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# PREPARATION OF CRYSTALLINE α-KOJIBIOSE OCTAACETATE FROM DEXTRAN B-1299-S: ITS CONVERSION INTO *p*-NITROPHENYL AND *p*-ISOTHIOCYANATOPHENYL β-KOJIBIOSIDE

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ABSTRACT

Acetolysis of dextran B-1299-S provides a preparative approach to crystalline α-kojibiose octaacetate. The latter was transformed via crystalline hepta-*O*-acetyl-α-kojibiosyl bromide into *p*-nitrophenyl and *p*-isothiocyanatophenyl β-kojibiosides.

## INTRODUCTION

For immunochemical studies we required several derivatives of kojibiose (2-*O*-α-D-glucopyranosyl-D-glucose), some of which could be covalently bound to macromolecular carriers such as a protein. Two such useful derivatives are *p*-aminophenyl and *p*-isothiocyanatophenyl β-kojibioside, both of which may be derived from *p*-nitrophenyl β-kojibioside. Hence, our primary objective was the synthesis of *p*-nitrophenyl β-kojibioside. This paper describes a preparative method for crystalline α-kojibiose octaacetate from dextran B-1299-S, and its subsequent conversion into the two title crystalline β-glycosides.

## EXPERIMENTAL

*General methods.* — Melting points were determined on a Fisher–Johns melting-point apparatus and are uncorrected. All evaporations were conducted *in vacuo* at 35–40° with a rotary evaporator. Silica gel (type G, Brinkmann) was used for t.l.c.; components were detected under u.v. light and by spraying with 50% (v/v) ethanol–sulfuric acid followed by charring at 120°. T.l.c. was performed with the following solvent systems: (*A*) 96:4 benzene–ethanol, (*B*) 1:1 ethyl acetate–benzene, (*C*) 9:5:3:1 butyl alcohol–acetic acid–ether–water, (*D*) ether, and (*E*) 1:1 chloroform–methanol.

Whatman No. 1 paper was used for descending paper chromatography, and Whatman No. 3MM paper for preparative work. The solvents used were: (*F*) 6:4:3 butyl alcohol–pyridine–water, (*G*) butanone–water azeotrope, and (*H*) 4:1:1 butyl alcohol–ethanol–water.

β-Glucosidase (emulsin, a β-D-glucosidase) was obtained from Worthington Biochemical Corporation, Freehold, New Jersey. The assay procedure was that outlined by Gatt[1] with the one exception that Triton X-100 and taurocholate were omitted from the reaction buffer. Thiophosgene was obtained from Matheson, Coleman and Bell, Norwood, Ohio. Hydrobromic acid (32% in acetic acid) was purchased from Eastman Kodak Co., Rochester, N.Y. *p*-Nitrophenyl α- and β-D-glucopyranosides were available in our laboratory as synthetic preparations or were purchased from Pierce Chemical Co., Rockford, Illinois.

Dextran B-1299-S was prepared from a lyophilized culture of *Leuconostoc mesenteroides* NRRL B-1299-S obtained from Dr. W. C. Haynes, A.R.S. Culture Collection Investigations, U.S.D.A., Peoria, Illinois. The procedures for activating the lyophilized culture, growing the organism, and producing the dextran were essentially the same as those described for *L. mesenteroides* B-512 (F) strain[2] (compare refs. 3,4). When the fermentation was complete, the viscous culture-fluid was diluted with an equal volume of water (mechanical stirring) and ethanol was added slowly with efficient stirring to a concentration of 35% by volume. The precipitate (mainly cells) was centrifuged off, and the concentration of ethanol was increased to the point where precipitation of dextran appeared to be complete (42–45% by volume). After 1 h the dextran had settled and was centrifuged off, redissolved in water (5% w/v), and reprecipitated. The procedure was repeated twice more, and the dextran was dried by solvent exchange.

*Isolation of α-kojibiose octaacetate.* — The acetolysis conditions of Suzuki and Hehre[5] were used. To a suspension of dry, finely pulverized dextran (8.0 g) in ice-cold acetic anhydride (80 ml) was added an ice-cold, colorless solution of acetic anhydride–sulfuric acid (64 ml, 3:1 v/v), both components being precooled to 0° in an ice–salt bath before mixing. (If the sulfuric acid and acetic anhydride were not precooled, a brown solution resulted upon mixing the two components.) The acetolysis reaction-mixture was stirred magnetically for 3–4 days at 25°, gradual darkening of the solution being observed. The resulting syrupy, brown liquid was poured into a mixture of ice (800 ml) and sodium hydrogen carbonate (175 g).

After the ice had melted, the brown gum that collected at the surface was removed with a spatula and dissolved in chloroform (100 ml). The aqueous solution was extracted with chloroform (3 × 100 ml) and the combined chloroform solution and extracts were washed successively with aqueous sodium hydrogen carbonate and water, dried over sodium sulfate, and evaporated to a clear, light-brown syrup (yield, 13–14 g). The syrup was dissolved in hot methanol (50–70 ml) and the cooled solution was centrifuged in a Sorvall RC-2 centrifuge at low speed. The clear supernatant solution was decanted into an Erlenmeyer flask, seeded with a few crystals of α-kojibiose octaacetate, and the flask allowed to stand uncovered at room temperature. Crystallization ensued during 3–4 days. The crystals were filtered off, washed with a small amount of cold methanol, and the crystallization procedure was repeated. Further extraction of the methanol-insoluble acetolyzate yielded additional material (100–300 mg). The yield of α-kojibiose octaacetate from 10 preparations varied from

0.8–1.4 g, m.p. 170°, $[\alpha]_D^{25}$ +149°, (*c* 1.0, chloroform). (Lit.[6] m.p. 166°, $[\alpha]_D$ +152°, (*c* 2.4, chloroform) (compare refs. 7, 8).

The crystalline compound was chromatographically pure by t.l.c. (solvents *A*, *B*, and *C*). A solution of α-kojibiose octaacetate in methanol was deacetylated by the addition of a small piece of sodium. After 24 h the solution was neutralized with concentrated acetic acid. The reaction solution gave a product that co-chromatographed with kojibiose on t.l.c. with solvent *C* and on paper in solvent *F*, and which failed to give a red color when sprayed with alkaline triphenyltetrazolium chloride (indicating[9] substitution at C-2).

*Anal.* Calc. for $C_{28}H_{38}O_{19}$: C, 49.51; H, 5.60. Found: C, 49.62; H, 5.63.

*Hepta-O-acetyl-α-kojibiosyl bromide.* — α-Kojibiose octaacetate (1.0 g) was suspended in 32% hydrogen bromide in acetic acid (8.0 ml) and the reaction mixture was stirred magnetically for 30 min at 4° and then at 25° until complete dissolution of solid material had occurred (2–3 h). (Heating caused by the magnetic stirrer had to be avoided or side products were formed.) At the end of this time, product analysis by t.l.c. in solvents *A* and *D* revealed complete conversion of the α-kojibiose octaacetate into a faster-migrating component. The reaction mixture was dissolved in chloroform, and the chloroform solution was washed successively with aqueous sodium hydrogen carbonate and water, and dried over sodium sulfate. Evaporation of the solvent gave a clear, colorless syrup that was crystallized from acetone and recrystallized from acetone–petroleum ether (b.p. 30–60°). The crystals (80 mg) migrated on t.l.c. in solvents *A* and *D* as a single component distinct from the parent octaacetate. Because of the somewhat unstable nature of the bromide, no attempt was made to maximize the yield of the crystalline product, and the syrupy compound was used directly in subsequent syntheses. Hepta-*O*-acetyl-α-kojibiosyl bromide had m.p. 141–142°, $[\alpha]_D^{25}$ +243° (*c* 0.72, acetone).

*Anal.* Calc. for $C_{26}H_{35}BrO_{17}$: C, 44.7; H, 5.04; Br, 11.4. Found: C, 44.7; H, 5.2; Br, 11.3.

p-*Nitrophenyl β-kojibioside.* — To syrupy hepta-*O*-acetyl-α-kojibiosyl bromide (1.3 g, 1.86 mmoles) was added a solution of *p*-nitrophenol (0.82 g, 5.88 mmoles) in acetone (5 ml) and aqueous 2.5M NaOH (2.5 ml, 6.25 mmoles). The solution was stirred magnetically for 25 h, at which time two-dimensional t.l.c. analysis in solvent *A* followed by solvent *B* showed the formation of a new, u.v.-absorbing component having a lower mobility than the starting material. Estimated conversion of the bromide into *p*-nitrophenyl β-kojibioside was 60–80%. The reaction mixture was evaporated to dryness and the yellow syrup obtained dissolved in chloroform. The chloroform solution was washed twice with sodium hydrogen carbonate and several times with water until the washings were colorless, and then dried over sodium sulfate. The solvent was evaporated, the resultant syrup was dissolved in dry methanol, and a small piece of sodium was added to effect catalytic deacetylation, after which step the solution was neutralized with acetic acid. On occasion, *p*-nitrophenyl β-kojibioside crystallized directly following concentration of this methanolic solution. However, isolation by crystallization was never complete, and the *p*-nitrophenyl β-kojibioside

was isolated in pure crystalline form by cellulose-column or thick filter-paper chromatography with solvent *G* for approximately 36 h. The u.v.-absorbing material just below the origin was excised, and eluted with aqueous methanol; it crystallized upon concentration of the methanolic solution. Recrystallization from 96% aqueous ethanol gave *p*-nitrophenyl β-kojibioside, chromatographically pure by t.l.c. in solvent *C* and by paper chromatography in solvent *G*; yield 266 mg (39%), m.p. 167.5–168.5°, $[\alpha]_D$ +152° (*c* 1.23, water).

*Anal.* Calc. for $C_{18}H_{27}NO_{14}\cdot H_2O$: C, 45.0; H, 5.62; N, 2.91. Found: C, 45.28; H, 5.68; N, 2.80.

Partial acid hydrolysis of the glycoside (15 mg) in 0.05M sulfuric acid (2 ml) for 30 min on a boiling water-bath gave, upon t.l.c. analysis in solvent *C*, components migrating with the mobilities of authentic kojibiose, *p*-nitrophenyl β-kojibioside, and *p*-nitrophenyl β-D-glucopyranoside, the last one being clearly discernible from the corresponding *p*-nitrophenyl α-D-glucopyranoside. In a second experiment *p*-nitrophenyl β-kojibioside (15 mg) was hydrolyzed for 1 h at 100° in a sealed tube with 0.25M sulfuric acid (1 ml). T.l.c. analysis in solvent *C* showed 3 components having the mobilities of authentic *p*-nitrophenyl β-D-glucoside (clearly distinguishable from the anomeric α-D-glycoside), kojibiose, and *p*-nitrophenyl β-kojibioside, the last component not being distinguishable from glucose in this solvent system. Isolation of *p*-nitrophenyl β-D-glucopyranoside from the hydrolyzate was accomplished by preparative paper chromatography in solvent *B*; the glycoside was eluted from the paper by aqueous methanol and shown to be pure by t.l.c. in solvent *C*. The eluted glycoside was hydrolyzed by β-glucosidase, as detected by release of *p*-nitrophenol and production of glucose. Neither *p*-nitrophenyl α-D-glucopyranoside nor *p*-nitrophenyl β-kojibioside were substrates for β-glucosidase. *p*-Nitrophenol was not released from *p*-nitrophenyl β-D-glucopyranoside in the absence of β-glucosidase.

The c.d. spectrum of *p*-nitrophenyl β-kojibioside was essentially superposable upon that of *p*-nitrophenyl β-D-glucopyranoside but was readily distinguishable from that of *p*-nitrophenyl α-D-glucopyranoside, all glycosides being at a concentration of ~22 μg/ml water.

p-*Isothiocyanatophenyl β-kojibioside.* — *p*-Aminophenyl β-kojibioside was prepared by catalytic hydrogenation at atmospheric pressure of a solution of *p*-nitrophenyl β-kojibioside (100 mg) in water (0.5–1.0 ml to dissolve the glycoside) and methanol (10 ml) in the presence of platinum oxide (Adams' catalyst). Conversion into the amine generally required 1–2 h and was monitored by t.l.c. in solvent *C*, by paper chromatography in solvent *H*, and by uptake of hydrogen. The reduced compound gave a yellow color when sprayed with an acid solution of *p*-(dimethylamino)benzaldehyde. Solutions of *p*-aminophenyl β-kojibioside turned yellow upon manipulation, hence the amino derivative was used directly.

To a solution of *p*-aminophenyl β-kojibioside in methanol (1 ml) and ethanol (8 ml) was added thiophosgene (0.16 ml) and the solution was stirred for 2 h at 25°. T.l.c. in solvent *E* showed almost complete conversion into a faster migrating species. The solvent was evaporated off and the crystalline mass that formed was triturated

with acetone and filtered to give 80 mg of crystals. Recrystallization from 95% ethanol gave the pure glycoside (50 mg), m.p. 179.5–180.5°, $[\alpha]_D^{25}$ +14° (*c*, 1.36, *N*,*N*-dimethylformamide).

*Anal.* Calc. for $C_{19}H_{25}NO_{11}S \cdot H_2O$: C, 46.2; H, 5.51; N, 2.84; S, 6.50. Found: C, 46.17; H, 5.47; N, 2.83; S, 6.46.

## RESULTS AND DISCUSSION

We selected dextran B-1299-S as starting material for these syntheses as it has been reported, both from immunochemical observations[5,10,11] and from optical rotational shifts[12] caused by the formation of cuprammonium complexes, that this dextran contains a high proportion of (1→2)-α-D-glucosidic bonds. Acetolysis of dextran B-1299-S leading to isolation in high yields (~20% by weight) of kojibiose, by Matsuda *et al.*[7] and by Suzuki and Hehre[5], confirmed these reports. Most recently, Bourne *et al.*[4] methylated dextran B-1299-S and showed that, on the average, every fifth α-D-glucopyranosyl residue is involved in a (1→2)-linkage.

The unexpected, direct crystallization of α-kojibiose octaacetate from a methanolic solution of the acetolyzate greatly facilitated this study. Yields of up to 1.4 g of crystalline α-kojibiose octaacetate from 8.0 g of dextran (~35% of theory, ~17% by weight) were obtained. Although these yields are certainly not as high as those reported by Hehre[5] and Matsuda *et al.*[7], nevertheless the direct crystallization procedure obviates the somewhat time-consuming deacetylation and column chromatographic isolation steps, and facilitates the preparation of glycosides by allowing the acetylated glycosyl bromide to be obtained directly.

Additional kojibiose can, of course, be obtained by deacetylation of the remaining acetolyzate followed by isolation of the disaccharide by column or preparative-paper chromatography.

Transformation of α-kojibiose octaacetate into hepta-*O*-acetyl-α-kojibiosyl bromide was accomplished in the usual way by the action of 32% hydrogen bromide in acetic acid. The conversion was complete after 2–3 h and the product was obtained crystalline from acetone. Its high specific optical rotation ($[\alpha]_D^{25}$ +243° in acetone) supports the assigned α-D configuration at C-1.

Conversion of the 1-bromide into the *p*-nitrophenyl glycoside by the method of Helferich[13] proceeded with Walden inversion to afford *p*-nitrophenyl β-kojibioside in ~40% yield. The β-D configuration at the *p*-nitrophenyl aglycon was assigned on the basis of the following evidence: (*a*) partial acid hydrolysis gave, among the products of hydrolysis, *p*-nitrophenyl β-D-glucopyranoside, which was readily discernible from the α-D-anomer on t.l.c.; (*b*) *p*-nitrophenyl β-D-glucopyranoside isolated from a partial acid hydrolysis of *p*-nitrophenyl β-kojibioside was hydrolyzed by β-glucosidase, whereas neither *p*-nitrophenyl α-D-glucopyranoside nor *p*-nitrophenyl β-kojibioside were substrates for the enzyme; (*c*) the specific optical rotation of *p*-nitrophenyl β-kojibioside ($[\alpha]_D^{25}$ −14° in water) is relatively low; and (*d*) the c.d. spectrum of *p*-nitrophenyl β-kojibioside displays a negative Cotton effect, with a peak at

~315 nm and is superposable on the c.d. spectrum of *p*-nitrophenyl β-D-glucopyranoside. *p*-Nitrophenyl α-D-glucopyranoside displays a positive Cotton effect, with a peak at ~310 nm.

Derivatives of *p*-nitrophenyl β-kojibioside that could be covalently linked to protein were prepared next. *p*-Aminophenyl β-kojibioside was readily obtained by catalytic hydrogenation of the *p*-nitrophenyl glycoside. Although the *p*-aminophenyl glycoside was not obtained crystalline, it was pure by t.l.c., and displayed all the properties expected of an aromatic amine (see Experimental). Subsequently, *p*-aminophenyl β-kojibioside was diazotized and conjugated to bovine serum albumin to give the typical reddish color characteristic of protein containing azophenyl groups[14].

Treatment of *p*-aminophenyl β-kojibioside with thiophosgene afforded crystalline *p*-isothiocyanatophenyl β-kojibioside. This derivative of Edman's reagent (phenyl isothiocyanate)[15] may be used for alkylation of the amino-terminal and lysyl ε-amino groups of a protein, and hence the introduction of carbohydrate moieties into a protein is readily achieved[14,16].

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REFERENCES

1 S. GATT, *Methods Enzymol.*, 14 (1969) 152.
2 A. R. JEANES, *Methods Carbohyd. Chem.*, 5 (1965) 118.
3 C. A. WILHAM, B. H. ALEXANDER, AND A. R. JEANES, *Arch. Biochem. Biophys.*, 59 (1955) 61.
4 E. J. BOURNE, R. L. SIDEBOTHAM, AND H. WEIGEL, *Carbohyd. Res.*, 22 (1972) 13.
5 H. SUZUKI AND E. J. HEHRE, *Arch. Biochem. Biophys.*, 104 (1964) 305.
6 S. A. BARKER, A. GÓMEZ-SÁNCHEZ, AND M. STACEY, *J. Chem. Soc.*, (1959) 3264.
7 K. MATSUDA, K. FUJIMOTO, AND K. ASO, *Tohoku J. Agr. Res.*, 12 (1961) 359.
8 B. HELFERICH AND J. ZIRNER, *Chem. Ber.*, 95 (1962) 2604.
9 K. WALLENFELS, *Naturwissenschaften*, 37 (1950) 491.
10 (a) J. M. NEILL, J. Y. SUGG, E. J. HEHRE, AND E. JAFFE, *Proc. Soc. Exp. Biol. Med.*, 47 (1941) 339; (b) J. Y. SUGG AND E. J. HEHRE, *J. Immunol.*, 43 (1942) 119; (c) E. J. HEHRE, *Proc. Soc. Exptl. Biol. Med.*, 54 (1943) 18; (d) E. J. HEHRE, *Bull. Soc. Chim. Biol.*, 42 (1960) 1581.
11 (a) P. Z. ALLEN AND E. A. KABAT, *J. Amer. Soc.*, 78 (1956) 1890; (b) *ibid.*, 81 (1959) 4382; (c) J. W. GOODMAN AND E. A. KABAT, *J. Immunol.*, 84 (1960) 347; (d) R. G. MAGE AND E. A. KABAT, *ibid.*, 91 (1963) 633.
12 T. A. SCOTT, N. N. HELLMANN, AND F. R. SENTI, *J. Amer. Chem. Soc.*, 79 (1957) 1178.
13 B. HELFERICH AND E. SCHMITZ-HILLEBRECHT, *Ber.*, 66 (1933) 378.
14 C. R. MCBROOM, C. H. SAMANEN, AND I. J. GOLDSTEIN, *Methods Enzymol.*, 28 (1972) 212.
15 P. EDMAN, *Acta Chem. Scand.*, 4 (1950) 277.
16 D. H. BUSS AND I. J. GOLDSTEIN, *J. Chem. Soc.*, (1968) 1457.