Vol. 104, No. 3, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 11, 1982 Pages 1114-1119

THE PREPARATION OF SPECIFICALLY DEUTERIUM- OR TRITIUM-LABELED N-ACETYLNEURAMINIC ACID AND CYTIDINE-5'-MONOPHOSPHO-β-N-ACETYL-NEURAMINIC ACID AS PRECURSORS FOR GLYCOCONJUGATE SYNTHESIS*

Lambertus Dorland, Johan Haverkamp, Roland Schauer[a], Gerrit A. Veldink and Johannes F.G. Vliegenthart
Department of Bio-Organic Chemistry, University of Utrecht, Croesestraat 79, 3522 AD Utrecht, The Netherlands
[a]Institute of Biochemistry, University of Kiel, D-2300 Kiel, F.R.G.

Received December 17, 1981

This report describes the specific exchange of H-3ax for a deuterium- or tritium-atom in free Neu5Ac in aquous solution under mild alkaline conditions (p^{2}H 9.0). This principle was applied to the synthesis of ^{3}H-labeled cytidine-5'-monophospho-β-N-acetylneuraminic acid. Subsequently, ^{3}H-labeled CMP-Neu5Ac was employed for the enzymic transfer of Neu5Ac to asialofetuin. The latter reaction is an example of the enzymic synthesis of sialoglycoconjugates, specifically labeled at C_3 of Neu5Ac

Introduction

During high-resolution ^{1}H-NMR spectroscopic studies on sialic acids and their derivatives [1-6] we discovered that in mild alkaline deuterium oxide solutions the axial proton at C-3 of free N-acetylneuraminic acid is exchanged for a deuterium atom. The exchange reaction is highly specific and the incorporated label is retained under neutral and acidic conditions. Obviously, in an analogous way tritium can be introduced at C-3 in axial position. As will be shown in this report, this finding can be applied to the preparation of specifically labeled sialic acid derivatives like CMP-Neu5Ac. Transfer of Neu5Ac from the latter nucleotide to carbohydrates or glycoconjugates with retention of label can be achieved by means of sialyltransferases. It is clear that the study of the functional role of sialic acid residues in biomolecules may be facilitated by the availability of specifically labeled compounds.

Abbreviations
Neu5Ac, N-acetylneuraminic acid; CMP-Neu5Ac, cytidine-5'-monophospho-β--N-acetylneuraminic acid; CTP, cytidine-5'-triphosphate; NMR, nuclear magnetic resonance; t.l.c., thin layer chromatography.

* This paper is dedicated to Professor Dr A. Rossi Fanelli on the occasion of his 75th birthday.

0006-291X/82/031114-06$01.00/0

Materials and Methods

a. Exchange experiments
Solutions containing 5 mg Neu5Ac (Serva, Heidelberg, F.R.G.) in 0.5 ml ^2H_2O (100% ^2H, Aldrich, Milwaukee, U.S.A.) at p^{2}H 5.5 and p^{2}H 9.0, respectively, were kept at room temperature for 6 h. After lyophilization, this procedure was repeated five times. For the preparation of N-acetyl-[3ax-^{3}H] neuraminic acid, 44.7 mg Neu5Ac was dissolved in 2 ml water, neutralized with 1 M NaOH and then lyophilized. The sodium salt of Neu5Ac was dissolved in 0.5 ml ^3H_2O (18.5 GBq), the pH brought to 9 with 1 M NaOH and kept at room temperature for 48 h. Subsequently, the solution was used for the synthesis of CMP--[3ax-^{3}H]Neu5Ac (see under c).

b. ^{1}H-NMR spectroscopy
^{1}H-NMR spectroscopy was performed at 360 MHz on a Bruker HX-360 spectrometer operating in the Fourier transform mode at a probe temperature of 25°C. Chemical shifts are given relative to sodium 2,2-dimethyl-2-silapentane-5--sulphonate (DSS) (indirectly to acetone in ^2H_2O : δ = 2.225 ppm).

c. Preparation of CMP-[3ax-^{3}H]Neu5Ac
In a total volume of 10 ml 0.4 M Tris/HCl buffer (pH 9.0; 0.01 M Mg^{2+}; 0.001 M 2-mercaptoethanol) the following reagents were present: 44.7 mg [3ax-^{3}H]Neu5Ac (in ^3H_2O; 18.5 GBq, see under a), 279.5 mg CTP (Boehringer, Mannheim, F.R.G.) and 1.2 units of a CMP-sialate synthase (EC 2.7.7.43) preparation from frog liver, which was partially purified by DEAE-cellulose chromatography [7,8]. After incubation for 4 h at 37°C, the reaction mixture was lyophilized. The residue was dissolved in 5 ml 1 mM NH_4OH and applied to a Dowex 1x8 column (HCO_3^- form, 1.5x43 cm, prewashed with 1 l 1 mM NH_4OH). After rinsing of the column with 250 ml 1 mM NH_4OH, the compounds containing Neu5Ac were eluted with 1 l of a linear gradient from 0.01-2 M triethyl-ammonium hydrogencarbonate, pH 7.8. The fractions containing CMP-[3ax-^{3}H]-Neu5Ac were pooled and lyophilized. For further purification the residue was dissolved in 1 ml 1 M NH_4OH and filtered over a Sephadex G-25 column (1x 90 cm), equilibrated with 1 mM NH_4OH [7]. The purity of the CMP-[3ax-^{3}H]Neu5Ac was checked as described previously [7], but using also thin-layer radio-scan detection [9]. The yield of CMP-[3ax-^{3}H]Neu5Ac was determined according to [10].

d. Stability test of CMP-[3ax-^{3}H]Neu5Ac
The lyophilized compound was dissolved in water, kept for 2 h at room temperature, lyophilized, counted for radioactivity by liquid scintillation and checked by t.l.c. [7,9]. This procedure was repeated twice.

e. Stability test of free [3ax-^{3}H]Neu5Ac
The retention of the ^{3}H label in Neu5Ac, liberated from CMP-[3ax-^{3}H]Neu5Ac by mild acid treatment (1 M HCOOH; 1 h; 37°C) was tested as follows. After lyophilization of the hydrolysate, samples each containing about 1 µg free Neu5Ac were dissolved in 1 ml water, 0.1 M HCOOH or 0.05 M triethanolamine bicarbonate buffer pH 8.0, respectively. The solutions were kept for 24 h at 0°C and lyophilized. Each of the residues was then dissolved in 1 ml of the original solvents and aliquots of 50 µl were counted for radio-activity. The remaining solutions were taken through the same procedure two more times with keeping the Neu5Ac concentration constant (maximum incubation time 72 h).
f. Transfer of [3ax-^{3}H]Neu5Ac from CMP-[3ax-^{3}H]Neu5Ac to asialofetuin.
Fetuin (Sigma, St. Louis, U.S.A.) was desialylated in 0.1 M HCl for 1 h at 80°C [11]. A microsomal preparation containing sialyltransferase activity (EC 2.4.99.1) was obtained from bovine submandibular glands [12, 13]. 25 µg CMP-[3ax-^{3}H]Neu5Ac (8 KBq) and 100 µg asialofetuin were incubated with 2 mg of the microsomal enzyme preparation in a total volume of 220 µl 0.1 M Tris/HCl buffer pH 6.9 for 1 h at 37°C. Subsequently, the microsomes were removed by centrifugation and the supernatants kept for 0, 2, 4, 8 or 42 h

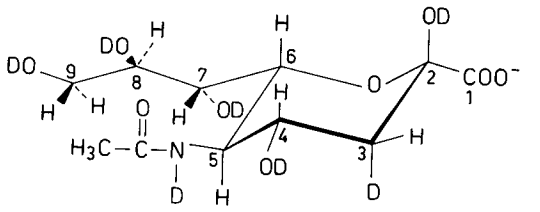
at 4°C before precipitating the fetuin on glass fibre filters by trichloroacetic acid and washing according to [13]. A control experiment was carried out using a heat inactivated enzyme preparation.

Results and Discussion

The 360-MHz ^{1}H-NMR spectrum of Neu5Ac in ^2H_2O at p^{2}H 9.0 is depicted in Fig. 1. The anomeric mixture of Neu5Ac contains predominantly the β-anomer. Slight differences in the chemical shifts of various proton resonances can be observed when compared to the spectra recorded from neutral or acidic solutions [3, 5, 14]. However, striking differences are the absence of the signal for H-3ax (normally at δ = 1.83 ppm) and the appearance of the signal for H-3eq as a doublet (see Fig. 1). As is evident from the observed coupling constants, the geminal coupling between H-3ax and H-3eq is missing. The pattern of the H-4 signal is also less complex, due to the absence of the coupling with H-3ax. Similar spectral features hold for the α-anomer, present for about 7% [2, 3, 5] in the equilibrium mixture. The absence of the H-3ax signal at δ = 1.62 ppm and the appearance of the H-3eq signal as a doublet at δ = 2.73 ppm are typical. The spectral data have led us to conclude that a virtually complete replacement of H-3ax by a deuterium atom has occurred in Neu5Ac. This stereo specific exchange reaction proceeds smoothly under mild alkaline conditions, most favourably at p^{2}H 9. No exchange takes place at p^{2}H $\leqslant$ 6. Therefore, label incorporated under alkaline conditions is retained at p^{2}H $\leqslant$ 6. Apparently this phenomenon is not restricted to Neu5Ac. In their discussion of the ^{1}H-NMR spectral data of Neu5Gc [p^{2}H $\sim$ 7), Jaques et al. [15] ascribe the reduced intensity of the H-3ax signal to saturation effects, which in the light of the foregoing results has to be conceived as the partial replacement of H-3ax by a deuterium atom. The exchange reaction is also suitable for the tritium labeling of H-3ax of Neu5Ac, simply by using ^3H_2O instead of ^2H_2O. ^{2}H or ^{3}H labeled Neu5Ac can be employed for the enzymic transfer to acceptor molecules via CMP-Neu5Ac as precursor.

CMP-[3ax-^{3}H]Neu5Ac was obtained from [3ax-^{3}H]Neu5Ac and CTP in the presence of CMP-sialic acid synthase in 37% yield (based on Neu5Ac). The product had a specific activity of 0.20 GBq/mmol. The product proved to be stable under alkaline conditions since the glycosidic linkage remained intact and no decrease of radioactivity was observed. It should be noted that if Neu5Ac is set free from CMP-[3ax-^{3}H]Neu5Ac by mild acid treatment, the radioactivity of free Neu5Ac remained unaltered at pH $\leqslant$ 6. However, in alkaline solutions the label is removed through the exchange reaction; for example at pH 8.0, 0°C, a decrease of the radioactivity to 1/3 of the initial value is observed within 72 h.

To demonstrate the transfer of labeled Neu5Ac from CMP-[3ax-^{3}H]Neu5Ac to an



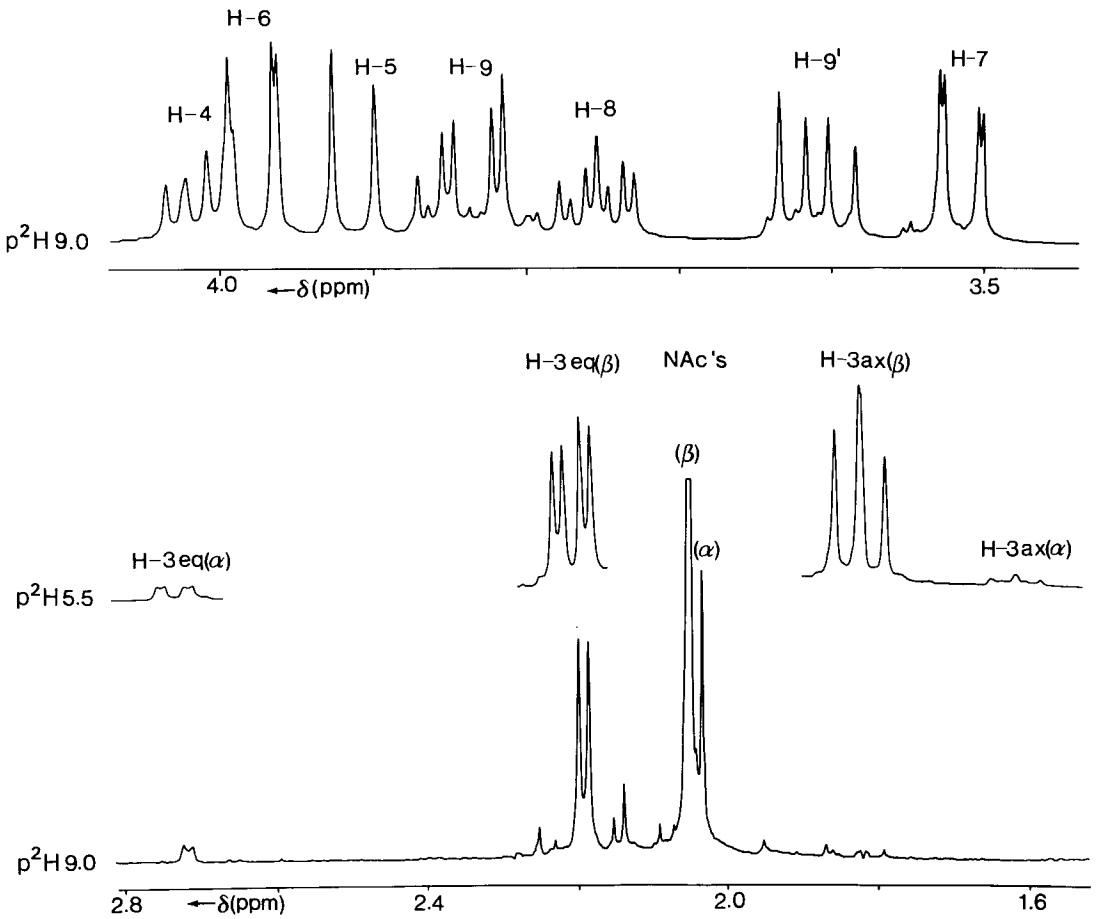


Fig. 1.
360 MHz ^{1}H-NMR spectrum of the anomeric equilibrium mixture of Neu5Ac in ^2H_2O solution at p^{2}H 9.0. For comparison the signals of H-3ax and H-3eq, obtained from the ^{1}H-NMR spectrum recorded at p^{2}H 5.5 are inserted. For clarity reasons, ^{2}H is represented by D in the structure of β-Neu5Ac.

acceptor molecule mediated by sialyl transferase, the nucleotide sugar was incubated with asialofetuin and a microsomal transferase preparation. The [3ax- ^{3}H]Neu5Ac appeared to be transferred in excellent yield. The ^{3}H label of fetuin is retained as long as Neu5Ac is glycosidically linked as was

tested under various conditions. Keeping the soluble enzymic reaction product for various periods of time in the incubation medium did not alter the amount of tritium in the resialylated fetuin. The radioactivity of the trichloroacetic acid-precipitated product was not affected by storage for different times in the washing solutions. By consequence, the radioactivity is incorporated into resialylated fetuin, and remains associated with this glycoprotein as long as Neu5Ac is glycosidically attached to it.

Concluding Remarks

The H-3ax of free Neu5Ac can specifically be exchanged for ^{2}H or ^{3}H in alkaline solutions. Under these conditions the exchange reaction is completely reversible. Glycosidically bound Neu5Ac is not subject to any exchange of the H-3ax, showing that only the free form undergoes this reaction. The precise mechanism of the reaction is not yet clear, but may be related to the GlcNAc — ManAc epimerization under alkaline conditions [16, 17]. Labeled Neu5Ac can via CMP-Neu5Ac be transferred enzymically to glycoconjugates in an efficient way. The availability of this new specific labeling technique constitutes a promising approach for studying the fate and metabolism of sialoglycoconjugates.

Acknowledgements

The authors are indepted to Mrs M. Wember for skilful technical assistance. This investigation was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO), the Netherlands Foundation for Cancer Research (KWF, grant UUKC-OC 79-13) and by the Deutsche Forschungsgemeinschaft.

References

[1] L. Dorland, J. Haverkamp, J.F.G. Vliegenthart, G. Strecker, J.-C. Michalski, B. Fournet, G. Spik and J. Montreuil, Eur. J. Biochem., (1978) 87, 323-329.
[2] J. Haverkamp, L. Dorland, J.F.G. Vliegenthart, J. Montreuil and R. Schauer, Abst. 9th Int. Symp. Carbohydr. Chem., London, (1978), pp 281-282.
[3] J.-M. Beau, R. Schauer, J. Haverkamp, L. Dorland, J.F.G. Vliegenthart and P. Sinaÿ, Carbohydr. Res., (1980), 82, 125-129.
[4] J.F.G. Vliegenthart, H. van Halbeek and L. Dorland, Pure Appl. Chem., (1981), 53, 45-77.
[5] J. Haverkamp, H. van Halbeek, L. Dorland, J.F.G. Vliegenthart, R. Pfeil and R. Schauer, Eur. J. Biochem., (1982), in press.
[6] J. Haverkamp, T. Spoormaker, L. Dorland, J.F.G. Vliegenthart and R. Schauer, J. Am. Chem. Soc., (1979), 101, 4851-4853.
[7] J. Haverkamp, J.-M. Beau and R. Schauer, Hoppe-Seyler's Z. Physiol. Chem., (1979), 360, 159-166.
[8] R. Schauer, J. Haverkamp and K. Ehrlich, Hoppe-Seyler's Z. Physiol. Chem., (1980), 361, 641-648.
[9] D.C. Comb, D.R. Watson and S. Roseman, J. Biol. Chem., (1966), 241, 5637-5642.
[10] E.L. Kean and S. Roseman, Methods Enzymol., (1966), 8, 208-215.
[11] J.P. Despont, C.A. Abel and H.M. Grey, Cell. Immunol., (1975), 17, 487-494.

[12] R. Schauer and M. Wember, Hoppe-Seyler's Z. Physiol. Chem., (1971), 352, 1282-1290.
[13] R. Schauer, E. Moczar and M. Wember, Hoppe-Seyler's Z. Physiol. Chem., (1979), 360, 1587-1593.
[14] E.B. Brown, W.S. Brey, Jr. and W. Weltner, Jr., Biochim. Biophys. Acta, (1975), 399, 124-130.
[15] L.W. Jaques, B.F. Riesco and W. Weltner, Jr., Carbohydr. Res., (1980), 83, 21-32.
[16] W.L. Salo, M. Hamari and L. Hallcher, Carbohydr. Res., [1976), 50, 287-291.
[17] M.A.E. Sallam, Carbohydr. Res., (1978), 63, 127-130.