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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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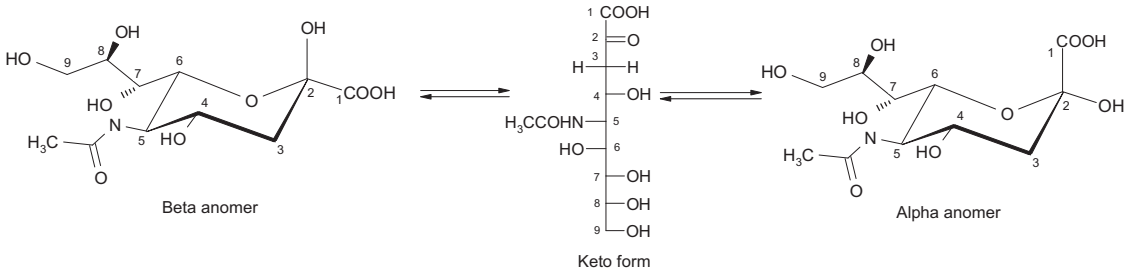


Figure 1. Anomeric forms of N-acetylneuraminic acid.

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.

1.4. Analysis

1.4.1. HILIC–LC–MS analyses

Oxidized and derivatized products were analyzed using HILIC–HPLC (hydrophilic interaction liquid chromatography) with UV detection (at 214 nm for the oxidized products and at 280 nm for the aniline derivatized products) coupled to ESI-MS (electrospray ionization mass spectrometry).

Samples were brought to an acetonitrile content of 80%. The liquid chromatography was performed with a Waters Alliance® system equipped with Diode Array Detector. A Luna® HILIC diol column (length 150 mm, diameter 4.6 mm, particles 3 μm, Phenomenex) was used. 10 μL sample was injected into the column. Fragments were separated with acetonitrile/ammonium formate (80:20, v/v, pH 6) at a flow rate of 0.8 mL/min, during 25 min. Detection was made through a MS/MS ion trap detector (Bruker Daltonics HCT Plus) using an ESI ion source, operated under negative mode. The control of the chromatographic system and data acquisition was achieved with the HyStar™ software Bruker Daltonics incorporating the MS acquisition program, Esquire Control.

1.4.2. NMR measurement

To follow the oxidation of NeuNAc during the reaction, samples were regularly taken from the solution, dialyzed against water to remove the salts and the excess of H_2O_2, lyophilized, and redissolved in D_2O.

^1H and ^{13}C NMR spectra were recorded at 30 °C with a Bruker Avance II spectrometer at 500 MHz equipped with a cryoprobe. The data were recorded using the Bruker TopSpin™ software. The spectra were referenced with respect to an internal standard TSP-d_4 (sodium tetra deuterated trimethylsilyl propionate), at 0 ppm (^1H) or −0.18 ppm (^{13}C).

2. Results and discussion

The mechanism of the hydrogen peroxide oxidation of carbohydrates has been thought to involve the highly reactive hydroxyl radical HO·.[21,22,30] In the present study, hydroxyl radicals were generated by the iron-catalyzed decomposition of H_2O_2 or by irradiation under ultraviolet light of H_2O_2 (homolytic fission). The experiments were repeated in the presence of EDTA (metal chelating agent) to suppress the influence of metal traces upon the oxidative degradation.[31]

2.1. Oxidation of SA with 1 molar equiv of H_2O_2

This experiment (a) confirmed the results from Ijima and collaborators. When SA and H_2O_2 were mixed together at a ratio 1:1 (at 66 °C, pH 6) a fast nucleophilic attack of H_2O_2 on the ketone of the open ring form of the SA happened, followed by a Baeyer–Villiger like rearrangement and an irreversible decarboxylation. The reaction was completed in 52 min (Scheme 1) and gave an acyclic acid, DSA (4-(acetyl-amino)-2,4-dideoxy-D-glycero-D-galacto-octonic acid), fully characterized by LC–MS and NMR (see Supplementary data). The DSA being very stable in its acyclic state, no lactone form was observed.

2.2. Oxidations of SA with excess of H_2O_2

Four different experiments (b–e) were carried out using large excess of H_2O_2 (90 molar equiv). For each of them, the first reaction was the decarbonylation of the SA, as seen previously. In less than 12 min, SA was entirely converted to DSA (no more SA detectable by LC–MS or NMR). Then, a large excess of H_2O_2 was still available to further oxidize the DSA (see Supplementary data). Use of LC–MS analysis showed the formation of selective oxidation products. The ions m/z of the new products were extracted from the total ion chromatograms and were compared.

The four experiments (b–e) seemed to follow the same degradation pathway but with different kinetics.

2.2.1. Excess of H_2O_2 with Fe^{2+}

During the experiment (c), the first peaks $[M-H]^-$ observed by LC–MS were those with m/z 278 (Fig. 2, peaks D, E, G). Then, these peaks tended to decrease and new peaks appeared: three of the new products formed were less hydrophilic than SA and DSA (shorter retention times by HILIC) with m/z of 248, 218, and 188, while three others were more hydrophilic (m/z 264, 234, and 204). After 5 h, a large amount of DSA was still present. Table 1 summarizes the spectral information of the detected peaks. Compared to the control experiment, experiment (b) using H_2O_2 in excess without any treatment, the obtained products were the same but their formation seemed to be slightly faster.

2.2.2. Excess of H_2O_2 with EDTA

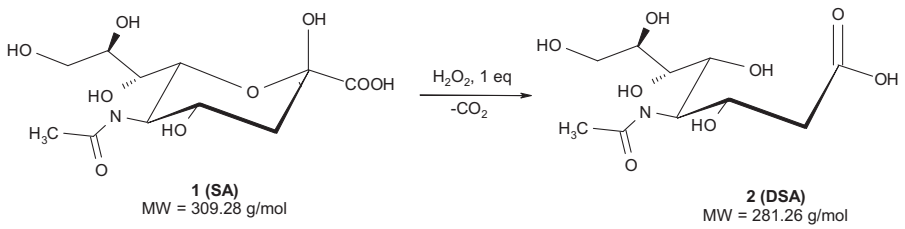
When EDTA was present in the solution, experiment (d), almost no oxidation of DSA occurred. The rise of peaks with a m/z of 278 $[M-H]^-$ was much slower than experiments (b) and (c). No other peaks were detected by LC–MS after 5 h.

2.2.3. Excess of H_2O_2 under UV

The experiment (e), under UV light, was significantly faster than the others. After 1 h, all DSA and the compounds with m/z of 278 disappeared. The reaction was stopped after 2 h in order not to totally degrade the oxidized products. The five predominant products detected by HILIC were those with m/z $[M-H]^-$ of 188, 218, 234, 204, and 264 (peaks A, B, H, I, and J, respectively, Fig. 2 and Table 1).

On the basis of these results, we can assume that the first reaction (decarbonylation of SA to DSA) would occur even without free radicals.

In the next oxidation reactions, the rapid increase in the number of peaks observed for the three experiments without EDTA was attributed to the reaction of DSA with hydroxyl radicals liberated by the Fe^{2+}/Fe^{3+} oxidation-reduction cycle (Fenton reaction) or by the homolytic cleavage of H_2O_2 under UV light. A metal contamination in the reaction mixture could explain the minor differences between the experiments with and without iron. The



Scheme 1. Reaction scheme of the decarbonylation of SA.

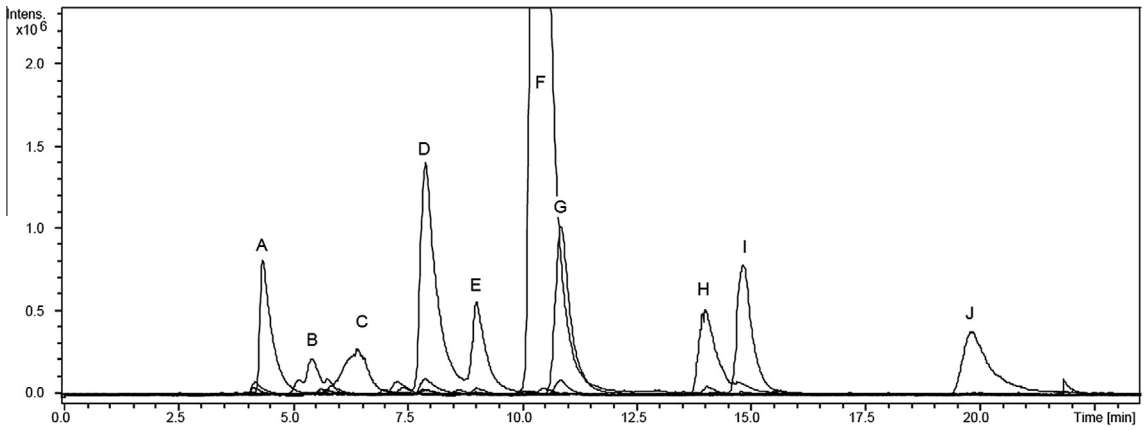


Figure 2. Extracted ion chromatograms of hydrogen peroxide oxidation of DSA in the presence of a trace amount of Fe^{2+}.

Table 1
Spectral information on H_2O_2 oxidative products from DSA in the presence of a trace amount of Fe^{2+}

Peak	A	B	C	D	E	F	G	H	I	J
RT (min)	4.5	5.4	6.4	8.0	9.2	10.5	11.0	14.0	14.9	19.8
$[M-H]^-$ (m/z)	188	218	248	278	278	280 (DSA)	278	234	204	264

presence of EDTA enhanced the quenching of radical hydroxyls,[31] which severely delayed the peroxide reaction, but did not stop it completely.

Studies on H_2O_2 oxidation[24] have shown that HO· can abstract hydrogen C–H moieties of an alcohol group, forming a radical intermediate, which then reacts with hydrogen peroxide affording a carbonyl group. In a same way, hydroxyl group of DSA could be oxidized to a carbonyl group, which would explain the formation of products with a m/z $[M-H]^-$ of 278. The hydrogen abstraction by hydroxyl radical being little or not selective, the five hydroxyl groups of DSA can potentially be oxidized. One of these reactions is shown in Scheme 2.

The further oxidation and degradation of these compounds could generate a series of acids and carbonyl derivatives.[24] A plausible reaction pathway would involve the nucleophilic addition of hydrogen peroxide to the carbonyl group, followed by a heterolytic alpha-hydroxy-hydroperoxide (αHHP) cleavage of the adduct. This would explain the formation of the products described in Table 1. The possible mechanism of formation of products with a molecular weight of 265 g/mol and 219 g/mol is depicted in Scheme 3.

A similar pathway could explain the formation of two other aldehydes (MW = 189 and 249 g/mol) and two other carboxylic acids (MW = 205 and 235 g/mol).

The aldehyde groups, almost completely hydrated to diols or cyclic hemiacetals, gave rise to several characteristic signals in the proton and carbon NMR spectra. The presence of carbonyl groups was confirmed by the addition of the carbonyl reductive agent $NaBH_4$ which caused a marked decrease of these NMR signals.

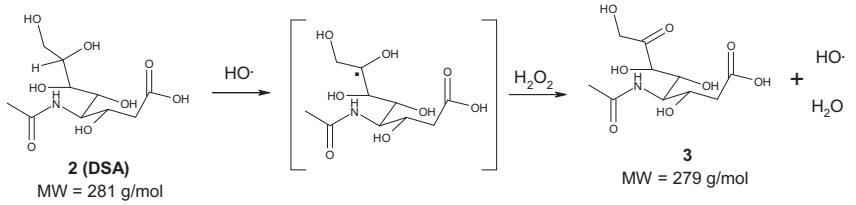
To summarize the hydrogen peroxide oxidation, the degradation products of SA and their relative concentrations observed by HPLC, according to the oxidation conditions, are presented in Scheme 4 and Table 2. Products **2**, **4**, **7,** and **8** were fully characterized by ^1H and ^{13}C NMR (see Supplementary data).

2.3. Derivatization of the oxidized compounds

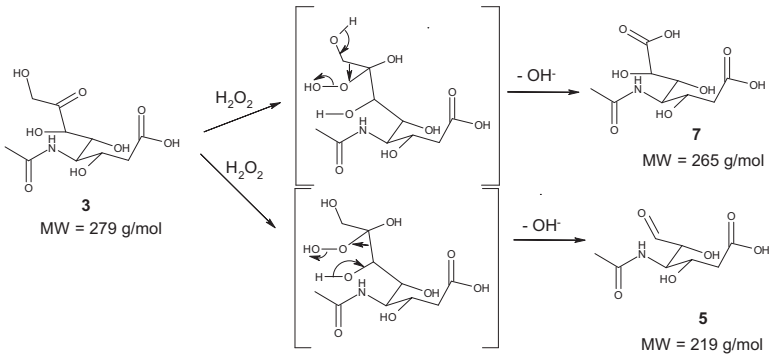
To confirm the presence of carbonyl groups, the degradation compounds for each oxidation were derivatized by reductive amination with aniline under the same conditions previously described and analyzed by LC–MS.

No reaction was observed between aniline and DSA or the di-carboxylic acids (products **7**, **8,** and **9**, Scheme 4), as this derivatization method was limited to carbonyl compounds. These four compounds were still present and visible by LC–MS at the end of the reductive amination.

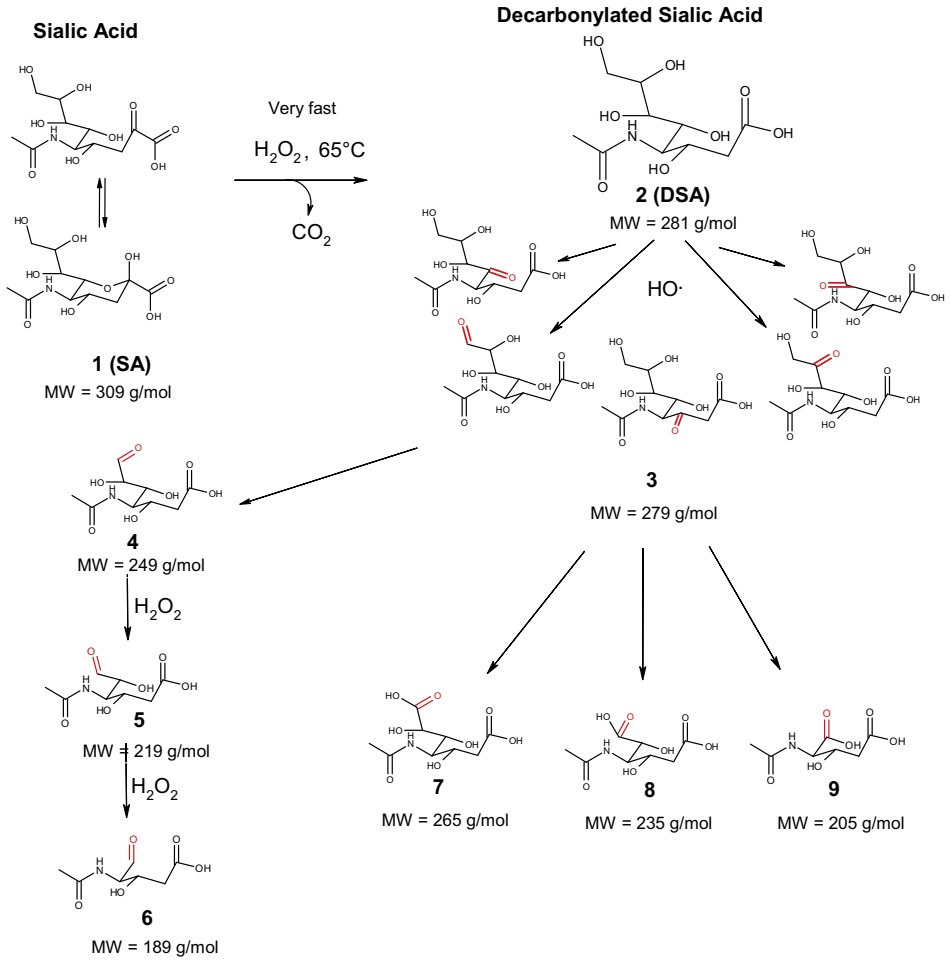
Four different structures absorbing at 280 nm (aniline absorption band) were identified (in different amounts according to the H_2O_2 oxidation condition), corresponding to the derivatization of the products **3**, **4**, **5,** and **6**. We were surprised to see that the derivatization kinetic was different according to the aldehyde. Product **3** (279 g/mol) and product **5** (219 g/mol) were reacting almost instantaneously with aniline and after $NaBH_3CN$ addition to form the derivatized structures with a mass of 356 g/mol and 296 g/mol, respectively, while it took more time for the other aldehydes to react. The derivatization of product **4** (249 g/mol) lasted around 20 h. A cyclization of product **4** by nucleophilic addition reaction between the carbonyl group and a hydroxyl group might happen



Scheme 2. Proposed reaction pathway for the formation of one of the products with a MW of 279 g/mol.



Scheme 3. Proposed mechanisms for the αHHP cleavage and the formation of new carbonyl and carboxylic groups.



Scheme 4. H_2O_2 degradation products of SA formed under various conditions.

and yield a six-membered ring (Fig. 3) more stable than a 7 or 5-membered ring.

This stable hemiacetal ring form could explain the slower derivatization reaction compared to the other products.

To verify the product structures obtained after H_2O_2 oxidation and derivatization, SA was decarbonylated with an excess of H_2O_2 in the presence of EDTA (to inhibit the oxidation reaction). Then, the produced DSA was submitted to periodate oxidation, a well known reaction where sodium periodate specifically cleaved at the adjacent hydroxyls of the non-reducing end (between carbon atoms 6, 7, and 8) of DSA to form three different aldehydes (with a mass of 189, 219, and 249 g/mol). These three intermediates were derivatized with aniline under the conditions previously used. The results (retention time and mass spectra) were similar than those observed with products **4**, **5,** and **6** (Scheme 4). Thus, this experiment confirmed the structure of the derivatized products obtained after the initial H_2O_2 oxidation.

The study (by HPLC at 280 nm) of the integrated peak areas of aniline excess and derivatized products helped us to estimate the derivatization yield of the carbonyl compounds according to the conditions of the prior oxidation reactions. The results are presented in Table 3.

Table 2
Degradation products of SA formed under various conditions and their relative concentration

Products	Reaction conditions				
	H_2O_2 equimolar	H_2O_2 ex[a]	H_2O_2 ex[a] + EDTA	H_2O_2 ex[a] + $FeCl_2$	H_2O_2 ex[a] + UV
2 (DSA)	+++	++	+++	+	0
3	0	++	+	++	0
4	0	+	0	++	0
5	0	0	0	+	+
6	0	0	0	+	+
7	0	+	0	+	+
8	0	+	0	+	+
9	0	+	0	+	+

('+++' indicates a compound present in higher concentration,'0' indicates an absent compound).
[a] ex: excess.

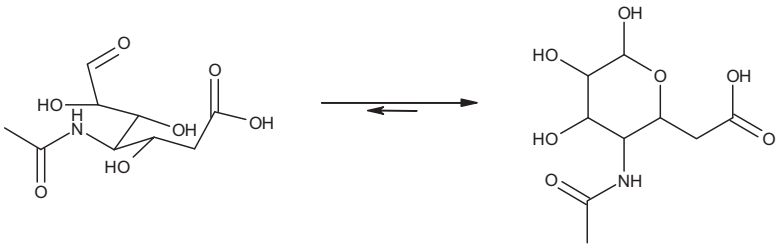


Figure 3. Proposed structures of product **4**.

Table 3
Aniline consumed during each derivatization reactions

Oxidation conditions	(a) H_2O_2 equimolar	(b) H_2O_2 excess	(c) H_2O_2 excess + EDTA	(d) H_2O_2 excess + Fe^{2+}	(e) H_2O_2 excess + UV	(f) H_2O_2 excess + $NaIO_4$	(g) No oxidative reagent
Aniline consumed	0%	20%	8%	16%	8%	40%	31%

The integrated peak areas of the derivatized products obtained after H_2O_2 oxidation were the highest for the experiments (b) and (d) (excess of H_2O_2 with or without Fe^{2+}). The amount of consumed aniline was also the most important for these two experiments (between 16% and 20%). As expected, the derivatization yields were lower for the experiments (a) and (c) (one equimolar H_2O_2 and with EDTA), but we were surprised to see that the consumed aniline for the reaction (e) (under UV) was almost three times less important than for the experiments (b) and (d). Probably during the H_2O_2 oxidation step, the quantity of very reactive hydroxyl radicals was too important. Thus, the carbonyl groups could undergo an over-oxidation resulting in unreactive smaller compounds like formic acid or carboxylic acid. As a comparison, 31% of aniline was consumed during the derivatization of the SA at its reducing end (without any prior oxidation reaction).

The sodium periodate oxidized compounds showed a significantly higher yield of derivatization as the aniline consumed was at least twice higher than the other derivatizations.

These results demonstrated that the periodate oxidation of DSA yielded a higher amount of carbonyl groups compared to the hydrogen peroxide oxidation. The modest derivatization yield of H_2O_2 oxidized compounds could be explained by an over-oxidation phenomenon responsible for the formation of underivatizable carboxylic acids. This fact was emphasized during the reaction under UV light, where a large amount of hydroxyl radicals were generated. It is also interesting to note that the formation of carbonyl groups on SA residues using H_2O_2 can be tuned by various oxidizing process conditions.

3. Conclusion

In summary, we developed a new method to activate sialic acid at its non-reducing end by generating carbonyl functions. Indeed, the chemical modification, by hydrogen peroxide-mediated oxidation, lead, via αHPP cleavages, to different carboxyl and carbonyl functions along the glycerol side chain of sialic acid (Scheme 4). The reducing activity was relatively modest compared to periodate mediated oxidation. It could be explained by the formation of undesired carboxylic acids and by over-oxidation reactions resulting in unreactive smaller compounds, which lower the derivatization yield.

Further research toward optimal oxidation conditions, such as reaction temperature, H_2O_2 concentration, and pH, might control SA reducing activity. Once these conditions were well defined, another study could be realized with oligosaccharide derivatives of SA.

Hydrogen peroxide appeared to be a non toxic and environmentally-friendly alternative to the use of sodium periodate as an activation agent of sialic acid residues.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carres.2014.01.007.

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