Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXXI, No. 9

Synthesis of N-Acyl-2-amino-2-deoxy-[1-^{14}C]-glucoses as Precursors for the Biosynthesis of Novel N-Acyl-neuraminic Acids

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SUMMARY

N-Propanoyl-, N-butanoyl-, N-pentanoyl-, N-hexanoyl-, N-heptanoyl- and N-crotonoyl-[1-^{14}C]-D-glucosamine were synthesized from [1-^{14}C]-D-glucosamine and their respective carbonic acid anhydrides as precursors for the biosynthesis of the corresponding N-acyl neuraminic acids. The N-acyl-glucosamines are phosphorylated to their respective phosphate by N-acetyl-glucosamine kinase in a rat liver homogenate. These precursors may become valuable tools to investigate the biological role of the N-acyl side chain of N-acyl-glucosamines and N-acyl neuraminic acids which are components of glycoconjugates.

KEYWORDS: N-acyl-2-amino-2-deoxy-[1-^{14}C]-D-glucoses, glycoconjugates, sialic acid.

INTRODUCTION

Labelled N-acetyl-D-glucosamine is known as a valuable precursor for the incorporation of radioactivity into the oligosaccharide portion of glycoconjugates. It is incorporated as N-acetyl-neuraminic acid and as N-acetylglucosamine itself [1]. These sugars of glycoconjugates play an important role in many biological processes such as cell adhesion and antigenicity [2]. Grünholz et al. [3] could show that N-propanoyl-D-glucosamine is converted to N-propanoyl neuraminic acid in a rat liver homogenate. This finding motivated us to synthesize different [^{14}C]-labelled N-acyl-2-amino-2-deoxy-glucoses with elongated N-acyl-side chains to investigate their metabolization in a rat liver

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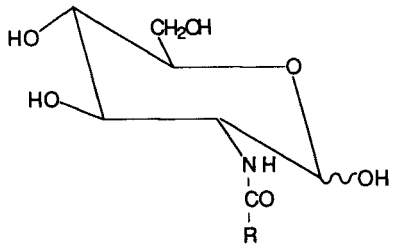
0362–4803/92/090711–05$07.50

Received 21 May, 1992
Revised 16 June, 1992

homogenate. The preparation of unlabelled N-acyl-2-amino-2-deoxy-glucoses was performed in order to check the reaction conditions and structures of the compounds for the radioactive synthesis. The preparation of the substances has usually been achieved by a procedure according to Roseman and Ludowieg [4] as outlined in Scheme 1.



Structure of N-acyl-2-amino-2-deoxy-glucoses
R = Propyl-, butyl-, pentyl-, hexyl-, heptyl- and but-2-enyl-

$$C_6H_{14}NO_5Cl \xrightarrow{\text{Dowex-1x2, carbonate form}} C_6H_{13}NO_5 \qquad [1]$$

$$C_6H_{13}NO_5 \xrightarrow[0\text{-}4\,^{\circ}C,\ \text{stirring}]{\text{carbonic acid anhydride}} C_7H_{12}NO_6R \qquad [2]$$

SCHEME 1

EXPERIMENTAL

Materials.

Chemicals and solvents of analytical grade were from Merck (D-6100 Darmstadt). [1-^{14}C]-Glucosamine (55 mCi/mM) was purchased from ARC (USA-St. Louis). Anhydrides of propanoic, butanoic, pentanoic, hexanoic acids, and D-glucosamine were from Merck (D-6100 Darmstadt). But-2-enoic acid and heptanoic acid anhydride were from Aldrich (Steinheim, Germany). For quantification of radioactivity a scintillation spectrometer, model 1900 CA (Packard Instruments Inc., CH-8038 Zürich) was used.

Synthesis of N-acyl-2-amino-2-deoxy-[1-^{14}C]-glucoses.

N-Acyl-2-amino-2-deoxy-glucoses were synthesized according to a modified method of Roseman and Ludowieg [4]. ^{1}H-NMR spectra were obtained on a Bruker 270 spectrometer (Bruker, Karlsruhe, Germany). For the synthesis of N-acyl-2-amino-2-deoxy-[1-^{14}C]-glucoses, 50 µCi [1-^{14}C]-glucosamine (0.9 µmol) were dissolved in 100µl water/methanol (1:1; v/v) then shaken with 20 µl of Dowex-1x2 (carbonate form) and 2µl of the respective alkanoic acid anhydride for 90 minutes at 0-5 °C. The mixture was centrifugated at 5000 x g for 10 minutes to remove Dowex-1x2 and the supernatant was passed through a column containing 1ml of Amberlite IR-120 (acid form). The effluent was

purified by descending paper chromatography as described above and the main product could be obtained by elution with water. Purity of the product was proven by rechromatography.

Chromatographic Methods.

Thin layer chromatography to check purity of reaction product was performed on plates coated with Kieselgel 60, thickness 0.2 mm (Merck, D-6100 Darmstadt), using n-butanol/ethanol/water (4:2:1.5, by vol.) as solvent system. Amino sugar derivatives were detected after spraying with sulfuric acid and then heating of the plate. Descending paper chromatography was performed on Whatman No. 3MM paper using a solvent system containing n-propanol/water/1mol/l sodium acetate (7:2:1; by vol.), pH 5.0. The chromatograms were usually developed for 16 h using UMP as internal standard. To determine radioactivity, paper strips from chromatograms were measured by liquid scintilation counting.

Metabolization of N-acyl-2-amino-2-deoxy-glucosamines in a rat liver homogenate.

In order to show in vitro metabolism of N-acyl-2-amino-2-deoxy-hexoses a rat liver homogenate was prepared as described previously [5]. The metabolization assay contained in a final volume of 350µl: rat liver homogenate (200µl), ATP (1.14 mmol/l), UTP (1.14 mmol/l), CTP (1.14 mmol/l), phosphoenolpyruvate (2.28 mmol/l), and pyruvate kinase (57mU/µl). The reaction performed at 37°C for 30 minutes was started by adding 100nCi of the respective N-acyl-2-amino-2-deoxy-glucose and stopped by addition of 500 µl ethanol (97%). The mixture was heated for 5 min at 65°C to stimulate the precipitation of proteins followed by centrifugation at 5.000xg for 3 min. The supernatants were analysed by descending paper chromatography as described above. The N-acyl-glucosamine phosphates were identified according to Grünholz et al. [3] by enzymatic disgestion with alkaline phosphatase (EC 3.1.3.1.).

RESULTS AND DISCUSSION

Synthesis of N-acyl-2-amino-2-deoxy-[1-^{14}C]-glucoses.

Transformation of the stable HCl-form into the free glucosamine is efficiently carried out using Dowex-1x2 anionic exchange resin (carbonate form) as shown in Scheme 1, [1]. Carbonic acid anhydrides easily react with D-glucosamine producing the respective N-acylated 2-amino-2-deoxy-glucose when educts were mixed in equimolar ratios (Scheme 1, [2]). The structure of the products were confirmed by the resonances of the methyl triplette at 0.8 ppm and the multiplets of the methylene groups in the high field of the acyl-side chain and the amino hydrogen duplet at approximately 7 ppm. The reaction with the labelled D-[1-^{14}C]-glucosamine lead to a second product which could be identified as the diacylated D-[1-^{14}C]-glucosamine. It can, however, easily be separated from the monoacylated derivative by paper chromatography. Purification of labelled N-acyl-D-glucosamine by descending paper

chromatography yields a compound with 99% purity containing less than 0.1% of D-[1-^{14}C]-glucosamine. Yields of acylated amino sugars, however, are decreasing with increasing length of the N-acyl-side chain obviously due to reduced solubility of the product in the reaction solvents used (Tab. 1).

Tab.1 Characterization of the N-acyl-2-amino-2-deoxy-[1-^{14}C]-glucoses synthesized
Synthesis of N-acyl-glucosamines, thin layer chromatography (DC) and descending paper chromatography (PC) were performed as described in the Experimental section.

substance	yield [%]	melting point [°C]	R_F-Value	
			DC	PC
D-glucosamine			0.05	0.40
N-Propanoyl-D-glucosamine	77	180-182	0.62	0.69
N-Butanoyl-D-glucosamine	51	204-207	0.68	0.75
N-Pentanoyl-D-glucosamine	42	209-211	0.71	0.79
N-Hexanoyl-D-glucosamine	51	190-191	0.76	0.83
N-Heptanoyl-D-glucosamine	11	n.d.	0.82	0.89
N-Crotonyl-D-glucosamine	25	n.d.	0.65	0.76

n.d. = not determined.

Metabolization of N-acyl-2-amino-2-deoxy-glucosamines in a rat liver homogenate.

By incubation of N-pentanoyl-[1-^{14}C]-D-glucosamine in a rat liver homogenate N-pentanoyl-D-glucosamine-6-phosphate is formed in analogy to the phosphorylation of N-acetylglucosamine to N-acetylglucosamine-6-phosphate by the N-acetylglucosamine kinase, an enzyme involved in the biosynthesis of N-acetylneuraminic acid (Fig. 1). N-Pentanoyl-D-glucosamine is not deacylated to D-glucosamine under the conditions applied. Identity of the N-pentanoylglucosamine was proven by the use of alkaline phosphatase. Disgestion with this enzyme yields the original precursor. Furthermore heating of the precursors synthesized with 1 mol/l HCl for 3h gave [1-^{14}C]-D-glucosamine, the original educt. In similar experiments metabolic formation of the respective 6-phosphate could be shown for all six precursors synthesized. Furthermore additional substances were formed which so far have not been identified; they may be metabolites belonging to the biosynthetic pathway of the

N-acetyl-neuraminic acid. By further experiments in vitro and in vivo with PC12 cells, rat and mouse, however, we were able to find hints that the labelled precursors are incorporated as their respective N-acyl-neuraminc acids into glycoconjugates [6].

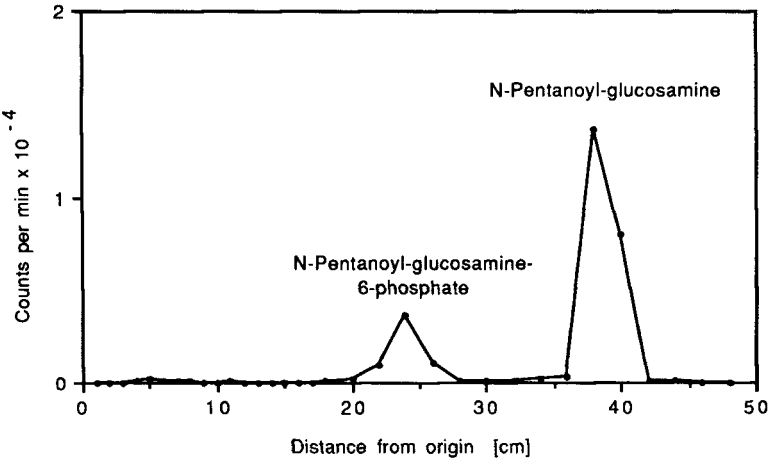


Fig. 1 N-Pentanoyl-[1-^{14}C]-D-glucosamine and its metabolites in a rat liver homogenate

The incubation time was 30 min. Metabolites were separated and identified as described in the experimental section. UMP moved 5cm from the origin.

ACKNOWLEDGEMENT

The authors acknowledge financial support from the Bundesministerium für Forschung und Technologie, Bonn, and the Fidia Pharma Forschung GmbH, München. Thanks are also due to the service section of the Institut für Organische Chemie der Freien Universität Berlin for carrying out the ^{1}H-NMR spectra.

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