BIOCHEMICAL PREPARATIONS

Volume 13

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Statement from The Editorial Board, Biochemical Preparations, May 1971

The Editorial Board of *Biochemical Preparations* has carried on extensive discussions about the status and future of this series. *Biochemical Preparations* has had a successful history and has continued to provide reliable descriptions of preparative methods. However, several competing efforts, including the excellent series of volumes of *Methods in Enzymology* and the journals *Analytical Biochemistry* and *Preparative Biochemistry*, have greatly reduced the number of preparations available to us. In addition, the commercial availability of many reliable reagents has improved to a highly satisfying extent, since the organization of *Biochemical Preparations*. While we still feel that the checked preparation has much value, it has become increasingly difficult to find willing submitters and checkers, and so we have regretfully decided to conclude the series with this volume.

PREFACE

The present collection of preparations reflects the increased interest in synthesis of peptides, using a variety of protected derivatives. It is hoped that the preparations included here will serve as appropriate examples of the available techniques. Some useful reagents, carbohydrate derivatives, and polypeptide hormones have also been included. A variety of synthetic and chromatographic methods are demonstrated.

Because this is the concluding volume of the series, I could not, as could editors of previous volumes, hold individual preparations over for the next volume and had to ensure that all preparations were checked before sending this final volume to press. This circumstance has delayed publication of some of the preparations by as long as four years. I wish to apologize for this delay and to thank many submitters for their patience. I also wish to express my gratitude to the checkers who donated time to confirm that each procedure was adequately and completely described.

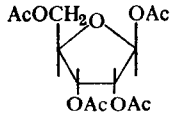
J. H. L.

May, 1971

COMPOUNDS OF BIOCHEMICAL INTEREST WHICH HAVE APPEARED IN *ORGANIC SYNTHESES* (THROUGH VOLUME 47)

CONTENTS

1,2,3,5-TETRA-*O*-ACETYL-β-D-RIBOFURANOSE



Mol. Wt. 318.1 ($C_{13}H_{18}O_9$)

Submitted by R. D. GUTHRIE and S. C. SMITH, School of Molecular Sciences, University of Sussex, Brighton, England
Checked by PETER M. BARNA, Calbiochem, Los Angeles, California

I. Principle

A new synthesis, based on the acetylation and acetolysis of the methyl ribofuranoside mixture obtained by the method of Barker and Fletcher,[1] has been developed.

Acetylation is performed using either pyridine and acetic anhydride or acetic acid and acetic anhydride with sulfuric acid as catalyst. In the latter case the triacetates are not isolated, but acetolysis is carried out directly by increasing the concentration of sulfuric acid.

II. Procedure

A. Base-catalyzed acetylation. A solution of D-ribose (32.0 g., 0.21 mole) in dry methanol (500 ml.) is treated with sulfuric acid (2.5 ml.) and left at 0–3° for 12–14 hours,[1,2] and then neutralized with dry pyridine (100 ml.). Evaporation gives the methyl ribofuranosides as a stiff syrup, of which a solution in dry pyridine (250 ml.) is treated with acetic anhydride (100 ml.) with cooling and left at room temperature for 2 days.[3] The usual work up procedure gives the methyl 2,3,5-tri-*O*-acetyl-β-D-ribofuranoside as a clear, pyridine-free syrup, which is dissolved in acetic acid (300 ml.) and acetic anhydride (70 ml). and treated with sulfuric acid (15 ml.)[4] with ice-cooling. After 12 hours

[1] R. Barker and H. G. Fletcher, Jr., *J. Org. Chem.*, **26**, 4605 (1961).

[2] After addition of sulfuric acid the checker left the solution at 0–8° overnight, ca. 17 hours.

[3] The checker observed that the acetylation mixture becomes carmine red on standing at room temperature for 2 days.

[4] The checker recommends that the H_2SO_4 addition be done very slowly because a considerable amount of heat can evolve.

at room temperature the red solution[5] is stirred with ice (400 g.), the mixture extracted with chloroform, and the extracts, after washing with water and then aqueous sodium hydrogen carbonate, dried and evaporated to give a syrupy solid. This material on treatment with ice-cold ethanol (40 ml.) gives tetra-*O*-acetyl-β-D-ribofuranose (28.7 g., 45%), m.p. 79–82°, $[\alpha]_D^{22}$ −13.0 (*c* 1.4 in $CHCl_3$). The mother liquor is evaporated to a syrup (15 g.) which is retreated as before, using scaled-down quantities, to give further tetraacetate (6.8 g., 10%), m.p. 79–82°.[6]

B. Acid-catalyzed acetylation. A methyl ribofuranoside syrup prepared as above, but using 50 g. of D-ribose, is dissolved in acetic anhydride (200 ml.) and acetic acid (150 ml.) and treated with sulfuric acid (6 ml.)[7] with ice-cooling and left at room temperature for 1 hour. More sulfuric acid (10 ml.) is then added with ice-cooling and the solution left at room temperature for an additional 2 hours.[8] The resulting dark red solution is then treated with an excess of sodium acetate (ca. 45 g.)[9] and the mixture coevaporated several times with ethanol to give a stiff, brown syrup to which is added chloroform (300 ml.). The mixture is washed with water, the chloroform solution dried and evaporated to give a syrupy solid, which, on treatment with ice-cold ethanol (60 ml.), yields the tetraacetate (44.5 g., 42%), m.p. 81–83°, $[\alpha]_D^{22}$ −13.1 (*c* 4.7, in $CHCl_3$). The ethanol washings are evaporated to a syrup (ca. 50 g.) which was reacetolyzed as above to give further tetraacetate, m.p. 81–83° (11.6 g., 11%).

III. Properties and Purity of Product

The samples of tetraacetate obtained are pure by multiple-pass, thin-layer chromatography on silica gel (Merck GF254) using 2% methanol-benzene as eluent, a system which separates the pyranose and furanose isomers. The physical characteristics obtained for the product are in agreement with those found by Kissman et al.[10] [m.p.

[5] The checker's solution was yellow, not red, probably because of efficient cooling during H_2SO_4 addition.

[6] The checker's yield was 36.7 g., 59%, m.p. 82.5–83°, $[\alpha]_D^{22}$ −13.1° (*c* 1.4, in $CHCl_3$).

[7] The checker again recommends very slow addition of H_2SO_4 or temperature may suddenly rise above 30°.

[8] The checker found that the acidic solution became dark if kept at room temperature overnight. He found that the solution remained almost white when kept for 2 hours only.

[9] The checker observed that on addition of Na acetate the color of the solution changes to cream and the temperature rises to 40°.

[10] H. M. Kissman, C. Pidacks, and B. R. Baker, *J. Amer. Chem. Soc.*, **77**, 18 (1955).

81–82°, $[\alpha]_D^{24.5}$ −12.9 (*c* 2.05, in chloroform)], and with those found by Zinner,[11] [m.p. 84°, $[\alpha]_D^{24}$ −12.6° (*c* 12.83, in chloroform)]. A mixed melting point with a sample, m.p. 81–82°, prepared by the method of Kissman et al.,[10] showed no depression.

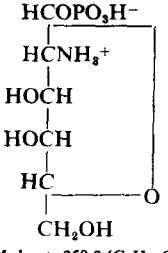
The mother liquors from both preparations were examined by thin-layer chromatography using the system described above and were found to contain both α- and β-tetra-*O*-acetyl-D-ribofuranose (the latter predominating) by comparison with authentic samples. An unidentified component of higher R_f and one near the origin were also found, but no tetra-*O*-acetyl-D-ribopyranoses were detected.

IV. Methods of Preparation

The available methods of synthesis are based on a tedious separation, either by partition chromatography[10] or by fractional crystallization[12] of the mixture of furanose and pyranose isomers that result from direct acetylation of D-ribose. Neither method was found convenient by the authors for the large-scale preparation of the tetraacetate. A recent patent describes a synthesis of the tetraacetate by simultaneous acetolysis and acetylation of various purine and pyrimidine nucleosides.[13]

[11] H. Zinner, *Chem. Ber.*, **83**, 153 (1950).
[12] G. B. Brown, J. Dovoll, and B. A. Lowy, *Biochem. Prepns.*, **4**, 10 (1963).
[13] Fr. pat. 1,498,005.

α-D-GALACTOSAMINE-1-PHOSPHORIC ACID
(2-Amino-2-deoxy-α-D-galactopyranosyl Dihydrogen Phosphate)



Mol. wt. 259.2 ($C_6H_{14}O_8NP$)

Submitted by D. M. CARLSON, A. SWANSON, and S. ROSEMAN, Rackham Arthritis Research Unit, The University of Michigan, Ann Arbor, Michigan[1]
Checked by P. PERCHEMLIDES and ROGER W. JEANLOZ, Massachusetts General Hospital, Boston, Massachusetts

[1] Present addresses: D. M. Carlson, Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio; A. Swanson, Department of Biochemistry and Biophysics, University of California, San Francisco, California; S. Roseman, Department of Biology, Johns Hopkins University, Baltimore, Maryland.

I. Principle

Incubation of galactosamine, adenosine triphosphate, and Mg^{2+} with crude yeast galactokinase preparations yields galactosamine 1-phosphate.[2] The product is isolated by ion-exchange chromatography and crystallized as the free acid.

II. Starting Material

Galactosamine hydrochloride is prepared as previously described[3] or obtained commercially. Phosphoenolpyruvate, 3-phosphoglycerate, and ATP are obtained commercially. A galactose-adapted strain of *Saccharomyces fragilis* is purchased from Sigma Chemical Co.

III. Procedure

A. Enzyme preparation. Crude galactokinase is prepared by autolyzing 2 g. of yeast in 6 ml. of 0.1 *M* $NaHCO_3$ for 12 hours at 25°. After centrifugation at 32,000 × *g* for 30 minutes, the supernatant fluid is decanted. The residue is washed with 6 ml. of bicarbonate, the mixture centrifuged, and the supernatant fluids combined. The crude extract contains approximately 50 mg. of protein per ml.

B. Preparation of D-galactosamine 1-phosphate. The incubation mixture contains the following components (mmoles) in a final volume of 140 ml.: galactosamine hydrochloride (6.0), phosphoenolpyruvate (10.0),[4] adenosine triphosphate (6.0),[4] 3-phosphoglycerate (10.0), potassium phosphate buffer, *p*H 7.8 (30.0), magnesium chloride (3.0), and 30 ml. of the crude galactokinase preparation. The *p*H is

[2] C. E. Cardini and L. F. Leloir, *J. Biol. Chem.*, **225**, 317 (1957).

[3] S. Roseman and J. Ludoweig, *J. Amer. Chem. Soc.*, **76**, 301 (1954).

[4] The checkers purchased the Ca salt of phosphoenol pyruvate from Sigma and converted it into the Na salt by passage through Dowex 50 and neutralization to *p*H 4.3 with NaOH. They used the trisodium phosphoenolpyruvate·6 H_2O and disodium adenosinetriphosphate·3 H_2O (Sigma).

maintained by adding 1 *N* NaOH as required.[5] After 6 hours at 30°, 0.2 ml. of toluene is added, and the mixture is maintained at 30° for an additional 12 hours. The reaction is terminated by heating at 100° for 5 minutes, and the precipitate is removed by centrifugation. These incubation conditions give an 80% yield (4.8 mmoles) of galactosamine 1-phosphate. The solution is placed on a column of Dowex 50 (H^+) (420 ml.,[6] 12% cross-linked, 100–200 mesh) and the column is washed with water to elute the desired product.[7] The product is eluted from the column following the nucleotides and other sugar phosphates, and is located by assaying the fractions for total phosphorus and galactosamine 1-phosphate.[8] The fractions containing the product are combined (yield 3.9 mmoles of galactosamine 1-phosphate) and lyophilized. The resulting white powder (1.1 g.) is dissolved in 20 ml. of water, and treated with 20 ml. of 95% ethanol. Crystals slowly form in the solution and are harvested after 4–6 days. The first-crop material contains 0.95 g.; approximately 0.1 g. is obtained in the second crop of crystals.

C. Assay of D-galactosamine 1-phosphate. This procedure, devised for the assay of galactokinase, is generally applicable to enzyme-catalyzed reactions where reducing sugars are converted into glycosides.

The assay mixture contains the following components (μmoles) in a final volume of 0.14 ml.: potassium phosphate buffer, *p*H 7.8 (200), magnesium chloride (2), D-galactosamine hydrochloride (40), adenosine triphosphate (40), phosphoenolpyruvate (10), 3-phospnoglycerate (10), and 0.01 ml. of the crude galactokinase preparation. After incubation

[5] The checkers found that the pH of the reaction mixture and the amount of enzyme used are critical. When the conditions described by Carlson et al. were used, a yield of only 30% was obtained; it was raised to 45% by doubling the amount of enzyme. When the *p*H of the substrate solution was adjusted to 7.45 before addition of enzyme, the initial pH of the mixture of enzyme and substrate was 7.15; it was adjusted to 7.20, and after a reaction time of 20 hours it had decreased to 7.07. In this case the yield of product was 60%, and it was increased to 90% by doubling the amount of enzyme (the yields were determined colorimetrically on the reaction mixture).

[6] The checkers were unable to obtain a good separation of galactosamine 1-phosphate with the amount of resin reported by Carlson et al. (420 ml. for 6 mmoles). They had to use 2.1 liters for 3 mmoles (ten times more). Using the modifications described above (control of *p*H, double amount of enzyme, 10-fold increase of resin absorbent), 1.38 g. (88%) of galactosamine 1-phosphate was obtained from 1.30 g. of galactosamine hydrochloride.

[7] G. W. Jourdian and S. Roseman, *Biochem. Prepns.*, **9**, 44 (1962).

[8] It was found preferable to detect the peak of galactosamine 1-phosphate with the ninhydrin reagent instead of with the Fiske-Subbarow reagent, since the first test is less time-consuming and more sensitive.

at 30° for 15 minutes the reaction mixture is heated at 100° for 2 minutes. After centrifugation, 0.05 ml. of the supernatant fluid is assayed for D-galactosamine 1-phosphate as described below.

1. *Reduction of the remaining substrate with sodium borohydride.* One drop of capryl alcohol is added, followed by 0.025 ml. of 1.0 *M* $NaBH_4$. After mixing, the solution is allowed to stand at room temperature for 5 minutes with occasional shaking, and the borohydride addition is repeated. After 5 minutes, 0.025 ml. of 2.0 *M* acetone is added, the solution is thoroughly mixed and allowed to stand at room temperature for 5 minutes.[9] Finally, the excess borohydride is completely destroyed by heating the mixture at 100° for 3 minutes.

2. *Acetylation.* The acetylation procedure involves the addition of 0.1 ml. of 0.5 *M* aqueous acetic anhydride while the *p*H is maintained between 7 and 9 with saturated $NaHCO_3$.[10] The mixture is allowed to stand for 10 minutes, although the N-acetylation is completed in a few minutes.

3. *Hydrolysis.* The *N*-acetyl-D-galactosamine 1-phosphate is hydrolyzed by adding 0.2 ml. of 2 *N* HCl, then heating at 100° for 10 minutes. After cooling, the reaction mixture is neutralized with 2 *N* NaOH using phenolphthalein as an indicator. Water is added to a final volume of 1.0 ml., and 0.5 ml. of the final mixture is assayed directly for *N*-acetyl-D-galactosamine by a modified Morgan-Elson method.[11]

IV. Properties and Purity of Product

After drying to constant weight over P_2O_5 under reduced pressure at 56°, the crystals showed the following chemical analyses. Calc. for $C_6H_{14}O_8NP$: C, 27.81; H, 5.45; N, 5.41; P, 11.95. Found: C, 27.67; H, 5.64; N, 5.43; P, 11.88. The optical rotation was $[\alpha]_D^{25}$ +142.6° (*c* 2.0% in water).

V. Other Preparations

After N-acetylation of the galactosamine 1-phosphate with acetic anhydride, the resulting *N*-acetyl-α-D-galactosamine 1-phosphate was

[9] The checkers omitted acetone and destroyed borohydride by heating at 100°.

[10] J. J. Distler, J. M. Merrick, and S. Roseman, *J. Biol. Chem.*, **230**, 497 (1958).

[11] C. T. Spivak and S. Roseman, *J. Amer. Chem. Soc.*, **81**, 2403 (1959).

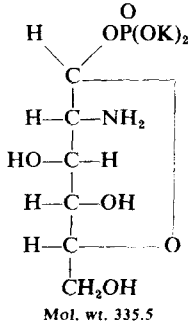
crystallized as the dipotassium salt which had $[\alpha]_D^{25}$ +112.4° (*c* 2.9% in water).[12] Uridine diphosphate-*N*-acetyl-α-D-galactosamine was then prepared as described previously:[13] $[\alpha]_D^{25}$ 81.7° (*c* 0.8% in water).

VI. Methods of Preparation

The procedure described for the synthesis of α-D-galactosamine 1-phosphate is a modification of that of Cardini and Leloir.[2] Galactokinase has a high K_m for galactosamine,[14] and by increasing the galactosamine concentration it is possible to increase greatly the yield of galactosamine 1-phosphate. Galactosamine 1-phosphate has been prepared by a chemical method.[15]

[12] D. M. Carlson, A. Swanson, and S. Roseman, *Biochemistry*, **3**, 402 (1964).
[13] S. Roseman, J. J. Distler, J. G. Moffatt, and H. G. Khorana, *J. Amer. Chem. Soc.*, **83**, 659 (1961).
[14] F. Alvardo, *Biochim. Biophys. Acta*, **41**, 233 (1960).
[15] T. Y. Kim and E. A. Davidson, *Fed. Proc.*, **22**, 239 (1963).

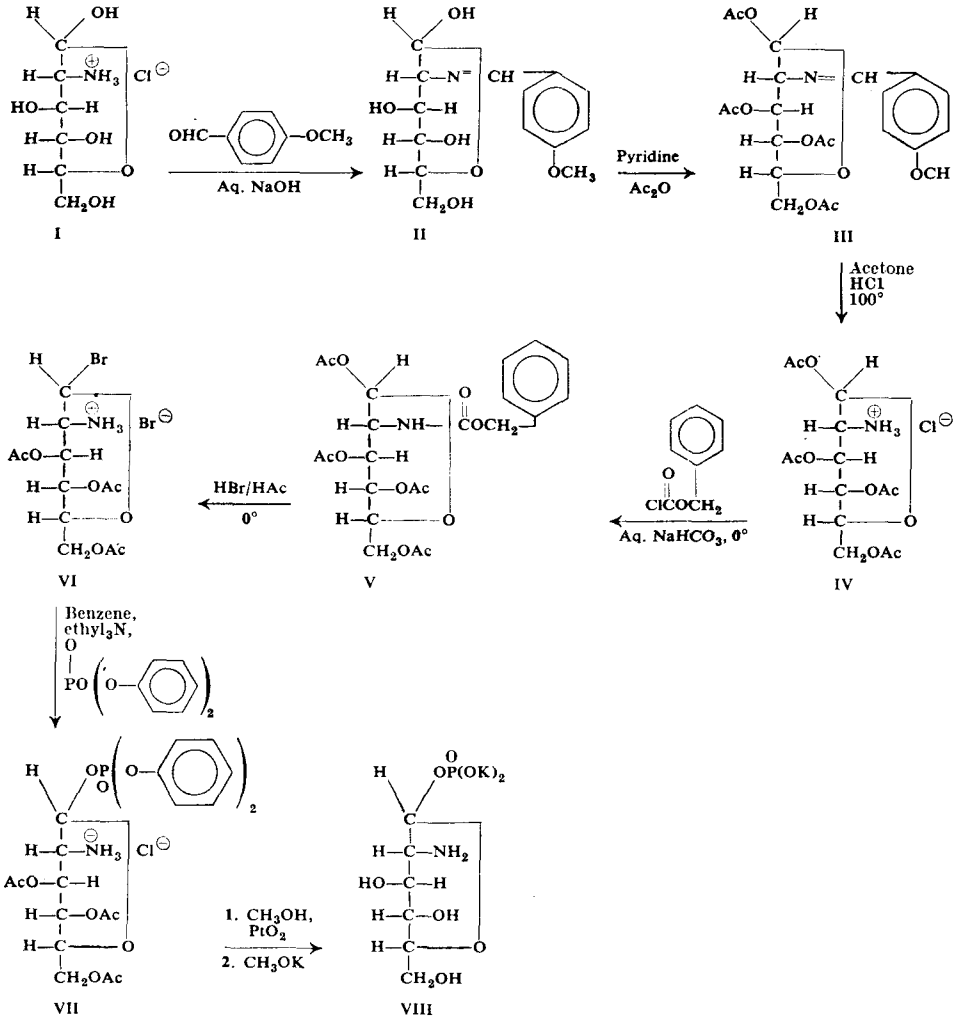
α-D-GLUCOSAMINE 1-PHOSPHATE



Mol. wt. 335.5

Submitted by ROBERT CHERNIAK, Department of Chemistry, State University, Atlanta, Georgia
Checked by FRANK MALEY, New York State Department of Health, Albany, New York

I. Principle



α-D-Glucosamine 1-phosphate is prepared by reaction[1] of α-1-bromotriacetyl-D-glucosamine hydrobromide with triethylammonium diphenyl phosphate as outlined in the accompanying scheme. All the intermediates are obtained in crystalline form without difficulty.

[1] F. Maley, G. Maley, and H. A. Lardy, *J. Amer. Chem. Soc.*, **78**, 5303 (1956).