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Ab initio calculations on various sialic acids provide valuable information about sialic acid-specific enzymes

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Abstract

This study presents ab initio calculations on six sialic acid derivatives (*N*-acetylneuraminic acid, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid, *N*-acetyl-4-*O*-acetylneuraminic acid, *N*-glycolylneuraminic acid, *N*-glycolyl-4-*O*-acetylneuraminic acid and *N*-acetyl-9-*O*-acetylneuraminic acid). The calculations were carried out using the GAMESS-UK-Program. Since the charge distribution of a ligand has an essential impact on the specific interaction with an enzyme or a receptor, the precise results of ab initio calculations lead to significant information concerning possible roles of the different functional groups occurring on sialic acids. In correlation to this, valuable conclusions about the activities of different sialic acid-specific enzymes, such as sialidases, trans-sialidases, lyases and *O*-acetyl- or *O*-methyltransferases can be drawn, since these activities strongly depend on the presence or absence of the various functional groups.

Keywords: Sialic acids; Enzyme reactions; Ab initio; NMR; Molecular dynamic simulation

1. Introduction

Sialic acids appear as terminal parts in the carbohydrate chains of glycolipids or glycoproteins mainly on cell surfaces and of secreted macromolecules. They are important in the regulation processes of many cellular events and thus are considered to belong to the most important molecules of life [1–9]. Modifications of the sialic acid moieties such as *O*-acetylation or *N*-acetyl hydroxylation are correlated with alterations in the recognition specificity by receptors (e.g. lectin or antibody) or enzymes binding or metabolizing sialic acids [3,5–10]. However, it is not completely understood whether these modifications are caused mainly by steric and structural effects or via an alteration in the charge distribution. A detailed structural and functional knowledge of such modifications on a submolecular level requires sophisticated experimental and computational methods. NMR-calculations and molecular dynamic (MD) simulations have successfully been carried out in order to address the question whether certain functional groups (e.g. *O*-acetyl groups) have a conformational influence on the carbohydrate part or are rather acting as direct contact points [6,11]. Ab initio calculations with their high degree of accuracy are applied to determine the impact of the different functional groups on the sialic acid derivatives under study. The results obtained by this approach were correlated with MD- and NMR-data [11] and thereby used to provide further insight into the basic principles of the effect of sialic acid modifications. A combination of NMR-experiments with molecular mechanic and MD studies have proven that a remarkable structural influence of the *O*-acetyl and/or *N*-glycolyl group on the overall conformation is not detectable in the case of GM3-gangliosides [11]. MD-studies of higher gangliosides show that this is also the case for 9-*O*-acetyl-GD1a when compared to

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unsubstituted GD1a [6]. These findings are supported by NMR-experiments of 9-*O*-acetyl-GD1a and an antibody fraction with preferential binding to *O*-acetyl sialic acid [6]. However, the interaction analysis of 9-*O*-acetyl-GD1a gave some indications that a few long-range interactions exist between several functional groups on different sugar residues of this ganglioside [6].

Ab initio calculations are the best tool to document an impact on the molecule's charge distribution, even in the case of a single atom variation in a panel of otherwise identical molecules, as for example demonstrated for several α, β-unsaturated ketones (β-O, β-N and β-S) [12]. In order to address still unanswered questions of biological relevance which are correlated with differences in one or more atoms of a sialic acid molecule, the electron-donating properties and further characteristics of the *N*-acetyl-, *N*- glycolyl-, *O*-acetyl- and other functional groups of sialic acid derivatives are computed and discussed in this paper. Neu5Ac (*N*-acetylneuraminic acid), Neu2en5Ac (2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid), Neu4,5Ac_2 (*N*-acetyl-4-*O*-acetylneuraminic acid), Neu4Ac5Gc (*N*-glycolyl-4-*O*-acetylneuraminic acid) and Neu5,9Ac_2 (*N*-acetyl-9-*O*-acetylneuraminic acid) were chosen as suitable model compounds. In combination with previous results from e.g. NMR-experiments and classical MD simulations, the knowledge about charge distributions, bond lengths and dihedral angles calculated in this study for four different sialic acids by ab initio methods shall therefore contribute to correlate the presence of special functional groups with their potential biological efficiency.

2. Computational details

The calculations were performed using the quantum chemical program GAMESS-UK [13] and a direct-SCF algorithm [14]. The calculations were run on a Silicon Graphics Power Challenge computer, an IBM RS/6000 43P model 260, a SUN Ultrasparc, and a Macintosh-G3 under LINUX. All machines except the SUN, which was located at the Pacific Northwest National Laboratory (PNNL) Richland (WA) USA, were located at the Theoretical Chemistry Group, Utrecht.

The starting geometries for the geometry optimizations were obtained with the help of a simulated annealing procedure, using the program Chem3D [15]. The geometries were optimized at the RHF (Restricted Hartree–Fock)/6-31G* [16] level employing no symmetry. The resulting structures are thus local minima. Different conformations were obtained for each molecule. The geometries with the lowest total energy are reported here. Electron distributions were calculated using a Distributed Multipole Analysis (DMA) [17] Mulliken populations [18] are employed to consider the bond-strengths.

3. Results and discussion

3.1. Data from ab initio calculations

In Table 1 a comparison between the crystal structure [19] and the ab initio optimized geometry of α-D-*N*-acetyl-2-*O*-methylneuraminic acid methyl ester (1,2diMeα Neu5Ac) is given. Probably, the ab initio bondlengths are slightly too short (since they are determined with the Hartree–Fock method) and the ab initio numbers refer to a free molecule in the gas phase. Still, the ab initio calculations which refer to the molecule (in its gas-phase), are in close agreement with those of the crystal structure. Distances are within 0.04 Å, angles within 8°, dihedral angles within 11°, and frequently the agreement is much better than this. These results suggest that the shape of the molecules in crystal, gas phase, or solution will be quite alike, so that our calculation addresses the natural situation in biological systems. The beta form of Neu5Ac ($E = -1155.831449$ Hartree) is slightly more stable than the alpha form ($E = -1155.829920$ Hartree). However, the difference is only about 1 kcal/mol and can be regarded as not very significant.

The alpha form is given in Fig. 1 including numbering. The charges over 0.3 electron are shown in Table 2. To determine the most probable place for a nucleophilic attack, we considered the charges of the nearby positive atoms in addition to the charges on the hydrogen atoms themselves. The results in this Table then suggest that the H(2–1) of the OH group attached to the rather positive C(2) atom is a prime site. Of course, the H of the carboxylic group close by is also similarly charged. The positive sites of Neu5Ac are more masked by negative, surrounding atoms. Such groups like the hydroxyls attached to C(7) and C(8) are thus the next possible candidates for proton abstraction.

The charge of C(3) is actually slightly negative (−0.2 for Neu5Gc and a bit less negative for Neu5Ac, Neu5,9Ac_2, Neu4Ac5Gc and Neu4,5Ac_2). The small but negative charge of Neu5Ac might aid the formation of the double bond when generating Neu2en5Ac. The Neu2en5Ac form, where water is extracted from the C(2)–C(3) site, is given in Fig. 2, with charges in the corresponding Table 3. The charges of Neu2en5Ac are even more pronounced than for the alpha-Neu5Ac molecule, leaving a very positive C(1) of the COOH group attached to the double bond. A comparison of the HOMO (Highest Occupied Molecular Orbital) representation of Neu2en5Ac and Neu5Ac is given in Fig. 3a,b. This figure shows that for Neu2en5Ac the double bond π-orbital indicates the most reactive site in the neutral (non-protonated) molecule. For Neu5Ac, which has a normal pyranose ring, the active sites in the neutral molecule are more distributed. The emphasis for reactivity of Neu5Ac is on the *N*-acetyl group.

Finally, protonated alpha forms have been investigated, where a methyl group has been attached to the O(2–1) in alpha-Neu5Ac. To this end the protonated structures were

Table 1
Comparison of the values for atom distances (Å) and angles (°) of *N*-acetylneuraminic acid methyl ester-α-methyl glycoside obtained from data of crystal structure[19] and the corresponding values determined by RHF/6-31G*, respectively

Distances	Cryst.	6-31G*	Angles	Cryst.	6-31G*
O(1−1)−C(1)	1.206	1.192	C(2)−O(2−2)−C(6)	114.4	118.1
O(4)−C(4)	1.419	1.400	C(1)−O(1−2)−H(1−2)	114.9	117.4
O(7)−C(7)	1.430	1.395	O(1−1)−C(1)−O(1−2)	124.7	132.6
O(1−2)−C(1)	1.329	1.313	O(1−2)−C(1)−C(2)	110.9	112.6
N(5)−C(5)	1.457	1.453	O(2−1)−C(2)−C(1)	108.2	106.9
C(2)−C(3)	1.531	1.526	O(2−2)−C(2)−C(1)	108.0	109.2
C(5)−C(6)	1.531	1.535	C(1)−C(2)−C(3)	116.0	114.2
C(8)−C(9)	1.511	1.520	O(4)−C(4)−C(3)	107.0	111.8
O(2−1)−C(2)	1.403	1.379	C(3)−C(4)−C(5)	109.5	110.4
O(2−2)−C(2)	1.414	1.381	N(5)−C(5)−C(6)	113.7	112.7
O(8)−C(8)	1.438	1.409	O(2−2)−C(6)−C(5)	107.9	109.0
O(1−2)−H(1−2)	1.458	1.422	C(5)−C(6)−C(7)	116.6	115.1
N(5)−C(10)	1.344	1.352	O(7)−C(7)−C(8)	106.8	107.4
C(3)−C(4)	1.513	1.523	O(8)−C(8)−C(7)	110.2	111.0
C(6)−C(7)	1.521	1.530	C(7)−C(8)−C(9)	111.7	112.0
C(10)−C(11)	1.501	1.512	O(10)−C(10)−N(5)	122.8	122.8
O(2−1)−H(2−1)	1.441	1.413	N(5)−C(10)−C(11)	115.6	115.7
O(2−2)−C(6)	1.434	1.415	C(2)−O(2−1)−H(2−1)	114.9	118.5
O(9)−C(9)	1.426	1.398	C(5)−N(5)−C(10)	122.6	123.2
O(10)−C(10)	1.243	1.206	O(1−1)−C(1)−C(2)	124.2	123.8
C(1)−C(2)	1.550	1.550	O(2−1)−C(2)−O(2−2)	107.1	109.4
C(4)−C(5)	1.531	1.532	O(2−1)−C(2)−C(3)	105.4	106.5
C(7)−C(8)	1.535	1.531	O(2−2)−C(2)−C(3)	111.6	110.5
			C(2)−C(3)−C(4)	115.7	112.3
Dihedrals	Cryst.	6−31G*	O(4)−C(4)−C(5)	114.2	112.2
C(10)−N(5)−C(5)−C(4)	−148.3	−142.7	N(5)−C(5)−C(4)	109.8	108.9
C(5)−N(5)−C(10)−O(10)	−9.1	−11.9	C(4)−C(5)−C(6)	106.0	108.6
O(1−1)−C(1)−C(2)−O(2−2)	−7.8	−18.8	O(2−2)−C(6)−C(7)	105.8	107.0
C(5)−C(6)−C(7)−O(7)	−57.0	−54.7	O(7)−C(7)−C(6)	111.6	112.1
C(6)−C(7)−C(8)−O(8)	−54.4	−51.4	C(2−2)−C(7)−C(8)	111.3	112.0
C(7)−C(8)−C(9)−O(9)	−170.7	−179.3	O(8)−C(8)−C(9)	107.4	106.0
			O(9)−C(9)−C(8)	111.8	110.8
			O(10)−C(10)−C(11)	121.6	121.5

fully geometrically optimized and proved to be local minima. It is with ~3 kcal/mol more favorable to protonate on the ring (O(2−2)) than on the carboxylic group (O(1−1)). Note that the charges on these oxygen atoms were calculated to be −0.6 and −0.8 before protonation, respectively. The effect of the protonation weakens the O(2−1)−CH_3 bond slightly as indicated by the Mulliken overlap population, which indicates the number of electrons between the nuclei and thus the strength of the bond. It goes from 0.185 electron (unprotonated) to 0.165 electron (protonated on ring) and 0.15 electron (protonated on the carboxylic group). This weakening is also visible from a slight elongation of the O−CH_3 bond by ~0.01 Å. The other CO bond O(2−1)−C(2) is weakened by the protonation of the COOH group (Mulliken overlap from 0.226 to 0.184) and made stronger on ring protonation (overlap population goes from 0.226 to 0.270).

Concerning the protonation of the ring a significant effect is observed on the C(2)−O(2−2) ring bond. The Mulliken overlap population reduces from 0.21 to 0.05 and the bond length goes concomitantly from 1.36 to 1.49 Å. In contrast, on protonation of the carboxylic group the ring is not affected. Thus, protonation of the ring, which is energetically most favorable, might induce ring opening.

Comparison of our results obtained from the ab initio calculations with those from MD-simulations [11] do not show any contradictions, for example, both conformations (both angle values) of −100 and +100° are possible according to the ab initio calculations.

In the following, the calculated physical data of the sialic acid molecules will be related to the reaction specificity of some sialic acid-metabolizing enzymes. From these considerations a better understanding of the corresponding enzymatic processes can be expected.

3.2. Enzymes modifying sialic acids

At least two different *O*-acetyltransferases have been found in some bacteria and many animal species including man [4,8,20−23], one for the sialic acid side chain and one for the pyranose ring. The acetyl-coenzyme A: sialate-4-*O*-acetyltransferase (EC 2.3.1.44) *O*-acetylates both Neu5Ac

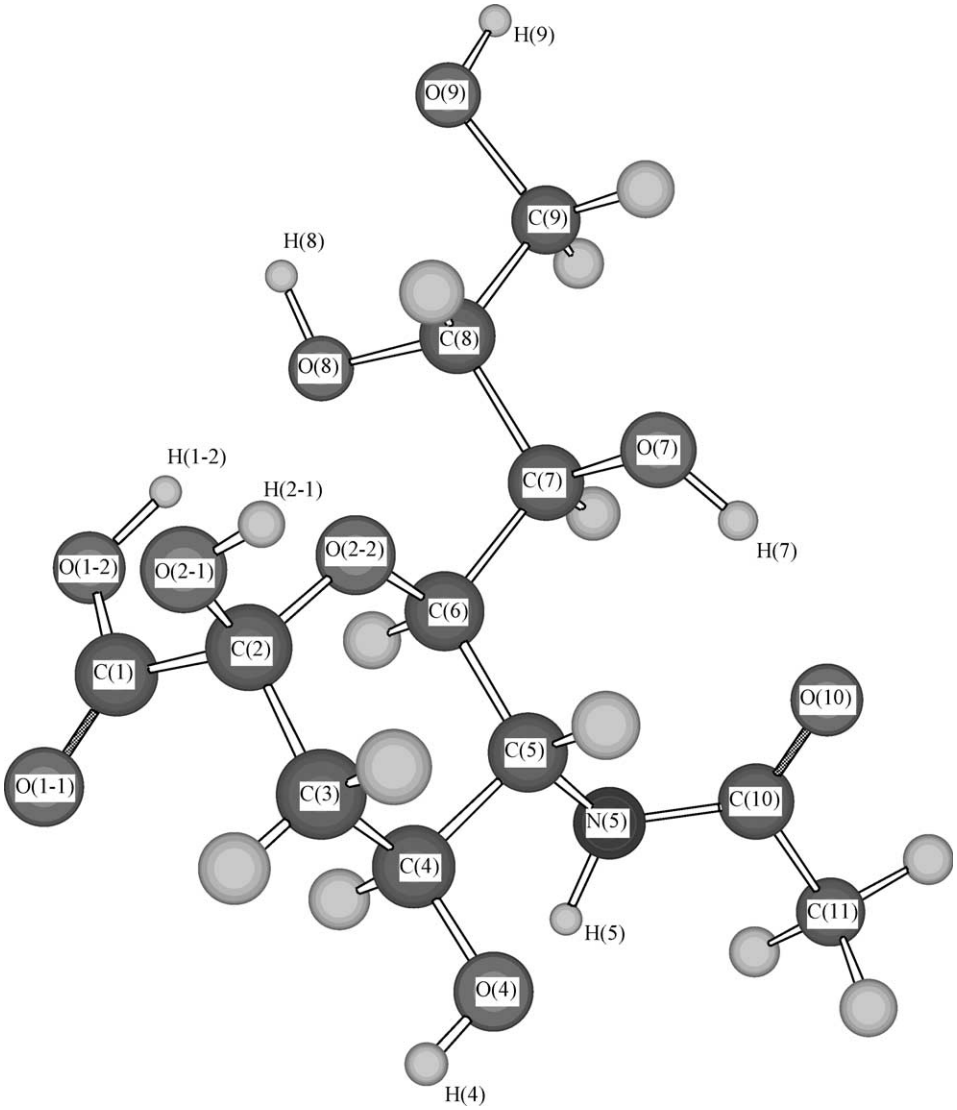

Fig. 1. The calculated structure of the alpha form of *N*-acetylneuraminic acid.

and Neu5Gc at C(4) exclusively, while the acetyl-coenzyme A: sialate-7(9)–*O*-acetyltransferase (EC 2.3.1.45) modifies the side chain of these sialic acids, preferably at C9. Remarkably, Neu5,9Ac_2 resulting from the activity of the latter enzyme is accompanied by *N*-acetyl-7–*O*–acetyl-neuraminic acid (Neu5,7Ac_2) and *N*-acetyl-8–*O*–acetyl-neuraminic acid (Neu5,8Ac_2). In various tissues additionally oligo-*O*-acetyl sialic acids occur, which are *O*-acetylated two- or three-fold in the glycerol side chain. The biosynthesis of the latter sialic acids is not yet completely understood, that is, whether one or more *O*-acetyltransferases are involved.

Among the possible candidates (hydroxyl groups) for enzymatic *O*-acetylation of Neu5Ac (C(4), C(7), C(8), and C(9)), C(4) and C(7) are the ones with the most positive charges. The charges of O(4), O(7) and O(8) are −0.7, −0.8, and −0.7, respectively, whereas that of O(9) is less negative with a value of −0.6. From the aspect of charge distribution the position at C(7)/O(7) is more preferred as acceptor for an acetyl group, as carbonium cation from acetyl coenzyme A, than that of C(9)/O(9). These theoretical findings are consistent with experimental results about that enzyme responsible for *O*-acetylation of the side chain showing that C(7)/O(7) may be the primary site of *O*-acetylation [8,24], (Lrhorfi et al., unpublished data). From this position the ester group migrates to C(8) and C(9) and presumably the *O*-acetyltransferase then re-*O*-acetylates O(7). Thus, finally all three hydroxyls of the side chain are esterified, as best observed in human colon mucosa and bovine submandibular gland [8,23,24], (Lrhorfi et al. unpublished data). However, comparing the calculated charge values of the different sialic acid derivatives (Table 2) it can be expected that in Neu5,9Ac_2 the position C(7)/O(7) has a reduced acceptor potency than in Neu5Ac, due to the significant lower charge of C(7) (electrophilic attack).

In enzymatic [24] and non-enzymatic studies [25] evidence for migration of *O*-acetyl groups from C(7) to

Table 2
DMA charges for Neu5Ac and four of its natural derivatives. The numbering of the atoms is shown in Fig. 1

Neu5Ac		Neu5.9Ac$_2$		Neu5Gc		Neu4Ac5Gc		Neu4.5Ac$_2$	
Atomnumber	Charge		Charge	Atomnumber	Charge	Atomnumber	Charge	Atomnumber	Charge
C(1)	1.0	C(1)	1.1	C(1)	1.1	C(1)	1.1	C(1)	1.1
C(2)	0.6	C(2)	0.6	C(2)	0.6	C(2)	0.5	C(2)	0.6
C(3)	<0.2	C(3)	<0.2	C(3)	−0.2	C(3)	<0.2	C(3)	<0.2
C(4)	0.3	C(4)	0.4	C(4)	0.4	C(4)	<0.2	C(4)	<0.2
C(5)	<0.2	C(5)	<0.2	C(5)	<0.2	C(5)	<0.2	C(5)	<0.2
C(6)	<0.2	C(6)	<0.2	C(6)	0.2	C(6)	<0.2	C(6)	<0.2
C(7)	0.4	C(7)	0.2	C(7)	0.3	C(7)	0.3	C(7)	0.3
C(8)	<0.2	C(8)	0.3	C(8)	0.3	C(8)	0.3	C(8)	0.3
C(9)	<0.2	C(9)	<0.2	C(9)	<0.2	C(9)	<0.2	C(9)	<0.2
C(10)	1.1	C(10)	1.0	C(10)	1.0	C(10)	1.0	C(10)	1.1
C(11)	−0.3	C(11)	−0.3	C(11)	<0.2	C(11)	<0.2	C(11)	−0.3
O(1−1)	−0.8	O(1−1)	−0.9	O(1−1)	−0.8	O(1−1)	−0.8	O(1−1)	−0.9
O(1−2)	−0.7	O(1−2)	−0.8	O(1−2)	−0.7	O(1−2)	−0.7	O(1−2)	−0.8
O(2−1)	−0.7	O(2−1)	−0.8	O(2−1)	−0.8	O(2−1)	−0.8	O(2−1)	−0.8
O(2−2)	−0.6	O(2−2)	−0.5	O(2−2)	−0.6	O(2−2)	−0.6	O(2−2)	−0.6
O(4)	−0.7	O(4)	−0.8	O(4)	−0.7	O(4)	−0.6	O(4)	−0.6
O(7)	−0.8	O(7)	−0.7	O(7)	−0.8	O(7)	−0.8	O(7)	−0.8
O(8)	−0.7	O(8)	−0.7	O(8)	−0.7	O(8)	−0.7	O(8)	−0.7
O(9)	−0.6	O(9)	−0.6	O(9)	−0.6	O(9)	−0.6	O(9)	−0.7
O(10)	−1.0	O(10)	−1.0	O(10)	−1.0	O(10)	−0.9	O(10)	−1.0
N(5)	−0.8	N(5)	−0.7	N(5)	−0.7	N(5)	−0.8	N(5)	−0.8
H(1−2)	0.5	H(1−2)	0.5	H(1−2)	0.5	H(1−2)	0.5	H(1−2)	0.5
H(2−1)	0.4	H(2−1)	0.5	H(2−1)	0.5	H(2−1)	0.5	H(2−1)	0.5
H(4)	0.4	H(4)	0.5	H(4)	0.4	group-C(22)	-	group-C(22)	-
H(5)	0.4	H(5)	0.4	H(5)	0.4	H(5)	0.4	H(5)	0.4
H(7)	0.5	H(7)	0.4	H(7)	0.5	H(7)	0.5	H(7)	0.5
H(8)	0.5	H(8)	0.4	H(8)	0.5	H(8)	0.5	H(8)	0.5
H(9)	0.4	H(9)	-	H(9)	0.4	H(9)	0.4	H(9)	0.5
				O(11)	−0.6	O(11)	−0.7		

Charges with an absolute value below 0.2 are listed as <0.2.

C(9) was obtained. Comparing the charges on O(7), O(8) and O(9) a systematic decrease of the charges from -0.8 to -0.6 is observed (Table 2). This shows that the position at C(9)/O(9) is a less active environment and gives an explanation that the *O*-acetyl group at this primary alcohol group is not likely to leave this position. This highest stability of the ester group at C(9) promotes isomerization of acetyl groups from C(7) and fits to the observation that in nature the side chain *O*-acetylation is most frequently found at C(9) [8,23–25], (Lrhofi et al., unpublished data).

Considering the *O*-acetyltransferase of the ring, the relatively high positive charge of the C(4) (0.3) and the high negative one of the O(4) (−0.7) are in favor of the C(4)/O(4) as acceptor side. However, the differences in the position specificity between the 4-*O*-acetyl- and side chain-*O*-acetyltransferases cannot be explained by charge distribution, but they are likely due to steric effects. Also the exclusive presence of methyl ether groups at C(8)/O(8) of sialic acid cannot be explained by charge differences alone. However, the relatively negative charge of this oxygen, although less than that of O(7), provides a suitable acceptor side. The same is valid for *O*-sulfation at this position. It must be assumed that the specificity of the enzymes involved in these modifications is influenced also by the sterical situation in this area of the sialic acid molecule. A *S*-adenosyl-L-methionine: sialate-8-*O*-methyltransferase (EC 2.1.1.78) has been isolated from the starfish *Asterias rubens* [10]. It is active with both Neu5Ac and Neu5Gc. Sialic acids sulfated at O(8) have also been found in echinoderms [8], but the enzyme involved in its biosynthesis has not yet been identified.

3.3. Sialyltransferases

The hydroxyl at C(8) is also a preferred target for sialyltransferases, especially for the polysialyltransferases, catalysing the formation of sialyl-α2,8-bonds [8,26]. The preferred nucleophilic attack of O(8) on the highly positively charged C(2) atom of the approaching sialic acid residue may have the same reasons as for the binding site of methyl and sulfate groups.

The introduction of an oxygen atom into the methyl of the *N*-acetyl group of Neu5Ac leads to a *N*-glycolyl group. Neu5Gc is a frequent modification of sialic acid [8]. Our calculations show that the *N*-glycolyl group does not change remarkably the charge distributions of neighbouring atoms after oxygen insertion into the sialic acid molecule (Table 2). This is in concordance with the observation that

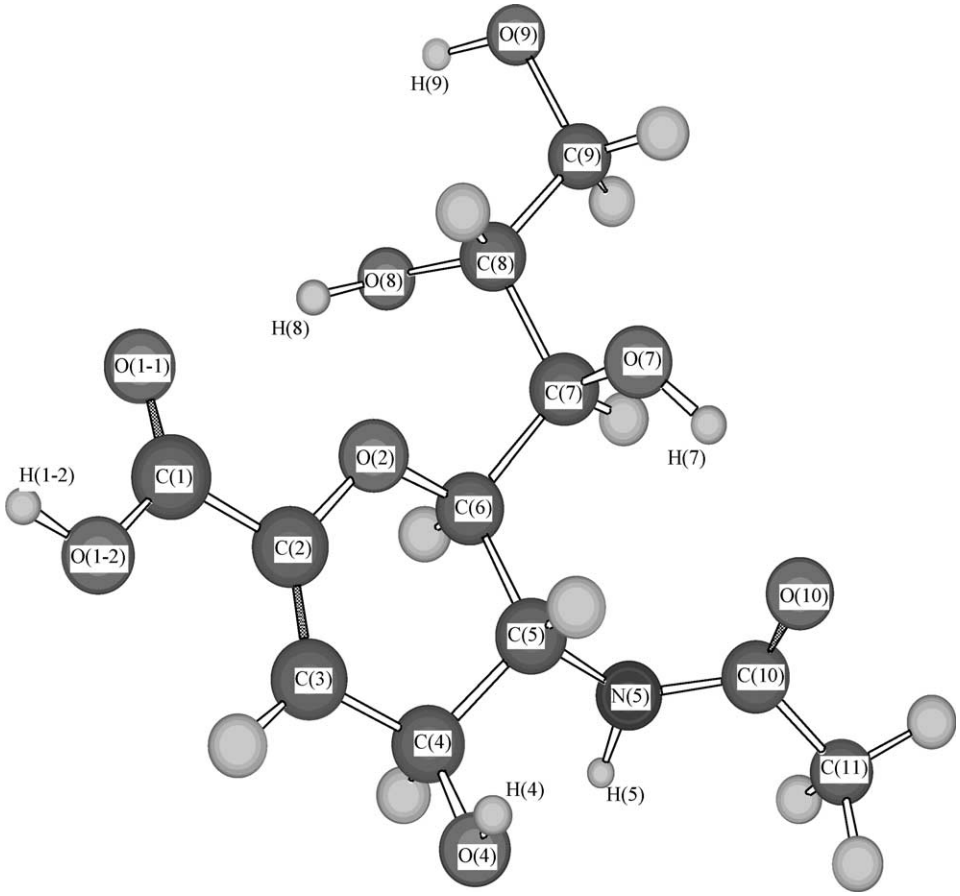

Fig. 2. The calculated structure of 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid.

Neu5Gc can be substrate for the modifications described above, most frequently the *O*-acetylation. However, the charge of C(11) is nearly neutral (<0.2) when it is part of a *N*-glycolyl group and it is −0.3 when C(11) belongs to a *N*-acetyl group. The oxygen atom of the *N*-glycolyl group O(11) has a charge of −0.7 in the case of Neu5Gc and a charge of -0.6 in the case of Neu4Ac5Gc. Neu5Gc originates from Neu5Ac by hydroxylation on the level of CMP-Neu5Ac [27]. For the CMP-Neu5Ac hydroxylase (EC 1.14.99.18) involved in this reaction, C(11) can be hydroxylated or oxygenated, perhaps since this C-atom has only little electronegative charge (−0.3) and the methyl group is in an exposed position and can get direct access to the active centre of the hydroxylase. As described above, the charge of O(11) in Neu5Gc was determined to be −0.7. This explains why the corresponding hydroxyl can be the acceptor site of other Neu5Gc residues in the starfish *Asterias rubens* resulting in homo-oligosaccharides with Neu5Gc(α2-O5)Neu5Gc-linkages [28]. The sialyltransferase responsible for this unique structure has not yet been characterized.

Sialic acids are frequently linked also to C(8) and more rarely to C(9) to form polysialic acids which regulate many events in the physiological and pathological events of

Table 3
DMA charges for Neu2en5Ac The numbering is shown in Fig. 2

Atom number	Charge
C(1)	1.2
C(2)	0.4
C(4)	0.3
C(8)	0.3
C(10)	1.1
C(11)	−0.3
O(1−1)	−0.8
O(1−2)	−0.8
O(2)	−0.6
O(4)	−0.7
O(7)	−0.7
O(8)	−0.8
O(9)	−0.7
O(10)	−1.0
N(5)	−0.7
H(1−2)	0.5
H(4)	0.4
H(5)	0.4
H(7)	0.5
H(8)	0.5
H(9)	0.4

Charges with an absolute value below 0.2 are not displayed.

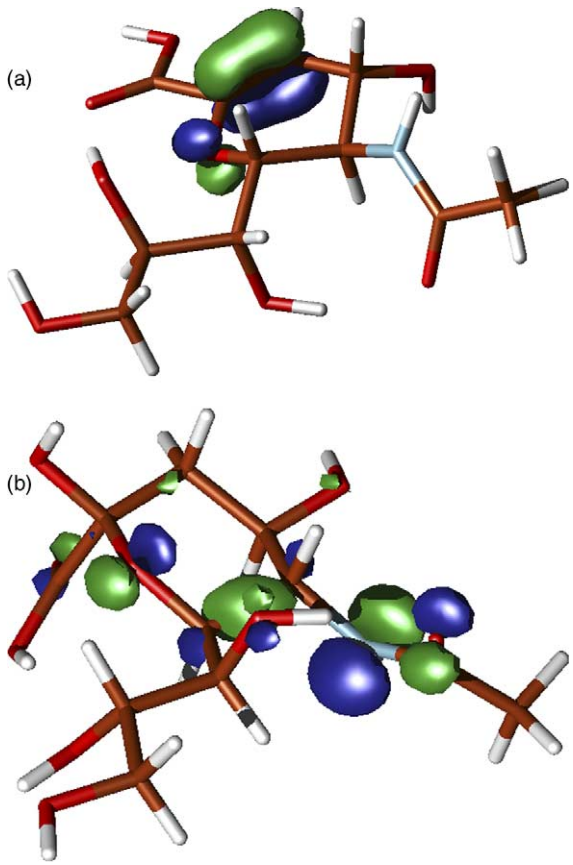

Fig. 3. A pictorial representation of the Highest Occupied Molecular Orbital (HOMO) for 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (a) and *N*-acetylneuraminic acid (b), respectively.

cells and tissues.[26] The electronegative charges of the corresponding *O*-atoms, especially O(8) (−0.7), are in favour of these reactions.

A large gene family codes for sialyltransferases transferring these monosaccharides from their CMP-glycosides in order to create α2-3-, α2-6-, α2-8- or α2-9-linkages to other sugars, mostly galactosides [29,30].

3.4. Sialidase and trans-sialidase

Sialidases (EC 3.2.1.18) are ubiquitous enzymes in animals expressing sialic acids, as well as in many microorganisms including viruses [8]. They are responsible for the hydrolytic release of sialic acids from oligosaccharides and glycoconjugates. The reaction product of sialidases is mostly free Neu5Ac, but also an unsaturated form (Neu2en5Ac), an anhydro derivative (2,7-anhydro-*N*-acetylneuraminic acid, Neu2,7an5Ac), or a new glycosidic linkage (Neu5Acα2-3galactose) can be the result of the action of sialidases and trans-sialidases, respectively.

The ab initio calculations presented here contribute to the understanding of the reaction mechanisms involved. If the proton goes to O(1−1) {O=C}, both O(2−1) and the *O*-glycosidic linkage O(2−1)−C(2) are weakened. If the proton goes to O(2−2) of the ring, the C(2)−O(2−1) bond is made stronger and the O(2−1)−CH_3 bond gets weaker. This corresponds to the most stable situation. This may lead to the possibility of an electrophilic attack to O(2−1) by a proton or a carbon atom at the second step of the sialidase and trans-sialidase reactions, outlined below. As discussed in the literature, the O(2−1) bond breaks probably after protonation in the sialidase reaction, followed by the release of the glycosidic partner and leading to an oxo-carbonium ion C(2) for sialidases and generally for glycosyl hydrolases [31–37]. Protonation is favoured by the relatively negative O(2−1) atom (−0.7). This ion represents a Neu5Ac transition state analogue in the sialidase reactions, having a distorted chair conformation [38,39]. It can bind a hydroxyl from a water molecule leading to free Neu5Ac. However, this molecule can also be stabilized by proton elimination from C(3) and the formation of Neu2en5Ac, which can be detected in small quantities after the sialidase reaction. It has to be mentioned that this is a reaction at low probability. Neu2en5Ac is an inhibitor of viral, bacterial and animal sialidases [8]. Our results suggest that protonation of the O(1−1)-position is most consistent with the formation of the unsaturated sialic acid (Neu2en5Ac). The O(1−1)-position is the less stable protonation site in vacuum. Sialidase reactions occur at slightly acidic pH (5–6) which favours protonation.

Such a generalized two-step mechanism is proposed not only for the hydrolytic sialidases, but also for the so-called trans-sialidases [36]. Accordingly, the C(2)−O(2−1) bond cleavage and oxycarbonium formation step 1 is shared by all enzymes of the sialidase superfamily. In the following reaction, for hydrolytic sialidases the nucleophile is a water molecule. For trans-sialidases, the nucleophiles are the hydroxyls of carbohydrates. Such enzymes, which occur in pathogenic trypanosomes [40], transfer sialic acids from one glycosidic linkage to the C(3) hydroxyl of a terminal β-linked galactose or *N*-acetylgalactosamine of oligosaccharides and glycoconjugates. The intramolecular trans-sialidase (IT-sialidase), found in the leech [36,41], uses as nucleophile the 7-hydroxyl of the same sialic acid molecule, which is released from the glycosidic linkage in this manner.

In all these cases the incoming hydroxyl is activated through hydrogen-bonding with an aspartic acid of the enzyme and forms the linkage with the oxycarbonium C(2). Based on this proposed mechanism, the proton of the hydroxyl is accepted by the aspartic acid and then released to the bulk solvent. In trypanosomal trans-sialidases the transfer to an external galactose moiety seems to be facilitated by an extra binding site for the acceptor galactose residue, which is made accessible by a conformational switch of the protein after sialic acid binding. Evidence for

this is coming from the recent crystallisation and X-ray analysis of the Trypanosoma cruzi trans-sialidase [42].

3.5. Sialate-Lyase

The second enzyme involved in the catabolism of sialic acids, liberated by sialidases, is the sialate-pyruvate-lyase (EC 4.1.3.3), which is also widely distributed in microorganisms and animals [8,43]. It splits the different sialic acid molecules into pyruvate and acylmannosamines. The enzyme belongs to the class I aldolases, in the reaction of which a Schiff base is formed, followed by an abstraction of the proton from OH at C(4). The ab initio calculations are in favour of such a reaction. Since C(2) is rather positive (0.6), C(3) nearly neutral and C(4) also positive (0.3), in combination with the OH-group (-0.3) of C(4), these atoms are properly lined for interactions with the lyase, where a lysine is known to start the reaction by a nucleophilic attack at C(2) [44,45].

From the structural point of view a ring opening before this Schiff base formation, which is discussed in the literature [8], seems not to be a prerequisite for the start of the lyase reaction and thus the model developed by Barbosa et al. (2000) is confirmed [45]. Furthermore, the pH optimum of the lyase is above 7, which is not in favour of protonation of the whole molecule. This would facilitate rapid opening of the ring. It is cited in the literature given that only the α-form of sialic acid is a substrate of the reaction. Studies with mutants of *N*-acetylneuraminate lyase [44], which were in some cases supported by CIDNP (Chemically Induced Dynamic Nuclear Polarizations) experiments in the same way as documented for sialidase mutants [5] were helpful for improvement of the insight into the enzymatic processes. The contribution of the ab initio calculations in special provides a detailed information concerning the charge distribution of the molecules under reaction circumstances, e.g. by comparing the molecules in protonated and unprotonated states.

3.6. Concluding remarks

Ab initio calculations are used to provide insight into reactions modifying and degrading sialic acids. The optimised geometry of α-methyl glycoside of *N*-acetylneuraminic acid methyl-ester is in good agreement with published crystallographic data.

The relatively negative charge on the sialic acid side chain O(7) supports the hypothesis that on *O*-acetyl transferase reactions this position is the primary insertion site of the acetyl ester group and not the less negative oxygen at C(9).

We show that upon ring-protonation the ring gets weakened, thus facilitating ring-opening and the glycosidic *O*-CH_3 bond gets more vulnerable to attack. In the case of protonation of the carbonyl group at C(1) on the other hand, the bond with the glycosidic oxygen is weakened. When this bond is broken a positive carbon site remains at C(2), which allows a nucleophilic attack in the (trans-)sialidase reactions.

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