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Direct Experimental Characterization of a Sialyl Cation

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Sialic acids are monosaccharide residues involved in several biological processes. Controlling the stereoselectivity of sialylation reactions is challenging and mechanistic studies on the structure of its intermediate, the sialyl cation, are scarce. Here it is shown that a sialyl cation can be generated and isolated from an ionized sialic acid precursor. This short-lived species is structurally characterized for the first time using cryogenic infrared spectroscopy. In combination with quantum chemical

Introduction

Sialic acids are monosaccharides found in glycolipids, glycoproteins, and human milk oligosaccharides.^[1] They often occupy the terminal position of the non-reducing end in glycan sequences and mediate recognition processes between hosts and pathogens.^[2] For example, it has been determined that the receptor-binding domain of the SARS-CoV-2 spike protein binds preferentially to monosialylated gangliosides.^[3] More than 50 sialic acids are known, which are all based on neuraminic acid.^[4]

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calculations, the results reveal that the positive charge at the anomeric carbon of the sialyl cation is stabilized by remote participation of the C5-NHAc group leading to the formation of a bridged structure. In this structure, the β -side is shielded from nucleophilic attack, potentially explaining the α -selectivity of this building block in S_N1-type sialylation reactions. Other modes of participation are energetically unfavored and cannot be observed experimentally.

The most common variant in mammalian cells is *N*-acetylneuraminic acid (Neu5Ac, Scheme 1). Biosynthetically, sialic acids are built up by a complex biochemical pathway. Sialyltransferes facilitate the attachment to glycans at the non-reducing end^[4–5] which exclusively yields α -glycosidic linkages with a 2,3 or 2,6 regiochemistry.^[6]

Sialylated glycans are promising candidates for drug and vaccine development.^[7] However, the *in vitro* synthesis of α -linkages for sialic acid donors remains challenging.^[8] Several methods and building blocks have been proposed to increase the α -selectivity in sialylations,^[9] including participating auxiliaries^[10] at the C1- or the C3-position, functionalization of the C5-NHAc group,^[11] assistance of nitrile solvents,^[12] and



Scheme 1. Sialylation reaction involving a sialyl cation intermediate. A = activator. Nu = nucleophile.

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constrained sialyl donors.^[6] We performed a literature survey of sialylation reactions using donor **1** with weak nucleophiles (Table 1). Traditionally, the mechanistic spectrum of glycosylation reactions is governed by an $S_N 1-S_N 2$ continuum (Figure S3).^[13] Depending on the reaction conditions, such as the strength of the nucleophile, the reaction is more likely to proceed via an $S_N 1$ - or $S_N 2$ -like pathway. With weak nucleophiles an $S_N 1$ -like behavior can be expected.^[14] Most listed sialylations utilize acetonitrile as solvent or cosolvent.^[15] Here,



 $\label{eq:LG} LG = leaving group. \ DCM = dichloromethane. \ MeCN = acetonitrile. \\ DMTST = dimethyl(methylthio)sulfoniumtetrafluoroborat. \ PhSOTf = benzene-sulfenyltriflate. \ DTBP = 2,6-di-tert-butylpyridine. \ TfOH = triflic acid. \ NIS = N-iodosuccinimide. \ TMSOTf = trimethylsilyl-trifluorometane-sulfonate. [a] These donors were used in a 1:1 anomeric mixture. \\ \ \end{tabular}$

the formation of α -glycosides with stereoselectivities ranging between 24:1 to 1.5:1 α : β is favored. Acetonitrile participation could lead to the formation of nitrilium ions, serving as explanation for the increased α -selectivity.^[15e] When acetonitrile is not used, such as in Koenigs-Knorr glycosylation,^[16] TMSOTf activation of a sialyl imidate,^[15e] and NIS activation of a thioglycoside,^[17] conflicting stereoselectivities can be observed. In the Koenigs-Knorr sialylation,^[16,18] only α -sialosides are formed. Previously, it has been invoked that Koenigs-Knorr glycosylations could proceed through positively charged intermediates, possibly stabilized by neighboring group participation.^[19] Likewise, using NIS as a promoter led to moderate α -selectivity (2:1 α : β).^[17] Contrary to these results, TMSOTf activation in DCM resulted in high β -selectivity (1:48 α : β).^[15e]

To gain a deeper mechanistic understanding of sialylation reactions, the structure of the putative sialyl cation intermediate (Scheme 1) is investigated in this study. Due to its short lifetime, it has so far only been characterized using computational^[20] and kinetic methods.^[21] In preparative carbohydrate synthesis, the sialyl cation, which carries a positive charge at the anomeric carbon (C2), is formed via ejection of a leaving group (e.g., departure of thiophenolate from sialic acid donor 1 under acidic conditions, Scheme 1). Neighboring^[22] or remote acyl groups^[23] can interact with the positively charged anomeric carbon to form a covalent bond that shields one side from nucleophilic attack, leading to an overall increased stereoselectivity. In theory, all five acyl groups in 1 can covalently stabilize the transient sialyl cation to form bicyclic structures. In this study, we investigate the structure of a sialyl cation generated from the fully protected Neu5Ac donor 1 using cryogenic infrared (IR) spectroscopy using both the messenger-tagging and the helium nanodroplet methods as well as quantum chemical calculations. In recent years, several glycosyl cations were characterized using IR spectroscopy in helium nanodroplets and IR multiple photon dissociation spectroscopy.^[22-24] Besides, glycosyl cations were stabilized in super acids and studied with NMR spectroscopy.^[25]

Experimental Section

Infrared Spectroscopy of Mass-to-Charge Selected Ions

The sialyl cation precursor 1 was synthesized according to a reported procedure.^[26] IR spectra were recorded on two custombuilt instruments. The Yale photofragmentation mass spectrometer^[27] and the Fritz Haber Institute (FHI) helium droplet instrument $^{\scriptscriptstyle [22c,23a,\ 28]}$ used for this study have previously been described and a detailed description can be found in the SI. Briefly, electrospray ionization and subsequent fragmentation leads to the formation of ions of interest, which are subsequently isolated and cooled to cryogenic temperatures. In the Yale instrument, the isolated ions are tagged with H₂ or D₂ at ca. 20 K in a cryogenic ion trap. When the IR frequency of the employed OPO/OPA laser system (1000-4000 cm⁻¹) is resonant with a vibrational transition of the ion, vibrational excitation causes the dissociation of the weaklybound tag through absorption of a resonant photon. A spectrum is obtained by monitoring the photofragmentation yield as a function of IR wavenumber.

In the FHI helium droplet instrument, the trapped ions are embedded in superfluid helium nanodroplets (ca. 0.4 K). After absorption of multiple resonant IR photons generated by the Fritz Haber Institute free-electron laser (FHI-FEL),^[29] the bare ions are released from the droplet and detected using a time-of-flight mass analyzer. The spectrum is generated by plotting the bare ion signal as a function of the IR wavenumber (700–2000 cm⁻¹).

Quantum Chemical Calculations

The experimental spectra were correlated to chemical structures by performing quantum chemical calculations, which are explained in detail in the SI. Briefly, the conformational space of the investigated sialyl cation was sampled using the computational tools FAFOOM^[30] and CREST.^[31] The conformational search with FAFOOM yielded structures of the oxocarbenium-type with no participation and participation of the C4, C5, and C7 groups, which were optimized at the PBE+vdW^{TS}/light⁽³²⁾ level of theory using FHI-aims.^[33] In addition, structural motifs incorporating no participation or participation of any acetyl group were sampled using CREST. The C7-, C8-, and C9-acetyl groups can interact either from the α - or the β -side, as they are located on the more flexible C6-arm. The structures generated by CREST are optimized either at the semi-empirical GFN2-xTB^[34] or the empirical GFN-FF^[35] methods. Representative structures, generated by both FAFOOM and CREST were reoptimized, and their harmonic frequencies were computed using Gaussian $16^{[36]}$ at the PBE0+D3/6-311+G(d,p)^[37] level of theory (Table S2). The harmonic frequencies are scaled by empirical scaling factor of 0.965 in the 1000-2000 cm⁻¹ region, which previously yielded good results for glycosyl cations, $^{\scriptscriptstyle [23b, \widetilde{2}4b]}$ and by 0.954 in the 2600–3600 cm⁻¹ region.^[38] More accurate electronic energies of the lowest-energy structures for each motif were obtained by carrying out DLPNO-CCSD(T)/def2-TZVPP^[39] single-point energy calculations using ORCA 5.0.3 (Table S3).^[40]

Results and Discussion

The mass spectrum of the sialyl donor 1 after electrospray ionization followed by in-source fragmentation is shown in Figures 1 and S4. Like the reactivity behavior observed in the condensed phase, the thiophenyl leaving group is readily cleaved upon protonation/sodiation (m/z 584 and 606) leading to the sialyl cation (m/z 474). The sialyl cation undergoes facile loss of acetic acid yielding the ion at m/z 414.

IR spectra (1000–2000 cm⁻¹) of the sialyl cation obtained using He nanodroplets and H_2/D_2 tagging are compared in Figure 2a, 2b, and S5. The two methods yield very similar band patterns. Typically, the absorption bands in the 1500–1900 cm⁻¹ region yield the most diagnostic information regarding the interaction between one of the acetyl groups and the positively charged anomeric carbon. For example, the absorption bands around 1750 cm⁻¹ (**#6**) originate from the carbonyl groups that do not interact with the anomeric carbon. The 1000–1500 cm⁻¹ region is less informative, as it mainly contains bands originating from C–H bending modes around 1400 cm⁻¹ (**#3**) and C–O stretching modes around 1250 cm⁻¹ (**#2**). The band around 1500 cm⁻¹ (**#4**), is observable as a weak absorption band in the messenger-tagging spectrum and only a minor signal in the



Figure 1. ESI-(+)-mass spectrum of the fully protected sialyl donor 1, identified as $[M + H]^+$ at m/z 584 and $[M + Na]^+$ at m/z 606. Fragmentation of the precursor leads to loss of the leaving group (–SPh) and the formation of a sialyl cation at m/z 474. The sialyl cation can undergo loss of acetic acid (–HOAc, m/z 414).

helium droplet spectrum. Such differences have been reported previously^[41] and are usually traced to the linear vs. multiple photon dependences on power.

The computational results reveal that the putative global minimum structure (Figure 2c, blue) features intramolecular stabilization of the positive charge by interacting with the C5-NHAc group (1.5 Å). Interaction with the C4-OAc group (1.5 Å) is disfavored by 34 kJ mol⁻¹ (Figure 2d, red), although it was the lowest-energy structure in a recent computational study,^[20b] where the C5-group is trifluoroacetylated. Electron-withdrawing substituents disable or decrease the ability of an acyl group to stabilize a positively charged anomeric carbon.^[23a] The lowestenergy oxocarbenium-type structure (Figure 2e, purple), which exhibits long-range stabilization by the C4-OAc group (2.1 Å), is disfavored by 50 kJ mol⁻¹. The lowest-energy oxocarbeniumtype structure without such interaction (Figure S6b) is disfavored by 72 kJ mol⁻¹. Intramolecular interactions with the C7-, C8-, or C9-OAc groups, which can interact with the anomeric carbon from either the α - or the β -side, are destabilized by 66– 143 kJ mol⁻¹ relative to the global minimum. Similar trends are observed when comparing Gibbs free energies (Table S3).

The predicted spectra for various structures (Figure 2) show a high degree of similarity in the 1000–1500 \mbox{cm}^{-1} region, whereas clear differences can be observed in the 1500-1900 cm⁻¹ region. Comparison of the experimental spectra (Figure 2b) with the scaled harmonic frequencies obtained for the global minimum structure (Figure 2c) indicates that it is indeed the best match. The bands 1628 and 1509 cm⁻¹ (#5' and #4'), which are associated with the oxazolidonium (NCO⁺) moiety, are particularly informative since only the global minimum structure recovers the two features. The 1628 cm⁻¹ band is mostly associated with the C=N⁺ stretching mode, while the feature around 1500 cm⁻¹ originates from the newly formed C-O bond by participation of the NHAc group. The carbonyl stretch at 1725 cm⁻¹ (#**6**_a') originates from the C9-OAc group, where the carbonyl oxygen interacts with the N-H hydrogen atom at relatively long range (2.8 Å). The feature at

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Figure 2. Experimental IR spectrum of the sialyl cation (gray) generated from 1 (a) in helium droplets and (b) using D_2 -messenger-tagging spectroscopy in the 1000–2000 cm⁻¹ region. The scaled harmonic frequencies of computed structural candidates are shown in the inverted traces for (c) participation of the C5-NHAc group (blue), (d) the C4-OAc group (red), and (e) no participation (purple). Simplified representations of each of the three structural motifs are shown on the right side with their puckers and relative energies indicated. The positively charged anomeric carbon (C2) is labeled by a red circle. Some vibrational bands are labeled to guide the reader through the text. The vibration #4 is labeled with an arrow to make it more distinguishable. 3D-representations and computed spectra of other structural candidates are shown in the supporting information.

1769 cm⁻¹ (**#6**_b') is composed of three carbonyl stretches of the C8-, C7-, and C4-OAc groups, whereas the frequency at 1814 cm⁻¹ (**#6**_c') originates from the carbonyl stretch in the COOMe group. Additionally, the computed harmonic frequencies in the 1000–1500 cm⁻¹ region, mainly originating from C–O stretching and C–H bending modes, match the experimental spectrum well.

The harmonic frequencies of the other computed structural motifs also exhibit matching vibrational bands, however, the experimental $C=N^+$ stretch (#5), is completely absent in these spectra. Additionally, some key features are predicted for these structural motifs, which are not reproduced in the experiment: i) for C4-OAc group participation (Figure 2d), symmetric and antisymmetric stretches of the O–C=O⁺ moiety are expected at 1474 and 1563 cm⁻¹, ii) for the oxocarbenium-type structure (Figure 2e), an oxocarbenium (C=O⁺) stretching and an amide II band are predicted at 1539 and 1492 cm⁻¹. The computed frequencies of the other predicted modes of participation, match the experiment less well and are energetically highly destabilized (Figure S6). Boltzmann-averaged computational spectra (Figure S7) look identical to the spectra of the lowestenergy structures, as higher-energy structures are hardly populated at the experimental temperature of 20 K.

Additionally, we used the H_2/D_2 tagging approach, to record IR spectra in the 2600–3600 cm⁻¹ region (Figure 3a). The experimental spectrum shows an isolated peak at 3394 cm⁻¹

(#8), which corresponds to the N-H stretching mode of the NHAc group. The broad feature with centroid at 2968 cm⁻¹ (**#7**) originates from multiple overlapping C-H stretching modes. Although the position of the computed frequencies for this mode are matching, the frequencies do not match the broad distribution of the absorption bands and significantly underestimate their intensity. To account for the anharmonicity of N-H and O-H bands, smaller scaling factors are used in the 2700-4000 cm⁻¹ region.^[38] Despite the adjusted scaling factor, the computed frequency for the N-H stretching mode at 3454 cm⁻¹ (**#8**') is still overestimated by 60 cm⁻¹ for the structure exhibiting C5-NHAc participation (Figure 3b). Even though, it is often assumed that the tag only has a small influence on the investigated structure,^[42] we considered the H₂-tagged structure (Figure 3c, 3d, and S8), due to the unsatisfactory match of the N-H stretching band. Two isoenergetic low-energy tagging isomers were sampled. In the IR spectrum of isomer a (Figure 3c), with side-on coordination of H₂ to the N–H hydrogen (Figure S13), the N-H absorption band is significantly shifted to 3396 cm⁻¹ and now aligns well with the experimental value of 3394 cm⁻¹. The IR spectrum of isomer b (Figure 3d), with unspecific H₂ coordination, on the other hand, is not perturbed by the tag. As both tagging positions are thermodynamically favored, the implications of the tag on the N-H stretch frequency are not clear. No significant absorption bands are

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Figure 3. (a) Experimental IR spectrum of the sialyl cation using D₂messenger-tagging spectroscopy in the 2600-3600 cm⁻¹ region. The scaled harmonic frequencies of computed structural candidates are shown in the inverted traces for (b) C5-NHAc group participation without and (c,d) with H₂-tag, (e) C4-OAc group participation, and (f) no participation. The spectra in c and d originate from tagging isomers.

Wavenumber [cm⁻¹]

observed in the 700-1000 and 2000-2600 cm⁻¹ regions (Figure S5).

The lowest-energy structures exhibiting C5- and C4-participation as well as oxocarbenium with and without C4-OAc longrange interactions were reoptimized in eight different solvents using an implicit solvent model (Tables S4–S5). In accordance with a recently published study,^[43] the relative energies are shown as a function of the relative permittivity of the respective solvents (Figure S9). The energies remain similar within a window of $\pm 10 \text{ kJmol}^{-1}$. In all cases, the structure exhibiting C5-NHAc participation remains the most stable motif by more than 25 kJ mol⁻¹.

The experimental and computational results suggest that the sialyl cation is stabilized by remote participation of the C5-NHAc group. This mode of participation might be favored due to the increased nucleophilicity of the NHAc group compared to OAc groups. In this structure, the β -side of the sialyl cation is shielded from nucleophilic attack, leading to increased α selectivity. Indeed, in a previously reported Koenigs-Knorr sialylation reaction, where a sialyl cation was the likely intermediate, α -sialosides were obtained as the major product (Table 1).^[16,44] When using acetonitrile as (co)solvent, the α product is generally obtained as the major product, with significant amounts of β -sialosides.^[15b] The α -selectivity has been rationalized by the formation of β -nitrilium ions.^[15e] However, our calculations indicate that the formation of $\alpha\text{-}$ and β -nitrilium ions is generally unfavored by 76/41 kJmol⁻¹ (ΔG_{233K}) compared to the structure exhibiting C5-NHAc participation. These results shift when using a solvent model (e.g. in acetonitrile 37/28 kJ mol⁻¹, Table S6). Despite these results, which show that the formation of the C5-NHAc structure is thermodynamically favored, the formation of nitrilium ions in sialylations cannot be fully ruled out, as the kinetics of nitriliumand bridged ion-formation are not understood yet. Using triflate promoters in pure dichloromethane leads to a high β-selectivity, which is likely due to the formation of anomeric triflates.^[15e,45] Excellent α -selectivities in sialylations have also been obtained with the C5-NHAc group modified in a way that prevents C5participation.^[11b,c, 46] Elucidating the mechanism behind the α selectivities in these specific cases is out of the scope of this work.

Conclusions

This study shows that cryogenic IR spectroscopy is an enabling technique to investigate short-lived reaction intermediates, such as sialyl cations. In combination with extensive DFT calculations, the experimental data reveals that the sialyl cation intermediate exhibits remote participation of the C5-NHAc group to stabilize the positively charged anomeric carbon. This mode of participation only allows for nucleophilic attack from the α -side. From all conceivable structures for this sialyl cation, the one exhibiting C5-NHAc remote participation aligns the most closely with the experimental spectrum. All experimental features are reproduced well, such as the one originating from the C=N⁺ stretching mode at 1628 cm⁻¹, which is missing in the other computed spectra. Thus, the herein reported data serves as basis for mechanistic understanding for the high amounts of *a*-products obtained in Koenigs-Knorr and possibly other sialylation reactions. The authors are aware that correlations between the gas and the condensed phase must be handled with caution. In solution, the mechanism of glycosylation reactions falls somewhere on the $S_N 1-S_N 2$ continuum, depending on the reaction conditions.[13] Under these conditions, acetonitrile molecules and triflate anions can form adducts to stabilize the intermediate. The gas-phase on the other hand represents one extreme side of the continuum and provides structural insight that are biased towards the S_N1-side of sialylation reactions. However, the results clearly show that an a-selective structure exhibiting C5-NHAc remote participation is favored. Future studies, focusing on nitrilium and triflate intermediates will help to provide a complete picture of sialylation reactions.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: glycosylation · infrared spectroscopy · mass spectrometry · carbohydrates · sialic acid

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