BBA 3851

THE PREPARATION OF SOME NEW DISACCHARIDES AND D-ALLOSE FROM 3-KETOGLYCOSIDES

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(Received July 11th, 1962)

SUMMARY

From 3-ketolactose, 3-ketomaltose, 3-ketomaltobionate and 3-ketosucrose the following new disaccharides have been prepared: gulosylglucose, allosylglucose and allosylfructose. Conditions are described in which the 3-keto function was reduced much faster than the hemiacetal group by Raney nickel. The yield of the reduction was 85–90%. The reduction of 3-ketolactose gave lactose and gulosylglucose. The former sugar was eliminated by oxidation with *Aerobacter cloacae*. Gulosylglucose was purified and crystallized as the monohydrate. The reduction of 3-ketomaltose gave maltose and allosylglucose. Better yields were obtained from 3-ketomaltobionate. Maltose was eliminated with a *Paracolobactrum* strain. Allosylglucose was prepared as a chromatographically pure syrup. The reduction of 3-ketosucrose gave allosylfructose, which was purified by charcoal chromatography. Arguments in favour of the proposed structures are given. The rare sugar D-allose can now readily be prepared from 3-ketosucrose after reduction with Raney nickel. The resulting mixture of sucrose and allosylfructose was oxidized with a *Paracolobactrum* strain, which hydrolyzed both disaccharides and oxidized both glucose and fructose, but left D-allose unchanged. The latter sugar was purified and crystallized.

INTRODUCTION

In previous papers[1–4]* a new pathway for disaccharide breakdown by some bacteria has been described. The primary step consists in the oxidation at C-3 of the glycosyl moiety of disaccharides and bionates with the formation of the corresponding 3-ketoglycosides. Several new carbohydrates have thus been prepared, such as 3-ketolactose, 3-ketomaltose, 3-ketosucrose, 3-ketolactobionate and 3-ketomaltobionate. With an improved preparative method[4] lactose, lacto- and maltobionate were converted nearly quantitatively into the corresponding 3-ketoglycosides, while maltose and sucrose gave yields of 12 and 20% respectively. 3-Ketolactose has been crystallyzed[4], the other compounds were obtained chromatographically pure[2,3]. The above mechanism appears to be limited to *Agrobacterium tumefaciens* and *Ag. radiobacter* (see ref. 5).

* In ref. 4 it was erroneously stated that 10^{14}–10^{15} cells/ml were used as inoculum. The correct value is 10^{8}–10^{9}. Similarly in Table I the correct value is 10^{7} cells/ml instead of 10^{13}.

The 3-ketoglycosides themselves can be used as the starting material for the preparation of hitherto unknown carbohydrates and derivatives. As an example we want to describe here the preparation and purification of gulosylglucose, allosylglucose and allosylfructose, from 3-ketolactose, 3-ketomaltobionate and 3-ketosucrose respectively. The main features of these preparations are the reduction by Raney nickel under controlled conditions and the selective elimination by appropriate bacterial strains of the undesirable 3-epimeric disaccharides.

Furthermore, the above new disaccharides can be used for the preparation of otherwise rare sugars such as D-allose and D-gulose. Previously we described[3] the preparation of the latter sugars after the reduction of 3-ketolacto- and 3-ketomaltobionate, followed by hydrolysis. We shall describe here the ready preparation of large amounts of D-allose, starting with 3-ketosucrose, by reduction with Raney nickel, followed by bacterial fermentation, which eliminates all carbohydrates except D-allose. The latter sugar can now no longer be considered as "rare".

MATERIALS AND METHODS

Bacteria used

We used the same strains as in our previous papers. Their taxonomic position was not clear and we tentatively called them *Alcaligenes faecalis*. We showed recently[5] that they belong in the genus *Agrobacterium*.

Preparation of 3-ketoglycosides

The preparation was carried out as previously described[4]. 10 l of culture medium containing 4% of the carbohydrate substrate, mineral salts, yeast extract, calcium carbonate and antifoam "A" (Hopkins and Williams) were used in a specially designed fermentor. After heavy inoculation with glucose-grown bacteria (10^9 cells/ml), the medium was aerated with an oxygen absorption rate of 6.

The concentration of 3-ketoglycosides was checked occasionally and the oxidation was stopped when the yield was maximal, usually after 20–23 h. The culture medium was centrifuged, deproteinized and the resulting clear solution was demineralized on ion-exchange resins or by precipitation of the mineral salts as previously described. The resulting clear and colourless solution was used as such as starting material for the reduction process.

Estimation of 3-ketoglycosides

The reaction with semicarbazide was carried out as follows: a 1-ml sample of the diluted 3-ketoglycoside solution, containing between 50 and 400 μg of the compound, was mixed with 1 ml of the reagent (1% semicarbazide–HCl and 1.5% sodium acetate trihydrate in water), incubated for 15 min at 30°, diluted to 5 ml with water and the absorption read at 230 mμ in the Beckman spectrophotometer model DU in 1-cm quartz cuvettes against a diluted blank. The law of Beer was followed in the range 0–400 μg/ml of 3-keto compounds. 200 μg/ml of pure crystalline 3-ketolactose gave an absorbancy at 230 mμ of 0.825. Nearly identical values were obtained with 200 μg/ml of chromatographically pure samples of other 3-keto compounds. Disaccharides, bionates and the other ingredients of the culture medium did not interfere with this reaction.

Preparation of Raney nickel

Active preparations were obtained according to the method of MOZINGO and his coworkers[6] and contained about 120 ml hydrogen/g. As these preparations lost their hydrogen and their activity upon standing, they were used as soon as possible. Their activity could be preserved to some extent by keeping them under absolute alcohol at 4°.

Paper chromatography

The following solvents were currently used to separate mixtures of carbohydrates: solvent A, water-saturated phenol; solvent B, collidine; solvent C, methylethylketone–acetic acid–water saturated with boric acid (9:1:1); solvent D, top layer of ethyl acetate–pyridine–water (100:45:100); solvent E, top layer of *n*-butanol–ethanol–water (4:1:5). Reducing carbohydrates were revealed with *p*-anisidine–HCl in *n*-butanol and lactones with the reagent of ABDEL-AKHER AND SMITH[7].

Chromatography on charcoal column

Two superposed columns (20000 π + 3250 π; diameter respectively 2.5 and 1.8 cm) according to CLAESSON[8] and containing a mixture of 50% of acid-washed Darco G-60 charcoal and 50% Celite No. 535 (Johns Manville) were used to separate gram quantities of monoses, sucrose and allosylfructose.

Crystalline 3-ketolactose

Crystalline preparations were obtained as previously described[3]. It was recrystallized twice from methanol and contained no lactose according to paper chromatographic analysis. Apparently it was the monohydrate, since upon drying over P_2O_5 *in vacuo* at 124° for 48 h it lost 4.92% of its initial weight (theoretically for $C_{12}H_{22}O_{11} \cdot H_2O$, 5.00%).

RESULTS

Reduction of the 3-keto function with Raney nickel

Previously[3] we showed that passing a stream of H_2 through the aqueous solution of either 3-ketolactose or 3-ketomaltose in the presence of freshly prepared Raney nickel, reduced both the 3-keto and the hemi-acetal functions. In the same conditions KARABINOS AND BALLUN[9] prepared maltitol and lactitol from the corresponding disaccharides. Since it was our aim to reduce only the 3-keto function, neither the latter procedure nor sodium borohydride could be used. The use of mildly reducing conditions (Raney nickel in low concentration at room temperature) gave entire satisfaction. A mixture containing 3% freshly prepared Raney nickel and 4% of any 3-ketoglycoside was vigorously stirred mechanically at 30°. Samples were taken at regular intervals and both the Benedict and semicarbazide reactions carried out. After about 3 h the 3-keto function had completely disappeared. However, the hemiacetal groups of the 3-ketodisaccharides appeared to be unchanged, as evidenced by the reduction of boiling Fehling reagent.

After the reduction two isomeric disaccharides or bionates were to be expected from each substrate. This was confirmed by paper chromatography. Solvent A showed that 3-ketomaltose had been transformed into two reducing sugars, one of them being

maltose (R_F 0.37) the other one presumably being allosylglucose (R_F 0.48). Similarly solvent B showed that reduction had transformed 3-ketolactose into lactose (R_F 0.24) and another reducing sugar, presumably gulosylglucose (R_F 0.39). 3-Ketosucrose after reduction showed with solvent A a spot corresponding to sucrose (R_F 0.40) and a second one, presumably allosylfructose with an R_F of 0.53. Paper chromatography of 3-ketobionates after reduction confirmed the absence of reducing compounds. The presence at this stage of two isomeric bionates from 3-ketomaltobionate, will be shown below by other methods.

The yield of the new disaccharides after the Raney nickel treatment was determined as follows. A solution of 3-ketolactose, containing 4 % of the anhydrous compound, was reduced with Raney nickel as above. The mixture of lactose and the presumed gulosylglucose was titrated with the LUFF AND SCHOORL method[10]. By comparing the latter value with a standard solution containing equal amounts of lactose and crystalline gulosylglucose (see below), it was calculated that the yield of both disaccharides after the reduction process was 85–90 %. The remaining 3-ketolactose, which was unaccounted for, had probably been reduced to lactitol and gulosylsorbitol.

The preparation of gulosylglucose

10 l of culture medium, containing 4 % 3-ketolactose and only traces of lactose, was prepared as mentioned in MATERIALS AND METHODS and used as starting material. To this clear and colourless solution 300 g of freshly prepared Raney nickel was added and the suspension thoroughly mixed with a mechanical stirrer. After about 3 h the concentration of the 3-ketoglycoside had fallen to practically zero. The catalyst was then immediately filtered off. Traces of dissolved nickel were removed by passing the filtrate through a column (height 50 cm, diameter 4 cm) of the strong cation-exchange resin Merck I.

The separation of gulosylglucose from lactose was realized by bacterial oxidation of the latter sugar. Preliminary Warburg experiments had shown that resting cells of several representative strains of the tribe *Escherichia* oxidized lactose to completion, while no oxygen was taken up with gulosylglucose. *Aerobacter cloacae*, strain K_3 of our collection, was selected for further work, because the assimilation of gulosylglucose was nearly negligible in a strongly aerated growing culture.

To 10 l of the above solution, containing the mixture of disaccharides, was added (in percent, w/v): $(NH_4)_2SO_4$, 0.2; KH_2PO_4, 0.1; $MgSO_4 \cdot 7H_2O$, 0.025; yeast extract (Difco), 0.175. Final pH, 6.8. This medium was sterilized by filtration and inoculated with *Aer. cloacae* K_3. A sterilized slurry of finely divided $CaCO_3$ and 200 p.p.m. antifoam (Hopkins and Williams) were added. Growth and aerobic fermentation were carried out with vigorous aeration (oxygen absorption rate of 6) in the same conditions in the fermentor as described for the preparation of the 3-ketoglycosides. Paper chromatography showed that after 48 h all lactose had disappeared, while gulosylglucose was still present. The reducing power, as determined with the LUFF AND SCHOORL method[10], had decreased to 45 % of its initial value. The culture medium was centrifuged, deproteinized and demineralized in the same manner as for the purification of 3-ketolactose. The remaining clear and nearly colourless solution was concentrated *in vacuo* until it became viscous (300 ml). It was poured under stirring in ten times its volume of methanol and a filamentous precipitate was filtered off.

The methanolic solution was distilled *in vacuo* to a viscous syrup, to which ethanol was added until a faint turbidity appeared. After some weeks in the refrigerator, white crystals appeared. The crystallisation was continued for some days and a first crop of presumed gulosylglucose of 120 g (60 %) was collected. It was recrystallized twice from 70 % aq. ethanol.

Structure and some properties of gulosylglucose

The component monosaccharides were identified after hydrolysis. 1 g of the presumed gulosylglucose in 100 ml 1 N H_2SO_4 was heated at 100° for 1 h. The solution was neutralized with $BaCO_3$ and the precipitate filtered off. The yellowish neutral filtrate contained two reducing compounds as revealed on descending paper chromatograms with solvent D for 24 h. The first spot had the same R_F value as glucose and the second one behaved in the same way as gulose. Both spots had the same intensity, indicating that both sugars were present in equal amounts. Neither lactose nor gulosylglucose was detectable.

Identification of glucose: The above filtrate was shaken overnight at 40° with 0.05 % (w/v) glucose oxidase (Boehringer, Mannheim) containing catalase. At the end of the reaction, finely divided $CaCO_3$ was added to neutralize the gluconic acid. After filtration, paper chromatography revealed that glucose had completely disappeared, while gulose was still present. Calcium gluconate was removed from the solution with ion-exchange resins first on a column of Merck-I resin (diameter 1.5 cm, height 8 cm) followed by a column of weak anion-exchange resin Merck II (diameter 2 cm, height 20 cm). Gluconic acid was desorbed from the latter column with 50 ml of 10 % Na_2CO_3. The alkaline eluate was neutralized by successive additions of the Merck-I resin in the H^+ form until no more CO_2 was evolved. The mixture was then poured on a column of Merck-II resin (diameter 2 cm, height 10 cm) and the recovered gluconic acid solution was concentrated *in vacuo* to about 10 ml. The lactone was prepared by dissolving the concentrate in an excess of dioxane, which was then distilled off *in vacuo*. This procedure was repeated three times. 400 mg of a syrup were finally obtained, still contaminated with traces of dioxane. It showed a strong Fe hydroxamate reaction according to HESTRIN[11]. Paper chromatography with solvent E showed with the spray reagent for lactones one spot with the same R_F as authentic gluconodelta-lactone.

Identification of gulose: The neutral solution which had passed through the above Merck-II column reduced the Fehling solution. By concentrating *in vacuo* 470 mg of syrupy material were recovered. The presumed gulose was converted into the phenylhydrazone according to FISCHER AND STAHEL[12], which was recrystallized from absolute ethanol and dried. The yellow crystals melted at 138° as expected.

Mode of linkage between glucose and gulose: Gulosylglucose was oxidized with bromine according to HUDSON AND ISBELL[13,14]. Its reducing properties completely disappeared. The presumed gulosylgluconic acid was hydrolyzed with 1 N sulfuric acid as above. The solution, which was now again reducing against the Fehling reagent, was shown to contain free gulose both by paper chromatography and by the melting point of its crystalline phenylhydrazone. Gluconic acid was isolated from the hydrolyzate as described above and characterized as the delta-lactone by paper chromatography. This experiment showed that in the new disaccharide, the gulose moiety is linked with C-1 to the glucose part, as expected from the starting material.

Some properties of gulosylglucose: The crystalline compound contained one molecule of water of crystallisation. After heating for 48 h over P_2O_5 at 140° *in vacuo*, the crystals lost 4.92 % of their weight; theoretically for $C_{12}H_{22}O_{11} \cdot H_2O$: 5.0 %. This water of crystallisation probably explains the variations in the melting point, which depended on the rate of heating. Melting points between 197° and 205° were frequently obtained.

Optical rotation: 1.4256 g in 25 ml in a 2-dm tube at 20° gave the following specific rotation: 6.3 min after solution, $[\alpha]_D^{20} = 40.7°$; 9 min: 39.2°; 16 min: 36.8°; 30 min: 33°; 60 min: 26.7°; 135 min: 18.1°; 26 h: 11.5° (constant).

The preparation of allosylglucose

A procedure identical to the above one was used *mutatis mutandis* for the preparation of allosylglucose from 3-ketomaltose. There was only one major change, consisting in the use of a *Paracolobactrum*, strain M 33, which was unable to use allosylglucose as a carbon source in growing cultures, while maltose disappeared completely in these conditions. The latter strain was selected from a collection of *Enterobacteriaceae* which, in preliminary Warburg experiments, oxidized maltose but not allosylglucose. The presumed allosylglucose was obtained as a yellow syrup which was still quite impure, since it contained only 50 % of the expected reducing sugar. The yield of 3-ketomaltose itself was already rather small to begin with, so that there were several reasons for discontinuing along these lines.

A better starting material was found in 3-ketomaltobionate. 10 l of the culture medium, in which 3-ketomaltobionate had been prepared from maltobionate with *Agrobacterium* and containing about 3.5 % of the 3-keto compound, was centrifuged and deproteinized as usual and treated with Raney nickel until all reducing power had disappeared. The catalyst was filtered off and the filtrate passed through a column of Merck-I resin (diameter 4 cm, height 50 cm). The acid solution was concentrated *in vacuo* to about 1 l. It contained maltobionic acid and allosylgluconic acid, which were reduced to the corresponding disaccharides after conversion into the lactones as follows. 1 l of dioxane was added and the distillation was continued to a syrupy consistency. The addition of dioxane, followed by distillation was repeated five times. The resulting syrup showed a strong Fe hydroxamate reaction. 250 g of this syrup, containing still some dioxane, were dissolved in 2 l of distilled water, cooled, acidified and treated with 250-g portions of 2.5 % sodium amalgam with vigorous stirring. The addition was repeated until the yield of reducing sugars was maximal. The solution was neutralized with sodium carbonate in order to hydrolyze the unreacted lactone. It was slightly acidified with sulfuric acid, concentrated *in vacuo* and ethanol was added to precipitate most of the sodium sulphate. The last traces of the dissolved salts (sodium sulphate and bionates) were removed from the solution by ion exchange on Merck-I and Merck-II columns. Upon concentration 55 g of a yellow syrup was obtained, expected to contain both maltose and allosylglucose. Indeed, paper chromatography with solvent A and *p*-anisidine as a spray reagent showed two spots of equal intensity, one of them with the same R_F as maltose, the other one identical to the allosylglucose prepared from 3-ketomaltose. Maltose was eliminated from the mixture of both disaccharides through selective oxidation and assimilation with the *Paracolobactrum* strain M 33 and the culture medium was purified in the same manner as described for gulosylglucose. After concentration *in vacuo* of the clear and

colourless allosylglucose solution, 21 g of a yellow syrup was obtained which gave only one spot on paper chromatograms and failed to crystallize up to now.

Structure of allosylglucose

The compound was hydrolyzed and glucose was identified as the glucono-δ-lactone exactly as described for gulosylglucose. The solution, after passing through the columns of ion-exchange resins, still reduced Fehling reagent and contained the presumed allose. It was concentrated to a syrup, treated with a small amount of methanol and inoculated with a minute crystal of pure D-allose. After a few days a first crop of 200 mg crystalline allose was obtained. Melting point (uncorrected), 128°; mixed melting point unchanged.

The preparation of allosylfructose from 3-ketosucrose

Sucrose was oxidized to 3-ketosucrose with *Agrobacterium*, followed by the reduction with Raney nickel as in the previous preparations. By paper chromatography two non-reducing sugars were detected, one being sucrose, the other one presumably allosylfructose. Sucrose was present in a large excess because the starting material itself contained 3.2 % sucrose and only 0.8 % 3-ketosucrose. A preliminary enrichment in allosylfructose was effected by fractional crystallisation of the mixture, dissolved in methanol. Chromatographically pure sucrose crystallized first. The process was stopped when the concentration of both sugars was nearly equal, because allosylfructose started to coprecipitate. The methanol was distilled off *in vacuo*. The resulting mixture of disaccharides contained small amounts of glucose, allose and fructose. Its separation was effected on a composite charcoal column. 1 g of the syrup, dissolved in 10 ml of water, was washed through the column with 60–70 ml of water until the monosaccharides, which were apparently not absorbed, had been eluted. The column was then developed with 4 % aq. phenol. The fractions were checked by paper chromatography. After 40 ml had passed through, pure sucrose was eluted in the next 80 ml, followed by the mixture of both disaccharides in 20 ml and by allosylfructose (350 mg) in 30 ml. By repeating this procedure three times 910 mg of allosylfructose was collected, still containing small amounts of sucrose. It was further purified by elution chromatography on an identical charcoal column using 5 % (v/v) absolute ethanol in water. After 270 ml had passed through, sucrose was eluted in the next 30 ml, followed by a mixture of both disaccharides in 110 ml and finally by chromatographically pure allosylfructose (600 mg in 170 ml). Attempts to crystallize this fraction failed up to now. Its structure was confirmed by showing that it contained allose and fructose. After hydrolysis according to CLERGET[15], two spots were detected on paper chromatograms after using solvent C for 48 h. One of them reacted as a hexulose and had the same R_F value as fructose. The other one reacted as an aldose and had the same R_F value as D-allose. Its m.p. was 129° (uncorrected) as expected for allose.

The preparation of D-*allose from 3-ketosucrose*

The mixture of sucrose and allosylfructose was prepared from 3-ketosucrose by Raney nickel treatment as mentioned above (*Structure of allosylglucose*). The catalyst was removed, the filtrate passed over the ion-exchange resins and concentrated *in vacuo* to syrupy consistency. An excess of methanol precipitated gum-like material,

which was filtered off. The methanol was removed by distillation *in vacuo* and the remaining syrup incorporated in 5 l culture medium as described above (*The preparation of gulosylglucose*). It was inoculated with *Paracolobactrum*, strain M 33, selected for its ready assimilation of sucrose. After 48 h of growth under vigorous aeration, neither sucrose, allosylfructose, fructose nor glucose were detectable by paper chromatography. There was only one spot, corresponding to allose. The culture medium was then further purified as described above. D-Allose was obtained as a colourless syrup which crystallized easily from 70 % aq. methanol. M.p. 127° as expected. For example in one particular experiment, starting with 400 g of sucrose, 60 g of 3-ketosucrose was obtained (15%). The first crop of crystalline D-allose was 10 g (63 % of the theoretical yield from 3-ketosucrose).

DISCUSSION

The structure of the new disaccharides

The structure of the new disaccharides can be partially inferred from the above results. Extensive qualitative analysis showed beyond doubt the nature of the constituent monosaccharides. Since no other sugars were detected except the 3-epimers of the glycosyl moiety and since the new disaccharides were obtained in an almost quantitative yield after the Raney-nickel treatment, the main event which thus happened during the latter treatment was the reduction of the 3-keto function. Therefore the new compounds must be disaccharides, which was confirmed by the observations that the intensity of the spots of the constituent monosaccharides on the chromatograms was the same. The new disaccharides are thus gulosylglucose, allosylglucose and allosylfructose. The linkage in the former two is most probably 1–4, while the latter has the 1–2 linkage, since it was indeed a non-reducing sugar. As yet, nothing can be said whether the allosyl and gulosyl moieties are in the pyranosyl or furanosyl forms. Neither is it known whether the α- or β-configurations are involved. Some of the above results also further corroborate the proposed structure of 3-ketosucrose. The analysis of the latter compound[4] was not carried as far as the other 3-keto compounds and its structure was proposed by the great similarities of its properties with those of the other 3-keto compounds. The formation of allosylfructose and the isolation of D-allose show that indeed the carbonyl function is located at C–3 of the glucose moiety and that the fructose part remains unchanged.

D-*Allose*

This sugar which has been rare and expensive up to now, will now be easily accessible on a large scale. We previously indicated the ready preparation of D-gulose from a guloside. It will not be possible to prepare other rare monosaccharides by our method from easily accessible disaccharides, since the latter are all either galactosides or glucosides.

The enzymic hydrolysis of the new disaccharides

The availability of the 3-epimeric disaccharides may now allow a further insight into the specificity of the hydrolases of different origin. Some preliminary information can be obtained from the fermentation experiments above.

Since allosylglucose was not attacked at all by several strains of the *Entero-*

bacteriaceae and assuming that it differs from maltose only by the configuration of the OH group at C-3 of the glucosyl moiety, this would mean that their α-glucosidase also requires the correct OH configuration at C-3. GOTTSCHALK[16] has already pointed out that "besides the glucosidic oxygen, the hydroxyl groups at C-2, C-4 and C-6 are involved in the formation of the enzyme–substrate complex". We can now also add C-3 to this description.

A similar conclusion can be drawn for the β-galactosidase of these bacteria. Again, since they attack lactose readily and gulosylglucose not at all, it is tempting to conclude that this enzyme is also specific for the correct OH configuration at the C-3 of the galactosyl moiety. A precedent is known already for the specificity of the sweet almond galactosidase which is unable to hydrolyze methyl-D-guloside.

Allosylfructose presents another interesting case. In contrast with the other two disaccharides it was readily attacked by our bacteria. As in the case of sucrose it could *a priori* be supposed that allosylfructose was hydrolyzed either by an α-glucosidase, by a β-fructosidase or by both. The former enzyme was present in our bacteria, but, as we have seen above, appears to be inactive with allosides. Therefore we are led to believe that the substrate is hydrolyzed by a β-fructosidase.

ACKNOWLEDGEMENT

The senior authors (M.J.B. and J.D.L.) are indebted to the Nationaal Fonds voor Wetenschappelijk Onderzoek for a grant.

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