises α-2,3-linked Neu5Ac, seasonal flu viruses are specific for α-2,6-linkage. Pandemic flu strains can recognize both α-2,6- and α-2,3-linked Neu5Ac.[9] Many organisms, such as the influenza virus, express neuraminidases, which hydrolyse the sialic acid linkages in glycoconjugates. Viral neuraminidases are therefore therapeutic targets for anti-flu treatment and high-throughput assays to determine their specificity are important tools in the diagnostics and development of new drugs.

In the past, the linkage selectivity of neuraminidases was determined using 3′SiaLac (Neu5Ac-α2,3Gal-β1,4Glc, **1**) and 6′SiaLac (Neu5Ac-α2,6Gal-β1,4Glc, **2**) as substrates and the amount of Neu5Ac released was determined using a thiobarbituric acid assay.[10] More recent and sensitive methods rely on fluorescently labelled sialyloligosaccharides *e.g.* BODIPY (4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene-3-propionic acid) derivatives,[11] however these methods are expensive. Glycoarray technologies using immobilised substrates have also been employed to detect the specificity and activity of influenza neuraminidases.[12] However, the complexity of the readout systems using lectins or mass spectrometry can make them unsuitable for quantitative studies so colorimetric assays are an attractive complementary tool to array techniques.

In the early 90's a colorimetric assay was reported by H. Kodama and co-workers;[13] the synthesis of chromogenic sialylated compounds using 4-nitrophenyl β-D-galactopyranoside as a substrate was achieved using an enzymatic approach. However, the high costs of the materials and enzymes required for the assay made this an unsuitable method to determine neuraminidase specific activity.



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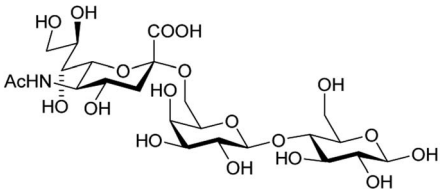

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Scheme 1 Structures of α-2,3- (**1**) and α-2,6-sialyllactoside (**2**), which are commonly found on animal cell surfaces.

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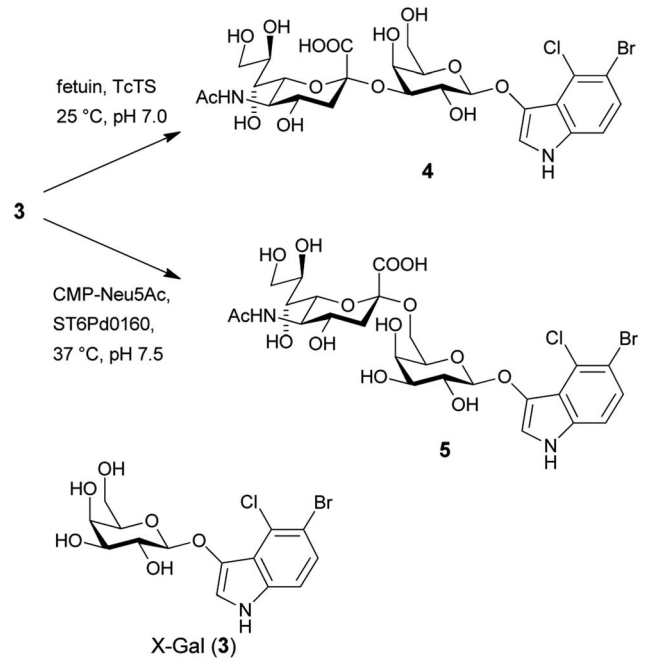
† Electronic supplementary information (ESI) available. See DOI: 10.1039/c3ra44791j



Scheme 2 Enzymatic synthesis of sialylated 5-bromo-4-chloro-3-indolyl-β-D-galactopyranosides **4** and **5**.

Here we describe the synthesis of two new chromogenic substrates, **4** and **5**, that allow us to determine the activity and linkage specificity of a diverse range of neuraminidases. The compounds were designed to release 5-bromo-4-chloro-indolyl-β-D-galacto-pyranoside (X-Gal **3**) upon neuraminidase activity.

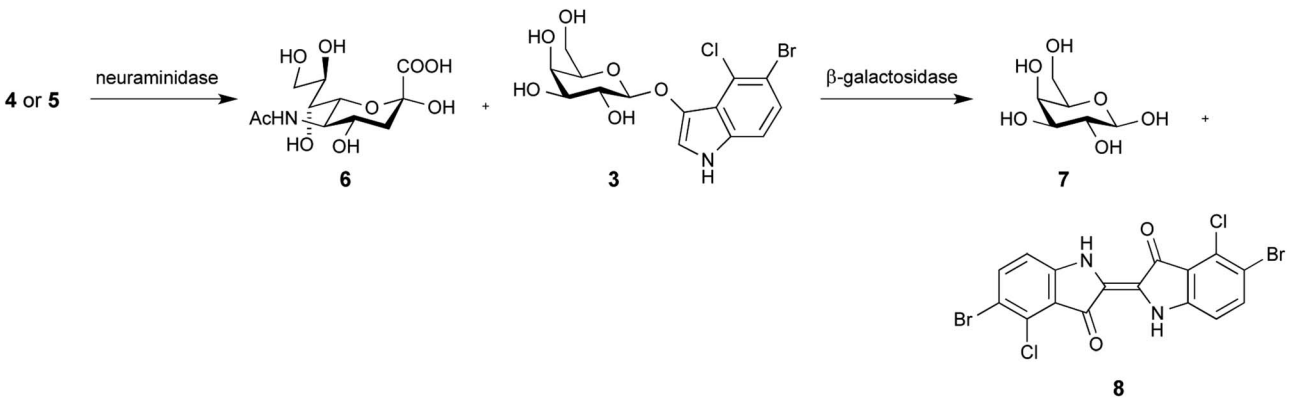
The chemical synthesis of sialosides such as **4** and **5** is a lengthy process requiring multistep protection, activation and coupling procedures.[14,15] As an alternative route, we investigated the use of biocatalysis for the synthesis of **4** and **5**, which offers a more effective and time efficient synthetic strategy. Oligosaccharides including sialosides have been synthesised in the past with high efficiency, regio- and stereo-specificity.[16–18]

For targets **4** and **5** the enzymatic route was particularly attractive since both regioisomers would potentially be accessible from commercially available X-Gal (**3**) through a one-step enzymatic glycosylations using either α-2,3- or α-2,6-selective sialyltransferases or *trans*-sialidases.

Using an enzymatic approach we prepared α-2,3- and α-2,6-Neu5Ac modified 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranosides **4** and **5** as novel substrates to test for sialidase activity assays. Both glycoconjugates (Scheme 2) were synthesised according to previously reported strategies using *trans*-sialidase from *Trypanosoma cruzi* (TcTS) for α-2,3-linked Neu5Ac[6] and sialyl-transferase from *Photobacterium damsela* (ST6Pd060) for α-2,6-linked Neu5Ac.[19] TcTS does not require CMP-Neu5Ac donor and can utilise sialoproteins such as fetuin. Due to reversibility of the *trans*-glycosylation by TcTS, an excess of fetuin was used for the synthesis of conjugate **4**. Formation of both **4** and **5** was confirmed by mass spectrometry (Fig. S1†, peak at *m*/*z* 722.76 for **4** and peak at *m*/*z* 722.80 for **5**) and HPLC (Fig. S4†).

To distinguish the glycosidic linkage in **4** and **5**, the amidation/lactonisation method was used. This method is based on the reaction of the sialylated compound with ammonium chloride in the presence of DMT-MM. Upon activation of the carboxylic group with DMT-MM an amide is formed in the case of 2,6-linked Neu5Ac. In the case of 2,3-linked Neu5Ac a lactone with the 2-OH group of the galactose residue is formed.[20] Upon permethylation, the two species give products that can be distinguished by MS analysis. Using lactose as an acceptor the characteristic peaks at *m*/*z* 838.19 *m*/*z* for Neu5Ac-α2,3Gal-β1,4Glc and 851.22 *m*/*z* for Neu5Ac-α2,6Gal-β1,4Glc were observed in MALDI-ToF MS spectra (Fig. S2†).

It is well known that sialic acids readily undergo fragmentation under the desorption–ionization process during MALDI MS analysis. To stabilise the sialylated analytes and significantly increase the signal intensity, a permethylation step prior to analysis is commonly used. The most widely used technique in glycomics is the NaOH and methyl iodide method. Powell *et al.*[21] developed a simple methyl esterification of the free carboxylic acid in sialosides which has also been shown to prevent cleavage. In our hands it has been found that also non-methylated Neu5Ac containing glycans can be analysed by MALDI MS when DHB (2,5-dihydroxybenzoic acid) is used as a matrix in positive, reflectron mode. For the analysis of both **4** and **5** respectively, no permethylation was needed.



Scheme 3 Two step activity assay: in the first step neuraminidase releases **6** (Neu5Ac) from the substrate **4** or **5**. In the following step β-galactosidase releases 5-bromo-4-chloro-indol from X-Gal **3** which quickly oxidises to form blue indigo derivative **8**.

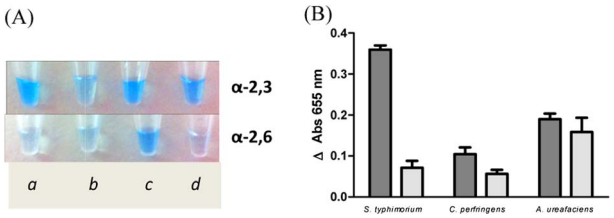


Fig. 1 Colorimetric determination of neuraminidase activity with α-2,3- (**4**) and α-2,6-sialylated 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside **5** as substrates. (A) Samples with bacterial neuraminidases (a) *S. typhimorium* neuraminidase, (b) *C. perfringens* neuraminidase, (c) *A. ureafaciens* neuraminidase, (d) TcTS (purified enzyme); (B) activity of three bacterial sialidases determined at 655 nm.

Using substrates **4** and **5**, we have established a colorimetric assay based on an indigo-derivative formation for a quick and easy detection of specific hydrolytic activity of neuraminidases. Upon incubation of compounds **4** and **5** with an active neuraminidase, an indolyl compound is released in the presence of β-galactosidase which quickly undergoes oxidation to form an indigo derivative (Scheme 3). The activity can easily be detected by blue coloration of the sample without need of any expensive equipment and complicated technical skills. Both compounds **4** and **5** respectively were used to test the neuraminidase activity of various bacterial sialidases in this two steps reaction sequence (Scheme 3). As the highly specific β-galactosidase only cleaves terminal Gal residues, compounds **4** and **5** are not accepted. After incubation of all reaction mixtures with bacterial neuraminidases, the blue coloration in positive samples could be observed by naked eye after the addition of β-galactosidase (Fig. 1A). For quantification of the enzymatic activity, maximum absorbance of the indigo derivative **8** was determined (Fig. S3†). The commercial neuraminidase from *S. typhimorium* with activity towards α-2,3-linked Neu5Ac and neuraminidase from *A. ureafaciens* with activity towards α-2,6-linked Neu5Ac were used as standards. Spectrophotometric analysis allowed us to determinate the activity and specificity of neuraminidases using absorbance measurements at 655 nm (Fig. 1B and 2). The data show that *S. typhimorium* neuraminidase is selective for α-2,3-linked Neu5Ac, whereas *A. ureafaciens* neuraminidase has broad selectivity towards both **4** and **5**.[22–24]

Neuraminidases have been found in many species, in particular microorganisms and they are useful tools for many biotechnological applications.[25] Sialidase activity has already been reported for more than 70 different microorganisms.[26] Kim *et al.* have reviewed characteristics of 30 microbial sialidases, with the majority of them belonging to the GH33 family in the CAZy database.[27] Genomic studies have shown that sialidase family sequences share a common domain of four or five copies of an aspartate box well known as the BNR/Asp-box motif.

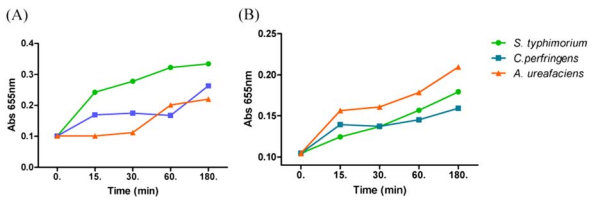


Fig. 2 Sialidase activities of three bacterial neuraminidases against **4** (A) and **5** (B).

Table 1 Bacteria with specific neuraminidase activity

	Activity against α-2,3-Neu5Ac linkage (**4**)	Activity against α-2,6-Neu5Ac linkage (**5**)
Bacillus pumilus	+	–
Arthrobacter aurescens	+	–

The bacterial genomes of *B. pumilus* and *A. aurescens* have recently been sequenced. The sequence analysis revealed the presence of BNR/Asp-box motif suggesting the possible existence of neuraminidases in this species, but the linkage specificity was unknown. We were interested in using our probes, **4** and **5**, to confirm activity and determine specificity of any sialidase activity in these two bacteria. Activity was tested on crude cell extracts. Interestingly, both bacteria showed neuraminidase activity, which was selective for α-2,3 (**4**) but not for α-2,6 (**5**) linkage (Table 1).

In conclusion, we have synthesised two new sialylated compounds **4** and **5** as substrates for specific α-2,3 or α-2,6 neuraminidase activity. Both compounds are accessible by a one step enzymatic synthesis from commercially available materials. **4** and **5** could be used to detect novel neuraminidase specificity in crude bacterial extracts and determine linkage specificity at the same time in a simple colorimetric assay. Compounds such as **4** and **5** will be useful to develop diagnostic tools for detecting neuraminidase activity and for developing screens for inhibitors of these enzymes, particularly in the context of viral and some parasitic infections.

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