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Note

Oxidation of sialic acid using hydrogen peroxide as a new method to tune the reducing activity

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ABSTRACT

Functionalized sialic acids are useful intermediates to prepare a wide range of biological products. As they often occur at a non-reducing terminal of oligosaccharides, the most used technique to activate them is by periodate-mediated oxidation of their glycerol side chain. Here, we describe an alternative, non toxic, and environmentally-friendly method to activate the sialic acid residues by hydrogen peroxide oxidation. Four oxidative systems involving H_2O_2, EDTA, iron chloride, and UV light were studied and the products obtained were analyzed by LC–MS and NMR, before and after a derivatization reaction. At first, we observed, for each system, an irreversible decarbonylation reaction at the reducing end. Then, the decarbonylated sialic acid (DSA) was oxidized and fragmented into a mix of carbonyls and carboxyl acids, more or less fast according to the experimental conditions. Analysis of the reaction indicated an apparent radical mechanism and heterolytic alpha-hydroxy-hydroperoxide cleavages. The modest reducing activity was mainly explained as a consequence of over-oxidation reactions.

Sialic acids consist of a large family of more than fifty acetylated, methylated, or sulfated 9-carbon carboxylated alpha-keto sugars.[1] The most common member of this family of natural products is N-acetylneuraminic acid (NeuNAc), which has an acetyl group attached to the 5-amino group. Its structure has been studied in detail in the literature[2] (Fig. 1). It exits predominantly in the β configuration.[3]

NeuNAc occurs in nature as carbohydrate chains of bacterial and animal glycoproteins,[4] glycolipids,[5] and polysaccharides.[6] Located at the terminus of numerous cell-surface oligosaccharides, NeuNAc has been used in a wide range of biomedical applications such as vaccines[7] or drug delivery systems.[8,9] They are ideally positioned to be covalently attached to proteins (glycoconjugate vaccines), polyethylene glycol (pegylated glycoproteins), or cytotoxic drugs (antibody–drug conjugates).

As most of the bioconjugations occur in aqueous solution, the ways to activate a carbohydrate present on a biopolymer are limited. The ketone at the reducing end of the NeuNAc can be derivatized, for example via a reductive amination.[10] But since the unreactive ring form dominates,[3] the reactivity of this function is relatively low. Furthermore, sialic acid (SA) often occurs at a terminal non-reducing oligosaccharide making its ring not capable of opening. Although alcohols are abundant in carbohydrates like NeuNAc, their reactivity in water is extremely low. In order to achieve a high conjugation reaction yield between this carbohydrate and another molecule, an activation step is necessary: number of known reactions exist to functionalize hydroxyl groups. A convenient method is to introduce a carbonyl group via an oxidation reaction. The most used technique is by periodate-mediated oxidation of the adjacent hydroxyls at C-7, C-8, and C-9 of sialic acid residues to obtain carbon–carbon bond cleavage and amine reactive aldehydes.[8,11] Other carbohydrate oxidation techniques involve the use of an oxidant such as sodium hypochlorite (NaOCl) to introduce carboxyl and carbonyl groups in polysaccharides.[12] Combined with TEMPO ((2,2,6,6-tetramethyl-1-piperidin-1-yl)oxyl), it selectively oxidizes primary hydroxyl groups in carbohydrates.[13] Such reactions with TEMPO and sialic acid[14] have been described leading to the formation of 9-carboxy-NeuNAc via several reactions. One drawback of these reagents is the difficulty to avoid over-oxidation of the produced carbonyl functions.

In a green chemistry context, it would be interesting to design an inexpensive oxidation reaction capable to selectively activate the SA moiety, at its non-reducing end, under mild reaction conditions. Hydrogen peroxide (H_2O_2), an environmentally friendly oxidant, might be an ideal candidate.

There have been a number of studies of carbohydrate oxidations with hydrogen peroxide as oxidant, like the depolymerization reaction of polysaccharides (chitosan,[15] starch[16] or hyaluronic acid,[17,18] *Neisseria meningitidis* capsular polysaccharides[19,20]), or the degradation of mono and di-saccharides[21,22] but only few

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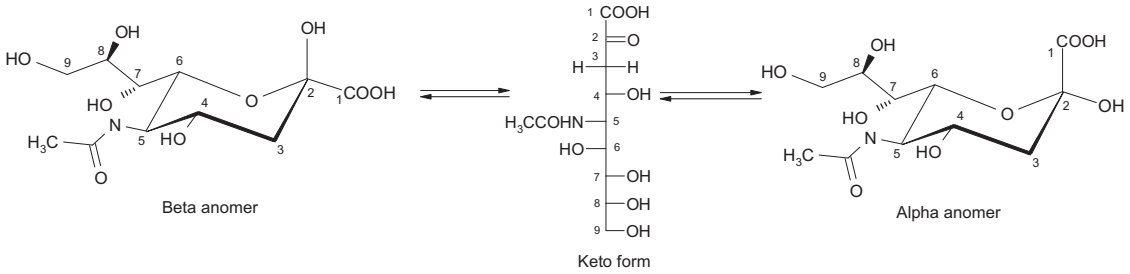

Figure 1. Anomeric forms of N-acetylneuraminic acid.

works have used H_2O_2 oxidation as an activation reaction through the introduction of carbonyl groups.[23]

Isbell and Frush[24] have proposed many mechanisms to explain the formation of various oxidation products formed from carbohydrates by the action of hydrogen peroxide. Under acid catalysis, carbohydrates seem to be inert to H_2O_2. But in alkaline conditions, in presence of traces of metal ions (as shown by Fenton[25]), or under UV light,[16] highly reactive species such as hydroperoxide anion, hydroxyl radical or hydroperoxyl radical can be generated and react with carbohydrates. While hydroperoxide anion will preferentially attack the carbonyl groups like the reducing ends, the radicals will be able to abstract hydrogen from C–H moieties. When hydrogen peroxide is in a large excess, Isbell[26] has postulated that the oxidative degradation of an aldose and a ketose proceeds by a nucleophilic addition of a peroxide anion on the reducing end of the sugar, followed by the fragmentation of the intermediate adduct by a free radical or an ionic mechanism. This alpha-hydroxy hydroperoxide (αHHP) cleavage gives formic acid and the next lower aldose. Repetitions of this reaction lead to the stepwise degradation of the aldose to formic acid.

Such reactions have been done almost entirely with 'simple' carbohydrates such as glucose or fructose but not with more complex sugars like carboxylated alpha-keto sugars. Various alpha-keto acids (similar to the acyclic keto form of the sialic acid) have been shown to react with a molar equiv of H_2O_2 at neutral pH via a nucleophilic attack of the hydroperoxide anion at the carbonyl group adjacent to the carboxylic group.[27] For all the keto-acids studied, the reaction with H_2O_2 gave rise to the formation of carboxylate anions of chain length one carbon shorter. The mechanism described involved the nucleophilic attack followed by a decarboxylation. A similar study has been done by Ijiama and collaborators[28,29] where the reaction between SA and H_2O_2 was characterized. They have found that an equimolar amount of H_2O_2 can oxidize sialic acid at pH 6 and provide its decarbonylated product via the same mechanism.

In order to understand the reaction mechanism of sialic acid residues with hydrogen peroxide, *N*-acetyl neuraminic acid was chosen as the simplest model to react with hydrogen peroxide. These results would be very valuable in case of a future investigation on the oxidation of glycoside derivatives of SA, such as oligosialic acids, with H_2O_2.

In this study we were interested in the degradation of NeuNAc when using common oxidative systems containing excess of H_2O_2 (in the presence of iron, EDTA or under UV light). We identified the main products by LC–MS and NMR of the obtained fragments and compared the data with results obtained from a well known mechanism (sodium periodate oxidation).

The aim of this study was to assess the introduction of carbonyl groups for each oxidative system. The reducing activity of these new carbonyl groups was evaluated by incorporating aniline to the oxidized fragments through a reductive amination. In this paper, we report identification of various structures by LC–MS and NMR.

1. Experimental procedures

1.1. Chemicals

N-Acetylneuraminic acid (NeuNAc) was purchased from Carbosynth. In this present work SA will refer to NeuNAc. All other reagent grade chemicals were purchased from Sigma-Aldrich. The solutions were prepared with Millipore-quality water (Milli-Q plus, Ultrapure water system, 18 MΩ cm).

1.2. Sialic acid oxidation experiments

In all reactions, NeuNAc (120 mg) was dissolved in 20 mL of sodium acetate (50 mM, pH 6). Five different oxidative systems with H_2O_2 were prepared.

The sialic acid solution was treated with the following entries: experiment (a) an equimolar quantity of hydrogen peroxide; experiment (b) a large excess of H_2O_2 (90 molar equiv); experiment (c) 25 μg of $FeCl_2$ were mixed to the NeuNAc solution and a large excess of H_2O_2 (90 molar equiv) was added to the resulting mixture; experiment (d) 50 μg of EDTA were mixed to the NeuNAc solution and a large excess of H_2O_2 (90 molar equiv) was added to the resulting mixture; experiment (e) a large excess of H_2O_2 (90 molar equiv) was added, then the solution was exposed to UV light (2 W/cm^2) using a photochemical device (system Omnicure® S2000).

All the reactions with H_2O_2 were conducted in the dark at 66 °C, during 6 h (except for the UV experiment, 2 h only) while maintaining pH 6 with sodium hydroxide and hydrogen chloride.

The experiments with sodium periodate (1.1 molar equiv) were carried out at 15 °C, pH 6, and stirred during 20 min and then quenched with glycerol.

The oxidized products were dialyzed three times against water (0.1 kDa membrane) and lyophilized.

1.3. Derivatization procedures

The formed carbonyl functions were characterized by derivatization with aniline. The lyophilized products were redissolved in 20 mL of sodium acetate (50 mM, pH 5). Aniline was added (1.1 molar equiv) and the pH was maintained at 5. After 2 h at 25 °C, 1.6 molar equiv of sodium cyanoborohydride ($NaBH_3CN$) was added to reduce the imine intermediates. The solution was stirred at pH 5 and 25 °C during 40 h.

Unoxidized NeuNAc was derivatized with aniline, as a control, to compare the reactivity of the reducing end with the new carbonyls functions formed through the H_2O_2 and sodium periodate reactions.