Synthesis of 2-Acetamido-2-deoxy-α-D-mannopyranosyl Phosphate and Uridine 5′-(2-Acetamido-2-deoxy-α-D-mannopyranosyl Dipotassium Pyrophosphate)*

Wilmar L. Salo† and Hewitt G. Fletcher, Jr.

ABSTRACT: The reaction of 2-methyl-(3′,4′,6′-tri-*O*-acetyl-1,2-dideoxy-β-D-mannopyrano)[2′,1′:4,5]-2-oxazoline (**1**) with dibenzyl hydrogen phosphate, followed by catalytic hydrogenolysis to remove the benzyl groups and transesterification to remove the acetyl groups, affords 2-acetamido-2-deoxy-α-D-mannopyranosyl phosphate which was isolated as its crystalline di(cyclohexylammonium) salt (**2**) in a yield (based on **1**) of 46%. The use of the oxazoline (**1**) here provides steric control, ensuring the formation of a product in which the phosphate group at C-1 is *trans* to the acetamido group at C-2. An alternate synthesis was carried out through the fusion (*in vacuo*) of 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-mannopyranose (**3**) with anhydrous phosphoric acid, **2** being isolated in 14% yield after deacetylation and salt formation.

Some aspects of the nuclear magnetic resonance spectrum of **2** are discussed briefly. Condensation of the tri-*n*-octylammonium salt of 2-acetamido-2-deoxy-α-D-mannopyranosyl phosphate with uridine 5′-monophosphoromorpholidate (**4**) in anhydrous pyridine solution yielded uridine 5′-(2-acetamido-2-deoxy-α-D-mannopyranosyl pyrophosphate) which was isolated as its dipotassium salt (**5**).

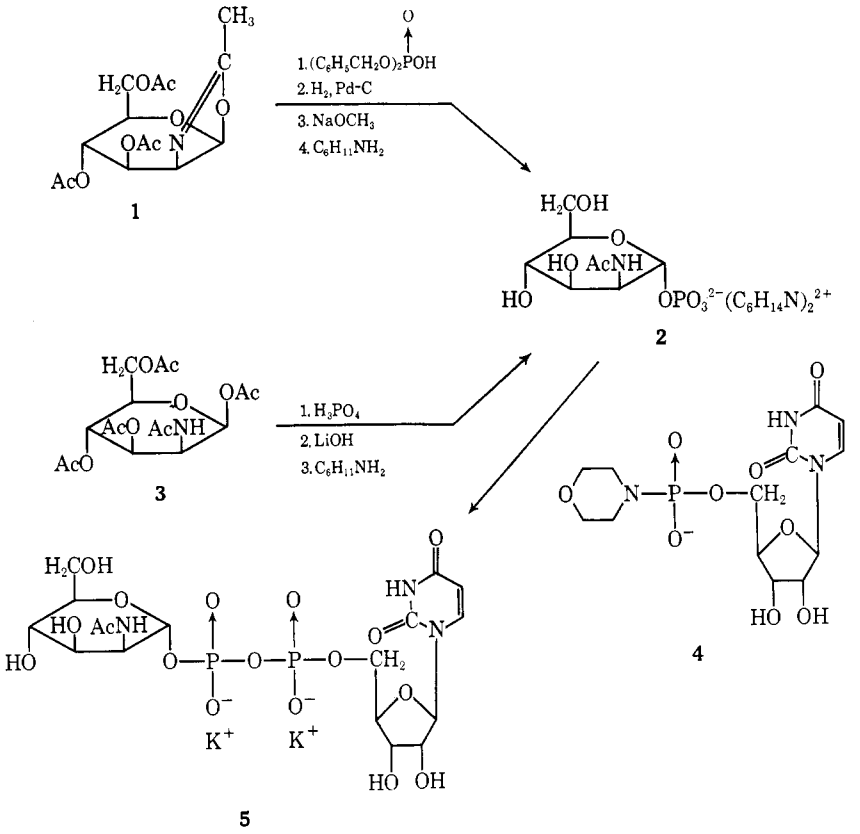
Cardini and Leloir (1957) showed that an enzyme from rat liver converted uridine diphosphate *N*-acetylglucosamine [uridine 5′-(2-acetamido-2-deoxy-α-D-glucopyranosyl pyrophosphate)] into an amino sugar which was later identified by Comb and Roseman (1958) as *N*-acetylmannosamine. While Spivak and Roseman (1966) have suggested that uridine diphosphate *N*-acetylmannosamine [uridine 5′-(2-acetamido-2-deoxy-α-D-mannopyranosyl pyrophosphate)] is a probable intermediate in the reaction, this sugar nucleotide has not as yet been isolated from biological media and we have, therefore, undertaken its syntheis by chemical means. This synthesis as well as that of the precursor substance, 2-acetamido-2-deoxy-α-D-mannopyranosyl phosphate, will be described here.

Earlier work in this laboratory (Pravdić *et al.*, 1967) showed that treatment of 2-acetamido-2-deoxy-D-mannose with a mixture of acetic anhydride and anhydrous zinc chloride readily affords 2-methyl-(3′,4′,6′-tri-*O*-acetyl-1,2-dideoxy-β-D-mannopyrano)[2′,1′:4,5]-2-oxazoline (**1**). Oxazolines of this type react very readily with nucleophiles (Micheel and Köchling, 1957; Pravdić *et al.*, 1967; Zurabyan *et al.*, 1969), the attacking species entering to form a product in which the substituent at C-1 is *trans* to the acetamido group at C-2. The simplicity and stereospecificity of this reaction strongly recommended it as the key step in the synthesis of 2-acetamido-2-deoxy-α-D-mannopyranosyl phosphate. Preliminary experiments, in which anhydrous phosphoric acid was allowed to react with the oxazoline (**1**) and the *O*-acetyl groups then removed, gave a complex mixture of products. Condensation of **1** (Scheme I) with dibenzyl hydrogen phosphate, however, yielded a single product from which the benzyl groups were removed by catalytic hydrogenolysis and the *O*-acetyl groups by transesterification. After chromatographic purification, a sugar phosphate was isolated in 46% yield (based on **1**) as its di(cyclohexylammonium) salt, having $[\alpha]_D^{20}$ +21.3° in water. The elemental composition of the material agreed with that of the di(cyclohexylammonium) salt of a 2-acetamido-2-deoxyhexosyl phosphate (**2**) and the substance gave 2-acetamido-2-deoxy-D-mannose on acidic hydrolysis. The nuclear magnetic resonance spectrum of the substance, dissolved in D_2O, showed a three-proton signal for the acetyl group; the signal for H-1 appeared as a quartet, being coupled with H-2 ($J_{1,2}$ = 2.0 Hz) and with phosphorus ($J_{1,P}$ = 8 Hz). For comparison, $J_{1,2}$ for both anomeric forms of 2-acetamido-1,3,4,6-tetra-*O*-acetyl-D-mannopyranose is 1.6 Hz (Inch *et al.*, 1966) while $J_{1,P}$ for the two anomeric D-glucopyranosyl phosphates is 7.5 Hz (Kochetkov *et al.*, 1969). α-D-Mannopyranosyl phosphate shows a $J_{1,P}$ of 8.5 Hz and $J_{1,2}$ of 1.5 Hz (Onodera and Hirano, 1966). From the above, it is evident that the H-1–H-2 coupling and H-1–P coupling of **2** are close to the expected values but that these values are not diagnostic of the anomeric configuration of **2**; evidence for this rests upon the mechanism of its formation from **1**.

As an alternative to the synthesis of **2** from **1**, we investigated the application of the fusion synthesis of aldosyl phosphates originally devised by MacDonald (1962). 2-Acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-mannopyranose (**3**) (O'Neill, 1959) was fused *in vacuo* with anhydrous phosphoric acid. Successive deacetylation, removal of inorganic phosphate and ion-exchange chromatography led to the isolation of the crystalline di(cyclohexylammonium) salt **2**. The physical properties of the product (melting point, specific rotation,

* From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare, Bethesda, Maryland 20014. *Received October 10, 1969.*

† Staff Fellow, National Institutes of Health, 1967–1969. Present address: Department of Chemistry, Division of Science and Mathematics, University of Minnesota, Duluth, Minn. 55812.



and nuclear magnetic resonance spectrum) clearly showed it to be identical with the product obtained earlier through the oxazoline (**1**). The yield of **2** from **3** was only 14% but this figure is of little significance since no effort was made to maximize the yield in the reaction. Such a low yield cannot, of course, be used to support mechanistic proposals but one would expect the α anomer to predominate on both kinetic and thermodynamic grounds.

With 2-acetamido-2-deoxy-α-D-mannopyranosyl phosphate in hand, we turned to the problem of the synthesis of the sugar nucleotide **5**. For this purpose, the procedure developed by Moffatt and Khorana (1961) and Roseman *et al.* (1961) was used as adapted by Nordin *et al.* (1965). The di(cyclohexylammonium) salt (**2**) was converted into the corresponding tri-*n*-octylammonium salt and this was condensed in dry pyridine solution with uridine 5′-monophosphoromorpholidate (**4**). The product, purified by ion-exchange chromatography, was isolated as an amorphous, hygroscopic potassium salt. Prepared thus, the uridine diphosphate 2-acetamido-2-deoxy-α-D-mannopyranose (**5**) behaved like its glucose analog on paper chromatography and paper electrophoresis. Dried *in vacuo*, it gave satisfactory elemental analyses; it showed the ultraviolet absorption spectrum of the uridine moiety and, on mild hydrolysis, afforded 2-acetamido-2-deoxy-D-mannose.

Experimental Section

General Methods. Melting points are equivalent to corrected values. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter. Nuclear magnetic resonance spectra were taken with a Varian A-60 spectrometer, using D_2O as a solvent and the sodium salt of 3-(trimethylsilyl)-propanesulfonic acid as an internal standard. Ultraviolet absorption measurements were made with a Beckman Model DU spectrometer. Gas–liquid partition chromatography was carried out with a Hewlett-Packard Model 5750 chromatograph using a flame ionization detector and a column (0.25 in. o.d. × 6 ft) of 3% SE-52 on Gas-Chrom A at 190°. Trimethylsilylation was performed with "Tri-Sil Z." Both Whatman No. 1 and No. 3MM papers were used for paper chromatography and paper electrophoresis, a Beckman Duostat power supply being used at 500–600 V and constant current for paper electrophoresis. Nucleotides and sugar nucleotides were located on chromatograms or electrophoretograms by viewing under radiation of 254 nm provided by a Gelman-Camag Universal ultraviolet lamp, Model 51402. Sugar phosphates were detected by the Hanes–Isherwood (1949) method as modified by Bandurski and Axelrod (1951). Reducing sugars were detected by the method of Trevelyan *et al.* (1950). For 2-acetamido-2-deoxy-D-mannose the modified Morgan–Elson method (Spivak and Roseman, 1959) was used; phosphate was assayed by the method of Fiske and Subbarow (1925).

Materials. 2-Acetamido-2-deoxy-D-mannose was purchased from Pfanstiehl Laboratories, Inc., while uridine 5′-monophosphoromorpholidate came from Calbiochem Corp. and uridine diphosphate *N*-acetylglucosamine was a product of Sigma Chemical Co. Dibenzyl hydrogen phosphate was kindly provided by Dr. D. E. Kiely. Palladium on carbon catalyst (10% palladium) was a product of Englehard Industries, Inc., and crystalline phosphoric acid was obtained from Matheson, Coleman & Bell.

Di(cyclohexylammonium) 2-Acetamido-2-deoxy-α-D-manno-

pyranosyl Phosphate (**2**). A. From 2-methyl-(3′,4′,6′-tri-*O*-acetyl-1,2-dideoxy-β-D-mannopyrano)[2′,1′:4,5]-2-oxazoline (**1**). The crystalline oxazoline (**1**, 805 mg, 2.44 mM) was dissolved in anhydrous benzene (70 ml) containing dibenzyl hydrogen phosphate (1.02 g, 4.08 mM). After storage at room temperature for 20 hr the reaction mixture, which was still clear and colorless, was poured into a hydrogen-saturated suspension of palladium on carbon (10% palladium, 800 mg) in anhydrous methanol (100 ml). The mixture was shaken with hydrogen at room temperature and pressure until absorption of the gas ceased (152 ml, *ca.* 2 hr) and was then filtered through a sintered-glass filter directly into a cold solution of sodium methoxide made from 200 mg of sodium and 50 ml of methanol. The solution was stored at +5° overnight, diluted with water, and then neutralized with carbon dioxide. After the solution had been concentrated to a small volume (10 ml), samples were removed for examination by paper chromatography using a mixture of 1 M ammonium acetate (pH 3.8) and 95% ethanol in relative volumes of 75:30 (Paladini and Leloir, 1952) and by electrophoresis in 0.1 M ammonium acetate (pH 7.5); both techniques showed the presence of an organic phosphate as well as of inorganic phosphate. The solution was therefore chromatographed on a column (2.8 × 45 cm) of Dowex 1 (HCO_3^-), elution being carried out with a linear gradient of triethylammonium bicarbonate (1575 ml of 0.05 M and 1575 ml of 0.3 M) (MacDonald, 1968), 15-ml fractions of eluate being collected. Fractions were assayed for inorganic phosphate and for 2-acetamido-2-deoxy-D-mannose and total phosphate after acid hydrolysis. Fractions 73–96 contained no P_i and liberated 2-acetamido-2-deoxy-D-mannose and P_i on hydrolysis; they were pooled and lyophilized. The residue was dissolved in water (10 ml) and the solution was passed over Amberlite IR-120 (pyridinium salt). Freshly distilled cyclohexylamine (2 moles/mole of sugar phosphate) was added to the solution and it was then evaporated to dryness. The resulting syrup was dissolved in water and the solution was concentrated; after this process had been repeated, 95% ethanol was added to the syrup. The product crystallized and was then recrystallized twice from 95% ethanol: yield 560 mg (46%), mp 159–163° (after yellowing at 145°), $[\alpha]_D^{20}$ +21.3° (*c* 0.93, water). Acidic hydrolysis of the product gave inorganic phosphate and 2-acetamido-2-deoxy-D-mannose, identified by paper chromatography using the upper phase of pyridine–ethyl acetate–water (2:5:7) (McFarren *et al.*, 1951) and by gas–liquid partition chromatography of its trimethylsilyl ether. The nuclear magnetic resonance spectrum of the product in D_2O solution included signals at δ 2.1 (singlet, 3 H, NAc), 3.85 (singlet, H-6), and 5.3 (quartet, H-1, $J_{1,2}$ = 2.0 Hz, $J_{1,P}$ = 8 Hz).

Anal. Calcd for $C_{20}H_{42}N_3O_9P$ (499.55): C, 48.09; H, 8.47; N, 8.41; P, 6.20. Found: C, 47.89; H, 8.56; N, 8.14; P, 5.98.

B. From 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-mannopyranose (**3**). Anhydrous phosphoric acid (5.5 g) was placed in a flask equipped with a side arm containing 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-mannopyranose (**3**, 5.0 g). The flask was evacuated with an oil pump and heated in an oil bath at 50°. After 1 hr, the sugar acetate was added to the molten phosphoric acid and mixing effected by repeatedly tilting the flask. After 2.5 hr at 50° the reaction mixture had ceased ebullition and acquired a dark red-brown color. The reaction conditions were maintained for 1 hr more and the cooled mixture was then dissolved in anhydrous tetrahydrofuran (50 ml). The solution was poured into ice-cold and vigorously stirred aqueous lithium hydroxide solution (1 N, 305 ml) and then stored at +5° overnight. Celite (10 g) was added and the solution was freed of suspended lithium phosphate by filtration through a sintered-glass filter of medium porosity. The filtrate was passed through a column of Amberlite IR-120 (pyridinium salt) and triethylamine (10 ml) was added to the effluent which was then concentrated to a volume of *ca.* 10 ml. This solution was placed on a column (15 × 2.8 cm) of Dowex 1 (HCO_3^-) and the column was washed with four bed volumes of water. The sugar phosphate was then eluted with 0.3 M triethylammonium bicarbonate (40 ml) and the eluate was lyophilized. Purified further by chromatography on a column of Dowex 1 (HCO_3^-) as described under A above, the product was obtained as the crystalline di(cyclohexylammonium) salt: 900 mg (14% yield), mp 155–160°, $[\alpha]_D^{20}$ +21.8° (*c* 1.53, water). The nuclear magnetic resonance spectrum of the product in D_2O solution was identical with that of the **2** prepared from **1**.

Uridine 5′-(2-Acetamido-2-deoxy-α-D-mannopyranosyl Dipotassium Pyrophosphate) (**5**). The crystalline di(cyclohexylammonium) salt of 2-acetamido-2-deoxy-α-D-mannopyranosyl phosphate (**2**, 200 mg) was dissolved in water and the solution was passed through a column of Amberlite IR-120 (pyridinium salt), the effluent being collected directly in pyridine (10 ml) containing tri-*n*-octylamine (121 mg). The resulting solution was concentrated to dryness on a rotating evaporator (35° bath) and three batches of pyridine were successively evaporated *in vacuo* from the residue. Finally, the material was dissolved in anhydrous pyridine (*ca.* 2 ml) and allowed to react with uridine 5′-monophosphoromorpholidate (**4**, 530 mg) as described by Nordin *et al.* (1965). After 4 days at room temperature, the sealed reaction vessel was opened and the pyridine was removed on a rotating evaporator. The resulting residue was dissolved in 0.05 M triethylammonium bicarbonate (10 ml) and the solution was extracted twice with dichloromethane (5 ml) to remove tri-*n*-octylamine. The solution was aerated to remove residual dichloromethane and it was then placed on a column (2.9 × 38 cm) of Dowex 1 (HCO_3^-). The column was eluted with a linear gradient of triethylammonium bicarbonate (1500 ml, 0.05 M and 1500 ml, 0.5 M), 15-ml fractions of eluate being collected. The uv absorption of the fractions at 262 nm was examined and they were also assayed for acid-labile phosphate. Fractions 100–110 contained 2-acetamido-2-deoxy-α-D-mannopyranosyl phosphate while fractions 210–240 contained the desired sugar nucleotide; electrophoresis in 0.1 M ammonium acetate at pH 7.5 provided supportive evidence for the identity of these components. Two ultraviolet-absorbing peaks, centered around fractions 40 and 160, were detected but not identified; they may have represented **4** and uridine 5′-phosphate, respectively. The fractions containing the sugar nucleotide (210–240) were pooled and lyophilized. The pale yellow residue was dissolved in the minimum volume of 0.05 M triethylammonium bicarbonate and the solution was placed on a column (1.8 × 33 cm) of Sephadex G-10 which had previously been equilibrated with the same solvent. The column was eluted with 0.05 M triethylammonium bicarbonate, 5-ml portions of eluate being collected and assayed for ultraviolet absorption at 262 nm.

Fractions 6–16 contained the sugar nucleotide; they were pooled and lyophilized. The now colorless product was dissolved in distilled water (2 ml) and the solution was passed through a small column (3 ml) of Amberlite IR-120 (pyridinium salt). It was then concentrated *in vacuo* at 30° (bath) until its pH dropped to *ca.* 4.5; the solution was then passed through Amberlite IR-120 (K^+) and lyophilized to give 67 mg of residue. The highly hygroscopic product thus obtained behaved like uridine 5′-(2-acetamido-2-deoxy-α-D-glucopyranosyl pyrophosphate) upon paper chromatography with ethanol–ammonium acetate (1 M, pH 3.8, 75:30) and on paper electrophoresis (0.1 M ammonium acetate of pH 7.5). It showed the uv spectrum of the uridine moiety and, upon mild acid hydrolysis, yielded 2-acetamido-2-deoxy-D-mannose which was identified by paper chromatography and by gas–liquid partition chromatography of its trimethylsilyl derivative. Prior to elemental analysis, the product was dried *in vacuo* at 50° for 1 hr, losing 5.65% of its weight.

Anal. Calcd for $C_{17}H_{25}K_2N_3O_{17}P_2$ (683.56): C, 29.87; H, 3.69; N, 6.15. Found: C, 29.64; H, 3.92; N, 6.04.

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