3-O-Methyl-D-mannose from *Streptomyces griseus*

BY D. J. CANDY* AND J. BADDILEY

Microbiological Chemistry Research Laboratory, Department of Organic Chemistry,
University of Newcastle upon Tyne

(Received 2 June 1965)

1. *Streptomyces griseus* was grown in a medium containing L-[Me-¹⁴C]methionine, and the labelled products from an ethanolic extract of the cells were examined. 2. Acid hydrolysis of one of the products gave a compound identified as 3-O-[Me-¹⁴C]-methylmannose by a series of degradative reactions. 3. Reduction of the radioactive compound gave 3-O-methyl-D-mannitol, indistinguishable from a synthetic sample.

It has been shown (Candy, Blumsom & Baddiley, 1964) that the methyl group of methionine is incorporated efficiently into the N-methyl-L-glucosamine moiety of streptomycin by *Streptomyces griseus*. During unsuccessful attempts to identify intermediates in the biosynthesis of streptomycin, *S. griseus* was grown in the presence of [Me-¹⁴C]-methionine and the labelled products were investigated. No such intermediates were identified, but during the investigation a highly radioactive fraction was obtained that gave on hydrolysis 3-O-methyl-D-mannose.

MATERIALS AND METHODS

Growth of organism. *Streptomyces griseus* (Glaxo strain L118) was grown on slopes as described by Candy *et al.* (1964). The resulting spores were used to inoculate 100 ml. of a medium containing Oxoid Nutrient broth no. 2 (2.5%) and glucose (1%), and the culture was grown for 24 hr. on a shaker (140 oscillations/min.). Portions (50 ml.) of this culture were used to inoculate each of two flasks (Glaxo pattern, 2.5 l. capacity), each containing 200 ml. of Carvajal's (1957) medium, 0.2 g. of soya-bean meal and 9.6 μ moles (0.15 mc) of [Me-¹⁴C]methionine (The Radiochemical Centre, Amersham, Bucks). The cultures were grown for 48 hr. on the shaker.

Preparation of extract. The culture was centrifuged at 10000g for 20 min. at 5° and the sedimented fraction was resuspended in 200 ml. of 0.6% NaCl solution and again centrifuged. The sedimented fraction was made into a slurry with water and adjusted to pH 6 with 0.2N-HCl. Boiling ethanol (2 vol.) was added, the mixture was rapidly brought to the boil and after 1 min. it was cooled and filtered.

Fractionation of extract. The extract was evaporated under reduced pressure to 20 ml. and passed through a column of Dowex 1 (Cl⁻ form) resin (9 ml.), then through a column of Amberlite CG-50 (Na⁺ form) resin (5 ml.) and

finally through a column of Dowex 50 (H⁺ form) resin (11 ml.). The solution was neutralized by stirring with Amberlite IR-45 (OH⁻ form) resin and was evaporated under reduced pressure to a small volume. This was called the 'neutral fraction'.

Hydrolysis of 'neutral fraction'. A sample of the 'neutral fraction' was hydrolysed in N-H₂SO₄ for 16 hr. at 100° in a sealed tube. The hydrolysate was diluted, neutralized with BaCO₃ and filtered. A paper chromatogram developed in solvent 1 showed that almost all the radioactivity was present in a spot with *R*_{GlC} 1.8. The radioactive compound was partially purified in the following manner.

The hydrolysate was mixed with a small amount of cellulose powder and applied to a column (1 cm. $\times$ 37 cm.) of Whatman cellulose powder, previously washed with propan-1-ol-ethyl acetate (7:1, v/v). Material was then eluted by successive application of propan-1-ol-ethyl acetate-water (7:1:1, by vol.) (50 ml.), propan-1-ol-ethyl acetate-water (7:1:1.5, by vol.) (40 ml.) and finally propan-1-ol-ethyl acetate-water (7:1:2, by vol.) (40 ml.). Almost all the radioactivity corresponded to a single peak eluted at 60 ml. The fractions containing the radioactivity were combined and concentrated under reduced pressure to a small volume.

3-O-Methyl-D-mannose from medium. The supernatant obtained by centrifugation of the original culture was deionized in the same way as described for the cell extract. On hydrolysis (N-HCl for 16 hr. at 100°) this neutral fraction gave a radioactive compound that corresponded chromatographically and electrophoretically to the product of hydrolysis of the 'neutral fraction'.

Paper chromatography. The following solvent systems were used: solvent 1, propan-1-ol-ethyl acetate-water (7:1:2, by vol.); solvent 2, butan-1-ol-ethanol-water (5:1:4, by vol.; top layer); solvent 3, butan-1-ol-ethanol-water-aq. NH₃ (sp.gr. 0.88) (40:10:49:1, by vol.; top layer). Whatman no. 1 paper was employed. Sugars were detected either by the method of Trevelyan, Procter & Harrison (1950) or by the periodate-Schiff reaction (Baddiley, Buchanan, Handschumacher & Prescott, 1956).

Determination of radioactivity. Radioactivity on paper chromatograms was detected either with a strip scanner (Atomic Accessories Inc.) or by radioautography with Kodirex X-ray film. Measurements were made with a

* Present address: Department of Biochemistry, University of Birmingham.

Packard Tri-Carb scintillation counter no. 3314, as described by Candy & Baddiley (1965).

Preparation of 3-O-methyl-D-mannitol. Methyl 4,6-O-benzylidene- α -D-mannoside was prepared from D-mannose as described by Buchanan & Schwarz (1962). The product (12g., m.p. 144°) was partially methylated by adding a mixture of methyl iodide (20 ml.) and methanol (5 ml.) and refluxing. Silver oxide (12g.) was added in several portions during 3 hr. and refluxing was continued for a further 3 hr. After being cooled, the reaction mixture was filtered and the residue was washed with chloroform and then methanol. The combined filtrate and washings were evaporated under reduced pressure, and the residue was hydrolysed in *N*-HCl (50 ml.) for 30 min. at 100°. After evaporation under reduced pressure the product was refluxed for 3 hr. in methanol containing 0.5% of HCl. The mixture was neutralized with Amberlite IR-45 (OH⁻ form) resin and filtered, and solvent was evaporated. The residue was dissolved in 0.05 M-sodium periodate solution (1 l.) and the solution was kept for 24 hr. at room temperature in the dark. Isomeric 2-O-methylmannoside was oxidized under these conditions. Spectrophotometric examination of the reaction mixture at 223 m μ (Dixon & Lipkin, 1954) showed that about 80% of the periodate remained. The reaction mixture was deionized by passage through an excess of Amberlite IR-120 (H⁺ form) resin and then Amberlite IR-45 (OH⁻ form) resin. The solution was evaporated and the residue was hydrolysed in *N*-HCl for 1 hr. at 100° and neutralized with an excess of Amberlite IR-45 (OH⁻ form) resin. The hydrolysate was passed through a column containing Dowex 1 (borate form) resin (50 ml.), the column was washed with water and elution was carried out with 0.5 N-boric acid solution. The eluate was collected in fractions, which were examined chromatographically for the presence of 3-O-

methyl-D-mannose. Appropriate fractions were combined and evaporated repeatedly with methanol to remove boric acid. Attempts to crystallize the sugar were unsuccessful.

The syrupy 3-O-methyl-D-mannose (150 mg.) was dissolved in water (10 ml.) and treated with sodium borohydride (100 mg.) in water (5 ml.). The reaction mixture was kept at room temperature for 16 hr., and then an excess of Amberlite IR-120 (H⁺ form) resin was added. The mixture was stirred for 15 min. and filtered. The filtrate was repeatedly evaporated with methanol to remove boric acid, and 3-O-methyl-D-mannitol was obtained from the residue by crystallization from butan-1-ol. The yield was 50 mg., m.p. 133–134°, $[\alpha]_D^{26} + 17.1 \pm 1^\circ$ (c 0.55 in water) (Bladon & Owen, 1950, give m.p. 132–133°, $[\alpha]_D^{20} + 16.5^\circ$).

RESULTS

Preliminary experiments. Since very small quantities of the labelled compound were available (the amounts used for each experiment were too small to detect chemically), the reactions were followed only by the observations of radioactive products (either by counting different fractions or by examining radioactivity on paper chromatograms of reaction mixtures). These preliminary experiments are summarized in Table 1 and illustrated in Scheme 1.

Identification of 3-O-methyl-D-mannitol. A sample of the labelled sugar was reduced with sodium borohydride to give a product (see Table 1, Expt. 6), chromatographically and electrophoretically identical with the synthetic 3-O-methyl-D-mannitol.

Table 1. Summary of experiments carried out on the labelled reducing sugar

Expt. no.	Treatment	Result
1	Solution treated with Dowex 50 (H ⁺ form)	¹⁴ C not adsorbed on resin
2	Kuhn–Roth oxidation (Milton & Waters, 1955)	No [¹⁴ C]acetic acid produced
3	Sugar treated with HBr (Hough, Jones & Wadman, 1950)	[¹⁴ C]Methyl bromide formed
4	Paper chromatography:	
	Solvent 1	
	Solvent 2	
	Solvent 3	
5	Paper electrophoresis in 0.05 M-sodium tetraborate	M_{Glc} 0.48
6	Reduced with sodium borohydride	Product chromatographically different from starting material
7	Heated at 100° with methanol containing 3% (w/v) of HCl	Product chromatographically and electrophoretically different from starting material
8	Oxidized with hypiodite (Willstätter & Schudel, 1918)	Product chromatographically different from starting material
9	Oxidized with periodate at pH 8 (Hough & Perry, 1956)	[¹⁴ C]Methanol produced
10	Oxidized with periodate at pH 4 followed by mild alkali hydrolysis (Huffman, Lewis, Smith & Spriesterbach, 1955)	Chromatography and electrophoresis showed same product (2-O-methylarabinose) given by similar treatment of 3-O-methylglucose
11	Periodate oxidation of methanolysis product (see Expt. 7 above) at pH 4	No oxidation

Scheme 1. Reactions of 3-O-methyl-D-mannose.

The product was mixed with synthetic 3-O-methyl-D-mannitol and the mixture was recrystallized from butan-1-ol containing 3% (v/v) of water. It is shown below that the added radioactive material crystallized with the authentic 3-O-methyl-D-mannitol, and that the specific activity remained constant through six recrystallizations.

Examination of the 'neutral fraction'. The 'neutral fraction' slowly diffused through a dialysis sac (Visking). It was somewhat retarded by Sephadex G-25, the radioactive material appearing in 1.1–1.5 times the void volume. These results suggest that the compound might possess a molecular weight of 1000–5000. Paper chromatography in propan-1-ol-aq. ammonia (sp.gr. 0.88)–water (6:3:1, by vol.) gave a fairly broad band with an R_f similar to that for a maltodextrin of chain length 7. Examination of the products of partial hydrolysis (0.1N-hydrochloric acid for 1 hr. at 100°) of the 'neutral fraction' by paper chromatography in solvent 1 showed a series of radioactive products with ascending R_f values up to and including that corresponding to the 3-O-methylmannose. This suggests that the radioactive component of the 'neutral fraction' is an oligosaccharide or small polysaccharide, or possibly a series of these covering a range of molecular weights. However, paper chromatography in a variety of solvents was not

entirely consistent with this view and it is not yet possible to classify this compound with confidence.

DISCUSSION

Although the nature of the compound in *S. griseus* containing a 3-O-methylmannose residue has not been studied in detail, the methylated sugar itself has been fully characterized. Evidence for the identity of the reducing sugar as 3-O-methyl-D-mannose is presented in Table 1 and below, and the reactions are illustrated in Scheme 1. The result of Expt. 3 (Table 1) suggests that the labelled atom might be present in an O-methyl group. The carbohydrate nature of the compound is likely from its susceptibility to oxidation with periodate, its chromatographic properties and its migration on electrophoresis in borate. Evidence that it is a reducing sugar was provided by the results of Expt. 6 (reduction by borohydride) and Expt. 7 (methyl glycoside formation). The results of Expt. 8 (hypoiodite oxidation) suggest that it is an aldose.

Treatment of 3-O-methyl-D-glucose with periodate at pH 4 (Expt. 10) oxidizes the sugar between C-1 and C-2; the product, 4-O-formyl-2-O-methyl-D-arabinose, is not further attacked, provided that the formyl ester group remains intact (Barker &

Smith, 1952). Mild hydrolysis in alkali removes the formyl group to give 2-*O*-methyl-D-arabinose (Huffman *et al.* 1955). When the radioactive sugar was treated in the same way as the 3-*O*-methyl-D-glucose a chromatographically identical product was obtained. Since the sugar is an aldose and yet is not identical with 3-*O*-methylglucose (Expt. 4), it must be the epimeric sugar 3-*O*-methylmannose. The result of Expt. 11 agrees with this, since a methyl mono-*O*-methylhexopyranoside with the ether group at any position other than at C-3 would be oxidized by periodate.

Proof of identity was obtained by co-crystallization of the reduced labelled sugar with synthetic 3-*O*-methyl-D-mannitol. For one to six recrystallizations the specific activities were respectively 686, 684, 690, 684, 680 and 686 counts/min./mg. The identification of the sugar, its derivatives and degradation products was achieved with radioactivity as the only means of detection. There were two reasons for adopting this procedure: (a) only very small quantities of material were available, and it was not possible to detect either starting material or products by chemical methods; (b) although the labelled reducing sugar was radiochemically pure (shown by chromatography, electrophoresis and behaviour in the various chemical reactions applied to it), it was not known whether it was chemically pure. Since such small amounts were available it would have been difficult to establish chemical purity.

Naturally occurring *O*-methyl sugars, although not common, have been isolated from a number of different sources. Thus certain antibiotics produced by Actinomycetes contain sugar residues bearing *O*-methyl groups. One example is the sugar noviose (Walton, Rodin, Stammer, Holly & Folkers, 1956; Hinman, Caron & Hoeksema, 1957). Cardiac glycosides frequently contain such residues, an example being 6-deoxy-3-*O*-methyl-L-mannose (Muhr, Hunger & Reichstein, 1954; see also Pigman, 1957), and they have been identified among the products of mycobacteria (Lee & Ballou, 1964). As far as we know, this is the first identification of 3-*O*-methyl-D-mannose from natural sources. Presumably, biosynthesis occurs by methyl group transfer from L-methionine to a sugar or sugar derivative. Such a process has also been demonstrated in the biosynthesis of noviose (Birch, Cameron, Holloway & Rickards, 1960).

The preparation of 3-*O*-methyl-D-mannose from D-mannose involves first protecting the hydroxyl groups at positions 1, 4 and 6, followed by partial methylation. Since methyl 4,6-*O*-benzylidene- α -D-mannoside is in the C-1 conformation (Reeves,

1949; Aspinall & Zweifel, 1957), the hydroxyl group at C-3 is equatorial and that at C-2 is axial. The equatorial hydroxyl group would be expected to be more readily methylated than the axial group, and partial methylation would produce a higher proportion of the 3-*O*-methyl derivative than of the 2-*O*-methyl derivative. Oxidation of the partially methylated methyl mannosides with periodate destroys methyl 2-*O*-methyl-D-mannoside and methyl D-mannoside, but methyl 3-*O*-methyl-D-mannoside is unaffected. 3-*O*-Methyl-D-mannose could be separated from 2,3-di-*O*-methyl-D-mannose and other impurities by chromatography on a borate column.

We thank Glaxo Ltd. for a grant and for cultures of *Streptomyces griseus*. Grants are also acknowledged from the Science Research Council and U.S. Department of Public Health (AI-05237-01 BIO).

REFERENCES

- Aspinall, G. O. & Zweifel, G. (1957). *J. chem. Soc.* p. 2271.
 Baddiley, J., Buchanan, J. G., Handschumacher, R. E. & Prescott, J. F. (1956). *J. chem. Soc.* p. 2818.
 Barker, G. R. & Smith, D. C. C. (1952). *Chem. & Ind.* p. 1035.
 Birch, A. J., Cameron, D. W., Holloway, P. W. & Rickards, R. W. (1960). *Tetrahedron Lett.* **25**, 26.
 Bladon, P. & Owen, L. N. (1950). *J. chem. Soc.* p. 604.
 Buchanan, J. G. & Schwarz, J. C. P. (1962). *J. chem. Soc.* p. 4770.
 Candy, D. J. & Baddiley, J. (1965). *Biochem. J.* **96**, 526.
 Candy, D. J., Blumsom, N. L. & Baddiley, J. (1964). *Biochem. J.* **91**, 31.
 Carvajal, F. (1957). *U.S. Patent* 2808364.
 Dixon, J. S. & Lipkin, D. (1954). *Analyt. Chem.* **26**, 1092.
 Hinman, J. W., Caron, E. L. & Hoeksema, H. (1957). *J. Amer. chem. Soc.* **79**, 3789.
 Hough, L., Jones, J. K. N. & Wadman, W. H. (1950). *J. chem. Soc.* p. 1702.
 Hough, L. & Perry, M. B. (1956). *Chem. & Ind.* p. 768.
 Huffman, G. W., Lewis, B. A., Smith, F. & Spriesterbach, D. R. (1955). *J. Amer. chem. Soc.* **77**, 4346.
 Lee, Y. C. & Ballou, C. E. (1964). *J. biol. Chem.* **239**, pc3602.
 Milton, R. F. & Waters, W. A. (1955). *Methods of Quantitative Micro-Analysis*, p. 118. London: Edward Arnold (Publishers) Ltd.
 Muhr, H., Hunger, A. & Reichstein, T. (1954). *Helv. chim. Acta*, **37**, 403.
 Pigman, W. (1957). *The Carbohydrates*, p. 552. New York: Academic Press Inc.
 Reeves, R. E. (1949). *J. Amer. chem. Soc.* **71**, 215.
 Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
 Walton, E., Rodin, J. O., Stammer, C. H., Holly, F. W. & Folkers, K. (1956). *J. Amer. chem. Soc.* **78**, 5454.
 Willstätter, R. & Schudel, G. (1918). *Ber. dtsch. chem. Ges.* **51**, 780.