LABELLED COMPOUNDS OF POTENTIAL BIOLOGICAL INTEREST

VII. Synthesis of 3-deoxy-D-*manno*-[1-^{14}C]octulosonic acid (D-*manno*-KDO) of high specific activity*

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SUMMARY

The preparation of the title compound from 2-deoxy-D-*manno*-heptose with potassium [^{14}C] cyanide followed by subsequent oxidation is reported. Frequent use of HPLC has made it possible to follow and optimize the different reaction steps. The synthesis was carried out on the millimole level and the product had a specific activity of 50 mCi/mmol.

Key words: D-*manno*-KDO, ^{14}C, HPLC.

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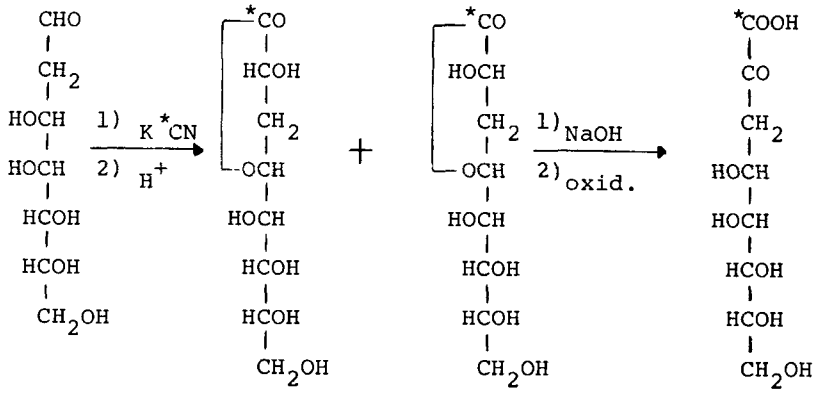
In connection with our studies on some enzymes involved in the biosynthesis of lipopolysaccharides in Gram-negative bacteria, radiolabelled 3-deoxy-D-*manno*-octulosonic acid (D-*manno*-KDO) was required for more accurate measurements of enzyme activities.

The preparation of ^{14}C-labelled KDO by an enzymatic method starting from D-arabinose and [1-^{14}C]pyruvate has been reported (1). In this procedure, however, access to an enzyme not available commercially is necessary, and it was therefore considered to be advantageous to develop a chemical procedure. By such a method it would also be possible to obtain KDO in a crystalline form, which is important as far as stability is concerned, and futhermore a high specific activity could be achieved. For the synthesis of unlabelled D-*manno*-KDO, two synthetic routes have been used. The first of these is mainly analogous to the biochemical method, and the starting materials are D-arabinose and oxalacetic acid or its di-*tert*-butyl ester (2,3,4). However, none of these compounds is commercially available in the ^{14}C-labelled form, and furthermore the reaction leads to a mixture of isomers of KDO. The yield in this procedure is low. In the second method D-mannose is converted in several steps to 2-deoxy-D-*manno*-heptose, which after addition of cyanide, hydrolysis and subsequent oxidation gives the desired product (5,6). We now report the preparation and characterization of D-*manno*-KDO labelled with ^{14}C at high specific activity essentially according to this latter procedure, but with some modifications and improvements.

SYNTHESIS

2-Deoxy-D-*manno*-heptose (1) was prepared from D-mannose and nitromethane by the method of Perry (5). According to HPLC analysis the final product contained an impurity, which was removed by column chromatography on cellulose. The next step, the addition of [^{14}C]cyanide in an equimolar ratio, was monitored by HPLC analysis and it was found that the reaction proceeded very slowly. The reaction time had to be extended to 13 days instead of 4 as published in order to obtain the addition product in high yield.

After hydrolysis and ion exchange chromatography the mixture of the 3-deoxyoctonolactones (2 and 3) was obtained in a crystalline form. Ring-opening of the lactones was effected by continuous addition of 0.5 M sodium hydroxide at pH 8, at the end of the addition the temperature was raised to 60°C. The final oxidation was performed according to the published procedure. The reaction was followed by HPLC and a reaction time of 12 h was needed. The greenish residue obtained after evaporation of the aqueous solution was subjected to chromatography on a short cellulose column with 70% aqueous acetone as eluent. After evaporation the yellow residue obtained was chromatographed on a second cellulose column and gradient elution with acetone-water was used. The fractions containing pure 3-deoxy- D-*manno*-octulosonic acid (4) (according to HPLC) were combined, neutralized with ammonia, and evaporated to give an oil. Recrystallization from ethanol-water then afforded the crystalline final product.



1 2 3 4

EXPERIMENTAL

Radioactivity was measured in a Packard-TriCarb liquid scintillation spectrometer (Model 3320) using internal standardization (n-[1-^{14}C]Hexadecane from Amersham International Limited, (Amersham).

Scanning of the paper chromatograms was carried out using a Berthold "Dünnschicht Scanner II".

HPLC

Apparatus

The liquid chromatograph consisted of the following components: a LDC 711-47 pump, an injection valve (Rheodyne Model 7120 syringe loading sample injector with 20 µl loop), a precision-bore stainless-steel separation column (length 150 or 200 mm, I.D. 3.8 mm and O.D. 1/4 in.), an Optilab Multiref 901 refractometer with 1 mm cell, and a Tekman TE 200 recorder. The column end-fittings were modified Swagelok® connections.

The chromatographic support was Bondapak Carbohydrate (particle size 10 µm), obtained from Waters Associates, Inc., (Milford, MA, USA). Acetonitrile (HPLC-grade) from Rathburn Chemicals Ltd., (Walkenburn, Scotland) was used for chromatography. Tetrabutylammonium hydrogen sulphate was supplied by Hässle, (Mölndal, Sweden), and was used after recrystallization from acetone and checking the purity.

The samples to be analysed were evaporated to dryness, and then dissolved in 50% aqueous acetonitrile and injected. The eluent was made up of 0.005 M tetrabutylammonium hydrogen sulphate in phosphate buffer with pH = 6.9 (25%) and acetonitrile (75%). The k'-values were calculated with tetrachloroethylene as reference. The flow rate was *ca.* 1.1 ml/min.

Comparisons with authentic samples refer to unlabelled compounds, which have been earlier prepared by the same route and characterized by physical data, unless otherwise stated.

Chromatographic technique

The separation column was packed by the balanced-density slurry technique with 1,1,2,2-tetrabromoethane-tetrachloroethylene (50:50, w/w) as the dispersion medium at an initial pressure of 400 bar. The packed column was washed with 250 ml of n-hexane, 100 ml of methylene chloride, and finally 100 ml of methanol before applying the mobile phase.

2-Deoxy-D-*manno*-heptose (1)

The title compound was prepared according to Perry (5). During the Nef reaction a minor amount of an impurity was formed (k' = 1.3 with 75% aqueous acetonitrile as eluent). Column chromatography on cellulose with acetone, 90 and 85% aqueous acetone as eluents afforded the heptose (1) in a pure form (k' = 1.6).

3-Deoxy-D-*glycero*-D-*galacto*-[1-^{14}C]octono-1,4-lactone (2) and
3-deoxy-D-*glycero*-D-*talo*-[1-^{14}C]octono-1,4-lactone (3)

2-Deoxy-D-*manno*-heptose (210 mg; 1.08 mmol) was dissolved in 2 ml of water and a solution of potassium [^{14}C]cyanide (65 mg: 1 mmol; 53 mCi; purchased from NEN Chemicals) in 1.5 ml of water was added. The mixture was kept in a refrigerator for 13 days. Samples of 20 µl were taken after 1, 5, 8, 11 and 13 days respectively, and analyzed by HPLC. The peak corresponding to the products ('k = 4.7) increased until the 11th day whereas that of the starting material (k' = 2.5) decreased. The ratio of products to starting material was in this sample about 94:6 and it did not change markedly during the last two days. The mixture of cyanohydrins was then hydrolysed by warming to 70°C (bath temperature) for 4 hours under a gentle stream of argon. Water was added from time to time in order to maintain the volume at about 3 ml. The solution was then cooled and applied to a short column of 6 ml of Dowex 50W X 2 (H^+) (50-100 mesh) cation-exchange resin. The column was washed with several portions of

water, in all 100 ml. The eluate and the washings were combined and evaporated to dryness under reduced pressure. The semi-crystalline residue was dissolved in 70 ml of warm methanol and kept in a refrigerator overnight. The crystalline product was collected, washed with a little ice-cold methanol and dried at room temperature *in vacuo*.

Yield: 154 mg (69.3% based on K ^{14}CN). M.p.: 151.8-154.4°C (uncorr.). HPLC showed a single peak with a retention time corresponding to that of an authentic sample (k' = 1.2). Specific activity: 240.8 μCi/mg = 53.5 mCi/mmol.

Ammonium 3-deoxy-D-*manno*-[1-^{14}C]octulosonate (4)

The mixture of lactone derivatives from the previous step (146.5 mg; 0.66 mmol) was dissolved in 0.7 ml of water and 0.1 ml of 0.5 M aqueous sodium hydroxide was added. The solution was kept at 60°C (bath temperature) and the pH was adjusted to 8 by adding 0.5 M aqueous sodium hydroxide whenever necessary. The total amount of sodium hydroxide solution needed was 1.1 ml. After about 2 h the hydrolysis of the lactones was complete as shown by HPLC. (k' for the octosonic acids = 4.5). The solution was cooled to room temperature and a suspension of an oxidation catalyst was added. The catalyst was made by dissolving 89.5 mg of vanadium pentoxide in 0.5 ml of conc. hydrochloric acid and adding 0.5 ml of pyridine to obtain pH = 3.2. Potassium chlorate (50.2 mg; 0.41 mmol) was added to the stirred reaction mixture and stirring was continued at room temperature for 12 h. Samples of 50 μl were taken after 2, 7, 8, 10, and 12 h respectively and analyzed by HPLC after removal of the catalyst and pyridine as described below. As the peak corresponding to the product (k' = 2.8) did not increase during the last two hours but the formation of a by-product (unidentified) raised from ca. 10% to ca. 17% compared to KDO, the reaction was stopped. The catalyst was removed by filtration, the pH of the filtrate was adjusted to 8 by adding 2 M aqueous sodium hydroxide (2 ml) and the excess of pyridine was removed by extraction with chloroform (3x7 ml). The aqueous solution was then evaporated to dryness at 30°C under reduced pressure. The residue was dissolved in 5 ml of 70% aqueous acetone, the

solution was passed through a column of microcrystalline cellulose (1x13 cm) equilibrated with 70% aqueous acetone, and the elution continued with 200 ml of the same solvent. This treatment removed most of the salts from the product. The eluate was evaporated to dryness and the residue was dissolved in 10 ml of 80% aqueous acetone. The solution was chromatographed on a column of the same sort of cellulose (2x27 cm) equilibrated with 90% aqueous acetone. The column was eluted first with 200 ml of 90% aqueous acetone followed by 300 ml 85% aqueous acetone and 200 ml portions of 80%, 75% and 70% aqueous acetone. Fractions of 3 ml were collected during the elution with the latter three solvent mixtures (fr. 1-63, 64-126 and 127-190, resp.) and were examined by measuring their radioactivity as well as by the use of the periodate-thiobarbiturate reagent (7). Both tests were carried out on Whatman No. 3 filter paper. The fractions which showed positive reactions were analyzed also by HPLC. By using this procedure 25 fractions (76-100) containing the desired product could be combined and they were evaporated to dryness under reduced pressure giving 35 mg of a greenish-yellow oil. This was dissolved in 3 ml of water and passed through a short column of 6 ml of Dowex 50W x 2 (H^+) (50-100 mesh) cation exchange resin. The pH of the eluate was adjusted to 8 by adding a few drops of 2 M aqueous ammonium hydroxide and the solution was evaporated to dryness under reduced pressure. The residue was taken up in 0.3 ml water at about 35°C and 0.3 ml of absolute ethanol was carefully added. On keeping the solution in a refrigerator overnight 23.5 mg of colorless crystalline ammonium 3-deoxy-D-*manno*-[1-^{14}C]octulosonate were obtained. Yield: 14% based on the lactone mixture (overall yield based on K^{14}CN 9.3%). Specific activity: 182 μCi/mg = 49.9 mCi/mmol. Paper chromatography on Whatman No. 3 paper in ethyl acetate-acetic acid-water (2:1:2) gave a single radioactive peak upon scanning as well as a single spot with the periodate-thiobarbiturate reagent at Rf = 0.59, which corresponds to the Rf value of an authentic sample and of commercial ammonium KDO (Sigma, Miss. USA). Also HPLC gave a single peak corresponding to the peak of these two samples. The mother liquor contained 8 mg, giving a single peak on HPLC.

The product was stored in a 30% aqueous ethanol solution at -20°C at a concentration of about 50 µCi/ml.

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