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Determination of the β-Anomeric Configuration of Cytidine 5'-Monophospho-*N*-acetylneuraminic Acid by ^{13}C NMR Spectroscopy[1,2]

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Abstract: The anomeric configuration of the NeuAc residue in enzymically prepared cytidine 5'-monophospho-*N*-acetylneuraminic acid was established to be β on the basis of the heteronuclear vicinal coupling constant $^3J_{C1\text{-}H3ax}$. For comparison the $^3J_{C1\text{-}H3}$ values of a number of α and β *N*-acetylneuraminic acid derivatives have also been determined. The biochemical implication of this finding is discussed.

The biosynthesis of cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-NeuAc) from CTP and NeuAc is catalyzed by the enzyme acylneuraminate cytidylyltransferase (EC 2.7.7.43):[4-7]

$$\text{CTP} + \text{NeuAc} \xrightarrow{\text{transferase}} \text{CMP-NeuAc} + \text{PP}_i$$

CMP-NeuAc is a key intermediate in the biosynthesis of glycoconjugates. The enzyme sialyltransferase (EC 2.4.99.1) transfers NeuAc residues from this donor molecule to oligosaccharides, glycoproteins, and glycolipids (in the following equation "R"),

$$\text{CMP-NeuAc} + \text{glycosyl-R} \xrightarrow{\text{transferase}} \text{NeuAc}\alpha(2 \rightarrow x)\text{glycosyl-R} + \text{CMP}$$

(x = 3 or 6 in the case of a hexosyl unit and 8 or 9 in the case of a neuraminyl unit).

To investigate the mechanism of these enzymic reactions the anomeric configuration of CMP-NeuAc has to be known. In the literature ambiguity exists about this configuration. Comb et al.[8] proposed a β-glycosidic linkage on the basis of optical rotation measurements. However, circular dichroism measurements led Stone and Kolodny[9] to the suggestion that an α-glycosidic linkage should exist.

In this paper the determination of the anomeric configuration of CMP-NeuAc by single-resonance ^{13}C NMR spectroscopy is described. The coupling constant $^3J_{C1\text{-}H3ax}$ is indicative of the anomeric configuration since its magnitude depends on the torsion angle between the coupled carbon and hydrogen atom (see Figure 1) according to a Karplus-type relation.[10-13]

Experimental Section

Synthesis of CMP-NeuAc. The incubation mixture (80 mL, pH 9) for the synthesis of CMP-NeuAc contained the following components: 0.8 mmol of NeuAc, 3.2 mmol of CTP (Boehringer, Mannheim), and 30 nkat enzyme preparation from frog liver.[14] The concentrations of Tris, Mg^{2+}, and mercaptoethanol were 0.4, 0.04, and 0.001 M, respectively. After 4 h of incubation at 37 °C the mixture was diluted tenfold with water and rinsed through a column of Dowex 2-X4, HCO_3^- form (0.8 L resin). After washing with 2 L of 1 mM ammonia the sialic acid derivatives were eluted by 3 L of a linear gradient from 0.01 to 2.0 M triethylammonium hydrogen carbonate buffer, pH 7.8. The fractions containing CMP-NeuAc[15] were pooled and lyophilized. The material was stored at −20 °C under NH_3 vapor. Thin layer chromatographic analysis of the product was carried out on cellulose plates using 95% ethanol–1 M ammonium acetate, pH 7.3 (7:3 v/v),[8] as solvent. The R_f value for CMP-NeuAc is 0.26 and for NeuAc 0.56.

Synthesis of Reference Compounds. NeuAc methyl ester β-methylglycoside (**2**) and the corresponding α anomer (**3**) were prepared according to Yu and Ledeen.[16] NeuAc α-methylglycoside (**4**) was obtained from **3** by saponification at 40 °C in D_2O, kept at pD ~11 with triethylamine. The reaction was followed by ^1H NMR analysis; after disappearance of the ester methyl signal at 3.88 ppm relative to sodium 2,2-dimethyl-2-silapentane-5-sulfonate the solution was lyophilized.

^{13}C NMR Spectroscopy. ^{13}C Fourier transform spectra were recorded in 12 mm o.d. sample tubes at 25.16 MHz on a Varian XL-100-15 spectrometer at 25 °C for compounds **1–4** and at ~15 °C for CMP-NeuAc. Samples of **1–4** were examined as neutral 0.7 M solutions in D_2O, and CMP-NeuAc as 0.3 M solutions in D_2O at pD ~8. Coupling constants were determined from the single-resonance ^{13}C spectra using 8192 data points over 250-Hz spectral width. The stability of CMP-NeuAc during the NMR experiments was checked by thin layer chromatography (see above).

Results and Discussion

^{13}C NMR spectra of CMP-NeuAc and the reference compounds β-NeuAc (**1**), NeuAc methyl ester β-methylglycoside (**2**), NeuAc methyl ester α-methylglycoside (**3**), and NeuAc α-methylglycoside (**4**) in D_2O were recorded under various conditions: (1) proton noise decoupled spectra for product control[17] and (2) single-resonance spectra of the carbonyl region (250-Hz spectral width) to determine the anomeric configuration. Chemical shifts and coupling constants of the carboxylate and *N*-acetyl carbonyl carbons are given in Table I. The assignment of these resonances was made on the basis of the various coupling constants (see also Figures 2 and 3). The resonance of C1 in **1** and in CMP-NeuAc (Figure 3) is a

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Table I. Chemical Shifts and Coupling Constants for NeuAc Carbonyl Carbons in Compounds 1–4 and CMP-NeuAc[a]

compd	C1				N-acetyl		
	δ	$^3J_{C1-H3ax}$	$^3J_{C1-H3eq}$	$^3J_{C1-H-ester}$	δ	$^2J_{CAc-HAc}$	$^3J_{CAc-H5}$
1	177.7	≤ 1[b]	≤ 1[b]		175.8	6.1	2.8
2	171.6	≤ 1[b]	≤ 1[b]	3.7	175.6	5.9	1.8
3	171.1	5.9	≤ 1[b]	3.4	175.9	6.2	2.2
4	174.4	5.4	≤ 1[b]		176.2	6.2	2.4
CMP-NeuAc	175.3	≤ 1[b]	≤ 1[b]		175.7	n.d.	n.d.

[a] For neutral solutions in D_2O (CMP-NeuAc: pD ~8); chemical shifts (δ) are given in parts per million with the *N*-acetyl methyl resonance taken at 23.25 ppm (i.e., the value in **1** relative to external tetramethylsilane). Coupling constants (J) are in hertz. [b] Estimated from the line width of the unresolved (double) doublet.

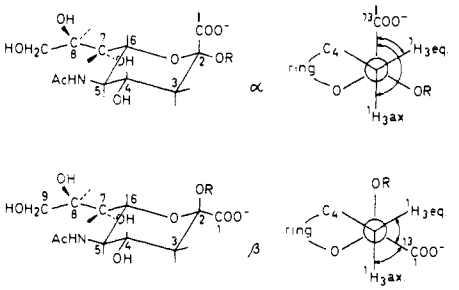

Figure 1. Structure of NeuAc α and β anomers and the torsion angles between C1 and the H3 protons (R = H or aglycon).

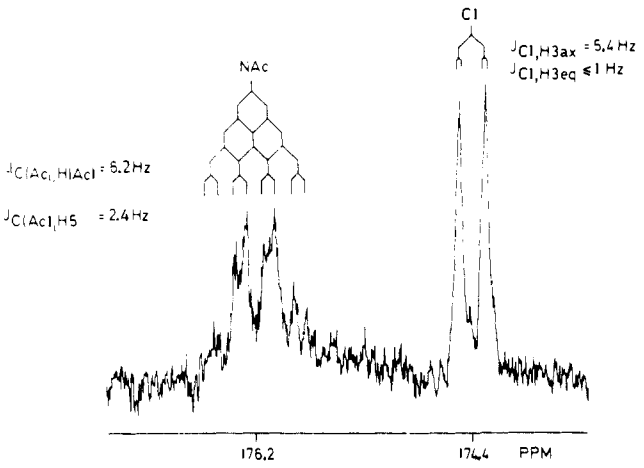

Figure 2. Carbonyl region of the undecoupled ^{13}C NMR spectrum of **4**.

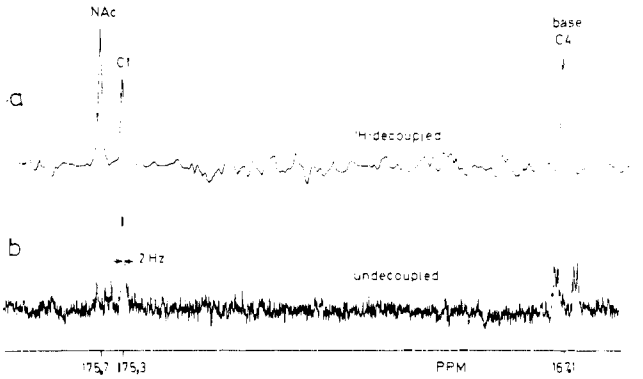

Figure 3. Partial ^1H-decoupled (a) and undecoupled (b) ^{13}C NMR spectrum of CMP-NeuAc. The *N*-acetyl carbonyl multiplet is not well developed.

narrow, unresolved multiplet with a line width of about 2 Hz. It has to be noted that the vicinal coupling $^3J_{C1-P} \leq 1$ Hz (Figure 3a) and does not significantly affect the estimation of the $^3J_{C1-H3}$ couplings. In the case of CMP-NeuAc the carbon resonances of the cytosine ring[18] (167.1 ppm, C4; 158.7 ppm, C2; 142.7 ppm, C6; 97.7 ppm, C5) do not interfere.

Vicinal $^{13}C-^1H$ coupling constants for polysubstituted systems are, in addition to the torsion angles, dependent on electronegativity, position and orientation of the substituents, the presence of heteroatoms in the coupling path, hybridization of the intervening and the coupled atoms, bond lengths, and bond angles.[11-13,19-21] With regard to the latter two parameters it has to be noted that all compounds (**1–4** and CMP-NeuAc) occur in the $^1C_4$ (=$^2C_5$) (D) chair conformation (see Figure 1). This could be concluded from the values of the vicinal proton–proton coupling constants $J_{3ax,4}$, $J_{4,5}$, and $J_{5,6}$ determined from 360-MHz ^1H NMR spectra.[22a] (Values for **1** are 11.8, 10.4, and 10.8 Hz, respectively; see also ref 22b. **2**: 11.7, 10.2, and 10.6 Hz. **3**: 12.5, ~10, and ~10 Hz. **4**: 12.3, 9.7, and 10.5 Hz. CMP-NeuAc: 11.6, 10.4, and 10.5 Hz, respectively[22c]). The large values for these coupling constants point to the antiperiplanar orientation of the coupled protons H_{3ax} and H_4, H_4 and H_5, and H_5 and H_6. The data in Table I show that the antiperiplanar orientation of C1 and H3ax present in the α anomers **3** and **4** gives rise to a relatively large coupling constant between these two atoms (see Figure 1; $^3J_{C1-H3ax}$ = 5.4–5.9 Hz). The coupling between C1 and H3 atoms in synclinal orientation, viz., $^3J_{C1-H3ax}$ and $^3J_{C1-H3eq}$ in **1** and **2** and $^3J_{C1-H3eq}$ in **3** and **4**, is not well resolved in the spectra (about 1 Hz). The values of these synclinal couplings in α and β anomers are about equal, since H3ax and H3eq in the β anomer and H3eq in the α anomer have the same orientation with respect to the three oxygen substituents at C2 and C4. The magnitudes of the synclinal couplings are in accordance with those described in the literature for the fragment $^-O-^{13}CO-C(R_1)NH_3^+-C(R_2)H-^1H$ of amino acids (1.3 ± 0.3,[23] 0.4 Hz[24]). However, the antiperiplanar $^3J_{C-H}$ couplings in **3** and **4** are significantly smaller than those reported for amino acids (9.8 ± 0.3,[23] 11.9 Hz[24]).

On the basis of the values for synclinal and antiperiplanar vicinal $^{13}C-^1H$ coupling constants of ~1 and 5.4–5.9 Hz, respectively (Table I), it is concluded that the NeuAc residue in CMP-NeuAc has the β-anomeric configuration. The dihedral angles between C1 and the respective H3 protons cannot accurately be inferred from the coupling constants $^3J_{C1-H3}$ since no reliable Karplus relation is known for the system $^-O-^{13}CO-C(OR_1)OR_2-CHR_3-^1H$. In this light the values for ∠C1–H3eq and ∠C1–H3ax of 45 and 155°, respectively, for **4** and of 55 and 55° for the corresponding β anomer[25] have to be considered as rough approximations.

The biochemical activation of NeuAc differs from that of other D sugars (e.g., Man, Gal, Glc, GalNAc, and GlcNAc) in several respects:

(1) A monophosphate group is present instead of a pyrophosphate.

(2) In the enzymic formation of CMP-NeuAc the nonphosphorylated monosaccharide and the nucleoside triphosphate are involved.

(3) The anomeric configuration is β, which is in contrast to the α configuration in other D sugar nucleotides.[6]

The existence of CMP glycosides of *N,O*-acylneuraminic acids has been demonstrated;[26] for analogous reasons it has to be expected that these have the β configuration too. Taking into account the fact that in glycoconjugates only α-NeuAc residues occur[27] the β-glycosidic configuration of CMP-NeuAc is in accordance with the assumption that NeuAc residues are transferred to the acceptor molecule via a "single displacement mechanism" with inversion of configuration.[6] So far, there is no evidence that a lipid intermediate is involved in this biosynthesis step since a twofold inversion of configuration would lead to sialic acid residues in β linkages to glycoconjugates. It has been reported that in the biosynthesis of colominic acid (an $\alpha(2 \rightarrow 8)$ linked NeuAc polymer) by *Escherichia coli*[28] sialylundecaprenyl phosphate plays a role. In view of the foregoing this intermediate probably only acts as an acceptor molecule for additional NeuAc residues, thus initiating the polymer formation.

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^{13}C NMR Relaxation Mechanisms in Methyl–Transition Metal Compounds

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Abstract: The ^{13}C NMR relaxation mechanisms in transition metal-methyl compounds have been investigated. The methyl carbons in *cis*-$Os(CO)_4(CH_3)_2$, $(\pi\text{-}C_5H_5)Mo(CO)_3CH_3$, $(\pi\text{-}C_5H_5)Fe(CO)_2CH_3$, $(\pi\text{-}C_5H_5)_2Zr(CH_3)_2$, and CH_3AuPPh_3 relax by the dipolar and spin-rotation mechanisms. The methyl carbon in $CH_3Re(CO)_5$ shows an additional contribution due to scalar relaxation of the second kind. The relaxation of the methyl and methylene carbons in the tautomeric clusters $Os_3(CO)_{10}(CH_3)(H) \rightleftarrows Os_3(CO)_{10}(CH_2)(H)_2$ is strictly dipolar. Estimates of the methyl rotation barriers from the spin-rotation relaxation times are reported.

Introduction

The use of ^{13}C NMR relaxation times as probes of the structures and dynamics of organic and main-group organometallic compounds is well established.[2] However, only a few studies involving transition-metal organometallic compounds have appeared,[3,4] and to our knowledge only one involving an alkyl carbon σ bonded to a transition metal.[5] We have thus undertaken studies to determine the ^{13}C(methyl) relaxation mechanisms of a number of representative transition metal-methyl complexes. In a preliminary communication[6] we reported the ^{13}C T_1's and η_{CH}'s for the methyl carbons in $(\pi\text{-}C_5H_5)Fe(CO)_2CH_3$, $(\pi\text{-}C_5H_5)Mo(CO)_3CH_3$, $Os(CO)_4(CH_3)_2$, and $(\pi\text{-}C_5H_5)_2Zr(CH_3)_2$. Herein we report variable-temperature (and in several instances variable-field) relaxation studies of the above compounds as well as of CH_3AuPPh_3, $CH_3Re(CO)_5$, and $Os_3(CO)_{10}(CH_3)(H)$.

Potential Relaxation Mechanisms for Methyl Carbons. A major contribution to the spin-lattice relaxation rate R_1 $(1/T_1)$ of the methyl carbons in transition metal-methyl complexes is expected to be dipole-dipole relaxation R_1^{DD}, due to the methyl hydrogens.[6,7] In the extreme narrowing limit this

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