CLXI. THE BIOCHEMISTRY AND PHYSIOLOGY OF GLUCURONIC ACID.

I. THE STRUCTURE OF GLUCURONIC ACID OF ANIMAL ORIGIN.

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The work described in this communication records the successful application to bornyl-*d*-glucuronide (borneolglucuronic acid) of the standard methods of elucidating the ring structure of sugar derivatives. Bornylglucuronide was isolated by Clement and Fromm [1902] from the urine of rabbits fed on borneol, and later Quick [1927] prepared it from the urine of dogs similarly fed. A full description of the general properties of this compound, however, was not given. Its formation was also detected by Hildebrandt [1909] after subcutaneous injection of a solution of bornylglucoside in rabbits. In identification Hildebrandt quotes only the melting-point, 174°. No previous investigation of the structure of a glucuronide synthesised in the animal body has been described. Whilst this study was in progress, two papers appeared dealing with the structure of uronic acid residues in naturally occurring products of plant origin. An aldobionic acid from gum arabic has been the subject of a study by Challinor, Haworth and Hirst [1931]. By methylation and hydrolysis they find it to be a 6-galactopyranose-*d*-glucuronic acid. The glucuronic acid residue was isolated, after hydrolysis of the methylated aldobionic acid, as syrupy trimethylglucuronic acid, which on methylation with methyl sulphate and alkali gave crystalline trimethyl-β-methylglucuronide. Then, by comparison of the rate of hydrolysis of the latter with that of β-methylglucopyranoside, these authors deduced a pyranoid structure for the glucuronide. Furthermore, Robertson and Waters [1931] have studied the structure of euxanthic acid, a compound of euxanthone and glucuronic acid. Euxanthic acid was methylated and the product hydrolysed. From the products of hydrolysis trimethylglucuronic acid [*cf.* Challinor *et al.*, 1931] was isolated. This was oxidised with nitric acid, and the products of oxidation, after esterification and distillation, yielded dimethyl *d*-dimethoxysuccinate and 2:3:4-trimethyl-δ-saccharolactone methyl ester. The saccharolactone was then prepared independently from a compound of known pyranoid structure, 2:3:4-trimethyl-α-methylglucoside, and hence its isolation from the oxidation products of trimethylglucuronic acid establishes a pyranoid structure for the glucuronic acid residue of euxanthic acid. It may be mentioned here that Robertson and Waters did not find *i*-xylotrimethoxyglutaric acid in the products of oxidation.

In the work here described it is proved by direct chemical evidence that the glucuronic acid residue of bornylglucuronide synthesised in the dog is a pyranoid compound. β-Bornyl-*d*-glucuronide was methylated with methyl iodide and silver oxide to give the methyl ester of 2:3:4-trimethyl-β-bornyl-*d*-glucuronide which was isolated as a white crystalline solid, M.P. 92–93° and $[\alpha]_{5461}$ − 30·7°

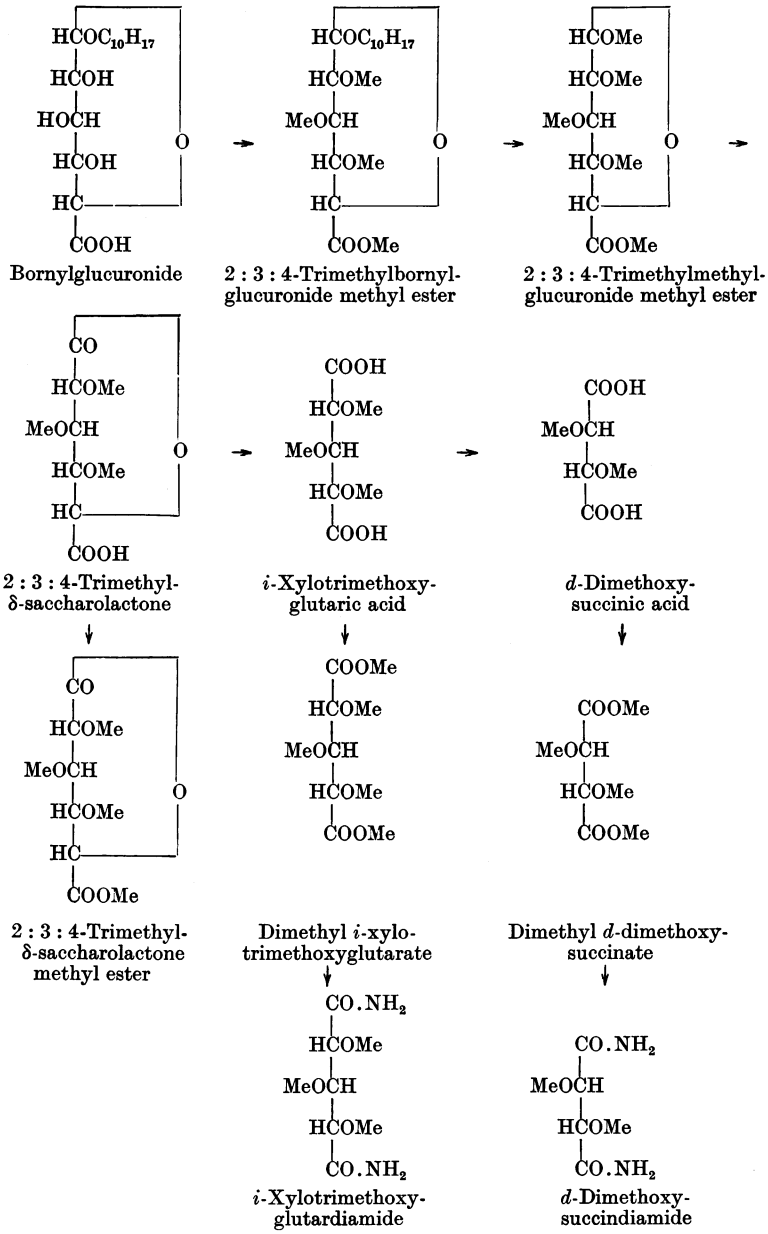
in alcohol. This ester was then subjected to the action of 0·2*N* sulphuric acid in methyl alcohol at 100° for 24 hours in a sealed tube, and a liquid mixture of the α- and β-isomerides of the methyl ester of 2:3:4-trimethylmethylglucuronide was isolated and purified by distillation in a high vacuum. This ester had $[\alpha]_{5461}$ + 98·9° in water. The fully methylated glucuronide was then carefully oxidised with nitric acid (sp. gr. 1·42) for 2½ hours, the temperature being regulated and never allowed to rise above 90°. After removal of the bulk of the nitric acid by continuous distillation with water, the products of oxidation were esterified with 3 % hydrogen chloride in methyl alcohol. After neutralising the acid with silver carbonate, the solvent was evaporated and the syrupy residue distilled in a high vacuum and fractionated.

The first and second fractions, from their rotations in methyl alcohol, were found to be mixtures of about 20 % of dimethyl *d*-dimethoxysuccinate and 80 % dimethyl *i*-xylotrimethoxyglutarate [see Hirst, 1926; Hirst and Purves, 1923]. The third fraction consisted mainly of 2:3:4-trimethyl-δ-saccharolactone methyl ester [Robertson and Waters, 1931]. On treatment of fractions 1 and 2 with ammonia in methyl alcohol at 0°, each gave two separate crops of crystals on standing. The first of these was collected after 2 days' standing and when purified was identified as *d*-dimethoxysuccindiamide by analysis and by comparison of the constants with those given by Haworth, Hirst and Miller [1927] for this compound. The second crystalline deposit was collected over a period of 14 days after the separation of the first crop and was identified as *i*-xylotrimethoxyglutardiamide by analysis and comparison with the constants given for this compound by Hirst and Purves [1923].

After 4 days' standing in a desiccator, the third fraction of the products of high vacuum distillation partially crystallised. On extracting the partially crystalline mass with small quantities of ether, the syrupy part was removed leaving a white crystalline solid which was only slightly soluble in ether. These crystals (platelets) were then identified as 2:3:4-trimethyl-δ-saccharolactone methyl ester by analysis, rotation, melting-point and titration; this lactone ester was described for the first time by Robertson and Waters [1931] and shown to possess a pyranoid structure.

The isolation of *i*-xylotrimethoxyglutaric acid derivatives from the products of oxidation can only be explained by ascribing to the original glucuronide a pyranoid structure, since carbon atoms 2, 3 and 4, with their attached methoxy-groups, have been isolated as crystalline *i*-xylotrimethoxyglutardiamide. The *d*-dimethoxysuccinic acid derivatives which were obtained in small yield are further oxidation products of the *i*-xylotrimethoxyglutaric acid. Additional evidence for the pyranoid structure of the uronic acid residue of bornylglucuronide is obtained from the isolation of the crystalline 2:3:4-trimethyl-δ-saccharolactone methyl ester, which, as has already been mentioned, is known to possess a pyranoid structure. The arguments therefore prove that bornylglucuronide has a similar structure to the normal glucosides and that the uronic acid residue follows the parent hexose, glucose, in possessing a pyranoid structure. The appended sequence of structural formulae (p. 1199) illustrates the above argument.

It will be observed from the following formulae that any trimethoxyglutaric acid formed by oxidation of glucuronic acid should be optically inactive since its stereochemical arrangement would give internal compensation; on the other hand, the dimethoxysuccinic acid formed by further oxidation, not being internally compensated, should be optically active. The experimental results are in conformity with these expectations.



Experimental.

Administration of borneol. Isolation of zinc bornylglucuronide.

3 to 4 g. of borneol[1] were fed daily to each of several dogs weighing from 20 to 30 lbs. Initially the borneol was fed smeared on meat, but since the dogs

[1] Borneol (Harrington's) $[\alpha]_{5461} + 20°$ (in EtOH).
Borneol (B.D.H.) $[\alpha]_{5461} + 26{\cdot}5°$ (in EtOH).

finally refused to eat any food tasting of borneol it was found easy and advantageous to administer it in small gelatin capsules. The capsules could be concealed inside pieces of meat which were rapidly swallowed by the dogs. Each capsule held about 0·5 g. of borneol. The dogs were kept in a large zinc-lined cage, which carried under the floor a large shallow zinc funnel, so that all the urine excreted could be collected in a beaker placed at the lower orifice of the funnel. The urine collected was worked up daily by a method based upon that of Quick [1927]. The urine was acidified with acetic acid and precipitated with the exact quantity of normal saturated lead acetate and filtered. The clear yellow filtrate was heated to the boiling-point and about 10 g. of solid zinc acetate were added to every 500 cc. of hot filtrate. The vessel was well shaken to initiate the separation of the zinc salt and, after standing for about 2 minutes, it was filtered under suction, washed with warm water and dried in the air. With stale urine the yield of zinc salt was low, and it had a brown colour and urinary odour. The salt develops a brown colour and urinary odour if, after precipitation, it is allowed to stand in contact with the mother-liquor for some time before filtering. Otherwise the salt is pure white and odourless. The yield was 0·5 to 0·6 g. per g. of borneol fed. The salt is insoluble in water and ordinary organic solvents, but is soluble with hydrolysis in dilute acids. This salt was first prepared by Clement and Fromm [1902] who gave it the formula $C_{32}H_{50}O_{14}Zn, 2H_2O$. In the present investigation it was prepared for analysis by boiling with distilled water, filtering under suction, washing with water, alcohol and ether and drying in a desiccator. It was then analysed for zinc by the zinc ammonium phosphate method. Found Zn, 8·56 %; $C_{32}H_{50}O_{14}Zn, 2H_2O$ requires Zn, 8·61 %.

Only a few experiments were carried out on human subjects, for reasons which will be dealt with in a later paper, but the zinc salt was isolated in the same way as for dogs and in about the same yield.

*The preparation of β-bornyl-*d*-glucuronide.*

The powdered zinc salt (50 g.) was dissolved in 80 cc. of hot 3·5*N* sulphuric acid and the solution filtered while hot on a glass filter. The filtrate was then allowed to stand in the refrigerator overnight whereby it became a semi-solid slightly brown crystalline mass. The liquid was filtered under suction and the solid washed with ice-cold water. It was recrystallised from hot water, decolorising with charcoal. The yield was 35 to 40 g. For titrations and optical rotations some specimens were recrystallised several times from hot water. β-Bornyl-*d*-glucuronide forms microscopic prismatic needles similar to those described by Clement and Fromm [1902] for the corresponding menthylglucuronide. It is odourless when pure and is easily soluble in hot water, ether and alcohol, slightly soluble in cold water, and practically insoluble in chloroform. Contrary to Quick [1927], who states that bornylglucuronide possesses water of crystallisation approximating to one molecule, the titrations described below show it to contain exactly 1·5 molecules of water; it is thus similar to the corresponding menthylglucuronide which also possesses 1·5 molecules. It melts at 174–175°. Its specific optical rotation will be dealt with in a later publication.

Titrations (microburette).

Several titrations were carried out with highly purified specimens.

1. *Air-dried specimen.*

0·1010 g. required 5·13 cc. of 0·055*N* NaOH.

5·14 cc. is required for $C_{16}H_{26}O_7 + 1{\cdot}5H_2O$,

5·28 cc. is required for $C_{16}H_{26}O_7 + H_2O$.

2. *Dried over sulphuric acid.*

0·1453 g. required 6·55 cc. of 0·0625*N* NaOH.
6·51 cc. is required for $C_{16}H_{26}O_7 + 1{\cdot}5H_2O$,
6·75 cc. is required for $C_{16}H_{26}O_7 + H_2O$.

3. *Dried at 137° over P_2O_5* in vacuo.

0·1009 g. required 4·91 cc. of 0·0625*N* NaOH.
4·89 cc. is required for $C_{16}H_{26}O_7$ (*i.e.* anhydrous).

*The methylation of β-bornyl-*d*-glucuronide. Isolation of 2:3:4-trimethyl-β-bornyl-*d*-glucuronide.*

Bornylglucuronide (3·3 g.) was methylated with silver oxide and methyl iodide in the usual manner. The reaction at first proceeded vigorously in the cold owing to esterification of the free carboxyl group, and later the methylation was continued at 45 to 50°. Four separate treatments with fresh silver oxide and methyl iodide were necessary to methylate the compound completely, and the methoxyl content of the product increased as follows: syrup after the second methylation had OMe, 25·46 %; after third methylation, OMe, 29·61 %; after fourth methylation, OMe, 30·63 %. The product of the fourth methylation was entirely crystalline; the yield was 3 g. It was recrystallised first from ether then from 50 % aqueous alcohol. It crystallised in white shining hexagonal platelets, M.P. 92–93°; it was very soluble in alcohol and ether, but insoluble in water; it did not reduce Fehling's solution and was neutral in reaction.

$[\alpha]_{5461}^{15°}$ − 30·7° in absolute alcohol (c = 0·717).

Found C, 62·4; H, 8·9; OMe, 31·3 %. $C_{20}H_{34}O_7$ requires C, 62·1; H, 8·9; OMe, 32·1 %.

*Simultaneous hydrolysis and methylation of trimethylbornylglucuronide methyl ester. Isolation of 2:3:4-trimethylmethyl-*d*-glucuronide methyl ester.*

Trimethylbornylglucuronide methyl ester (6 g.) was dissolved in 100 cc. of 0·2*N* sulphuric acid in methyl alcohol and the solution heated for 24 hours at 100° in Carius tubes. After cooling, the tubes were opened and the solution, which smelled strongly of borneol, was neutralised with silver carbonate and filtered. The methyl alcohol was removed at 40° under diminished pressure. Some crystals of borneol separated from the syrupy residue, but the bulk of it was precipitated by adding water. It was then filtered. After thorough extraction of the borneol with water, the combined aqueous extracts were concentrated *in vacuo* at 45° to a syrup. The syrup, however, still contained silver salts and these were removed by dissolving the syrup in ether and filtering the precipitated silver salts. The ethereal solution on concentrating gave a faintly yellow clear mobile syrup which was thoroughly dried and distilled (yield 3·8 g.). The syrup distilled at 131°/4 mm. giving a mobile and perfectly colourless syrup in a yield of 3·3 g. It was non-reducing and proved to be the expected 2:3:4-trimethylmethyl-*d*-glucuronide methyl ester: $n_D^{17°}$ 1·4469, $n_D^{14·5°}$ 1·4480; $[\alpha]_{5461}^{18°}$ + 98·9° in water (c = 0·531). Found C, 49·7; H, 7·7; OMe, 57·6 %. $C_{11}H_{20}O_7$ requires C, 50·0; H, 7·6; OMe, 58·7 %.

The borneol which separated was dissolved in ether and the solution filtered and evaporated to dryness. The residue was then purified by distillation in steam and dried in a desiccator. It melted at 206° and had $[\alpha]_{5461}^{16°}$ + 30·1° in absolute alcohol (c = 1·164). The rotation of the original borneol fed to the dogs was $[\alpha]_{5461}$ + 20° in absolute alcohol; M.P. 207°.

Oxidation of 2:3:4-trimethylmethylglucuronide methyl ester with nitric acid. Esterification of the products of oxidation.

The ester (2 g.) was dissolved in 20 cc. of nitric acid (sp. gr. 1·42). The reaction was initiated by heating on a water-bath to 65°. Very vigorous evolution of oxides of nitrogen took place and the mixture had to be cooled to moderate the reaction. After half an hour the temperature was raised to 90° and this temperature was maintained for 2 hours. At the end of this period the evolution of gas had ceased and the solution was diluted with an equal volume of water. The nitric acid was removed by distillation with water at 40° under diminished pressure, using a continuous feed arrangement to supply fresh quantities of water without interrupting the distillation. Finally, the solution was concentrated to a pale yellow stiff syrup with a strong acid reaction, due in part to the presence of residual traces of nitric acid. This syrup was taken up in methyl alcohol and the solvent evaporated to remove traces of water with it. The syrup was then thoroughly dried *in vacuo* over phosphorus pentoxide at 70–80°. The residual nitric acid did not cause any decomposition during drying, nor did it interfere with the subsequent esterification.

Esterification of the acid syrup was accomplished by boiling for 7 hours with 30 cc. of 4 % hydrogen chloride in methyl alcohol. The solution was next neutralised with silver oxide and dried by standing overnight over anhydrous sodium sulphate. After filtering and extracting the residues with methyl alcohol the solution was concentrated to a pale yellow mobile syrup. It still contained silver salts, derived from the residual nitric acid, and these were removed by dissolution in ether and filtering. The ethereal solution was now concentrated and the resulting syrup thoroughly dried *in vacuo*. The yield was 1·5 g.

High vacuum distillation of the esterified syrup. The dried syrup was distilled in a high vacuum and three fractions collected as follows:

Fraction	Bath temperature	Pressure mm.	Yield g.	Remarks
1	115–119°	0·24	0·531	Colourless mobile syrup
2	120–136°	0·25	0·358	Ditto
3	140–150°	0·34	0·497	Viscous yellow syrup

A small amount of a dark residue remained in the distilling flask and was neglected. Each fraction was then examined separately.

Examination of fraction 1.

The refractive index was $n_D^{14°}$ 1·4409; $[\alpha]_{5461}$ + 18·72° in methyl alcohol (c = 1·602). Found OMe, 61·56 %. These values accord with those required for a mixture consisting of 80 % of dimethyl *i*-xylotrimethoxyglutarate [Hirst and Purves, 1923] and 20 % of dimethyl *d*-dimethoxysuccinate [Haworth, Hirst and Miller, 1927] (the former has $n_D^{15°}$ 1·4402 and $[\alpha]_D$ 0°, and the latter has $n_D^{20°}$ 1·4340 and $[\alpha]_D$ 81° in methyl alcohol). A mixture of these two esters in the above mentioned proportions gives $n_D^{14°}$ 1·4398, $[\alpha]_D$ + 16·2° and OMe, 61·64 %.

Action of ammonia on fraction 1. 0·4 g. of the syrup was dissolved in 10 cc. of dry methyl alcohol, and the solution was saturated with ammonia at 0°. The solution developed a light pink colour and deposited crystals in 24 hours; these were filtered, washed thoroughly with methyl alcohol followed by ether and dried. They formed tufts of needles and were identified as *d*-dimethoxysuccindiamide [Haworth, Hirst and Miller, 1927]. The solution was then retreated with dry ammonia, and a second crop of crystals, differing in appearance from the first, was collected over a period of 14 days. The solution had also

developed, in one experiment, a deep purplish colour, typical to the formation of the xylotrimethoxyglutardiamide [see Hirst and Purves, 1923]. On evaporating the solution to dryness a further amount of the second crystalline material was collected. Some difficulty was experienced in purifying the last crop of crystals, but this was eventually accomplished by dissolving in ethyl alcohol, decolorising with charcoal and then filtering the solution. The solution was then evaporated to dryness and the resulting sticky crystals washed with cold methyl alcohol followed by ether, to remove adhering syrup. These crystals were identified as *i*-xylotrimethoxyglutardiamide [Hirst and Purves, 1923].

Analysis, etc.

1. *d*-Dimethoxysuccindiamide. Yield 0·08 g.; M.P. 272°; $[\alpha]_{5461}^{16°}$ + 114·6° in water ($c = 0·193$); $[\alpha]_D$ = + 97·1° (calc.). $[\alpha]_{5461}^{19°}$ + 113·2° in water ($c = 0·159$); $[\alpha]_D$ 95·9° (calc.). Found OMe, 35·8; N, 15·9 %. $C_6H_{12}O_4N_2$ requires OMe, 35·2; N, 15·9 %.

2. *i*-Xylotrimethoxyglutardiamide. Yield 0·1 g.; M.P. 195° to a blue melt. It was optically inactive. Found OMe, 42·1; N, 12·97 %. $C_8H_{16}O_5N_2$ requires OMe, 42·3; N, 12·73 %.

Examination of fraction 2.

The refractive index was $n_D^{14°}$ 1·4430; $[\alpha]_{5461}^{17°}$ 18·37° in methyl alcohol ($c = 1·143$). Found OMe, 59·26 %. These values show it to possess almost the same composition as fraction 1.

Action of ammonia on fraction 2. 0·25 g. of the syrup was treated in the same way as fraction 1. The solution turned a pale greenish-blue colour indicating the presence of the xylo-derivative. A small amount of dimethoxysuccindiamide was isolated followed by the xylotrimethoxyglutardiamide and was examined as in fraction 1.

Examination of fraction 3.

Isolation of 2:3:4-trimethyl-δ-saccharolactone methyl ester. The refractive index of the syrup was $n_D^{14°}$ 1·4570 and the methoxyl content OMe, 55·63 %. The methoxyl value suggested that it was, most probably, a mixture of trimethyl-saccharolactone methyl ester and dimethyl *i*-xylotrimethoxyglutarate. After standing for 4 days in a desiccator the syrup became partially crystalline. It was then treated with ether in small quantities, and the adhering syrup was removed, leaving a mass of white, shining platelets sparingly soluble in ether. These were thoroughly washed with ice-cold ether, dried in a desiccator and identified as 2:3:4-trimethyl-δ-saccharolactone methyl ester [*cf.* Robertson and Waters, 1931; Charlton *et al.*, 1931]. The crystals melted at 106·4° and had $[\alpha]_{5461}^{15°}$ + 175·9° in benzene ($c = 0·216$).

Titration of total carboxyl. 9·55 mg. of the lactone were boiled for half an hour with 10 cc. *N*/70 NaOH and then the solution was titrated with *N*/70 sulphuric acid. Found 5·23 cc. of *N*/70 NaOH; $C_{10}H_{16}O_7$ requires 5·39 cc. Found C, 48·4; H, 6·6; OMe, 50·6 %. $C_{10}H_{16}O_7$ requires C, 48·4; H, 6·5; OMe, 50·0 %.

Robertson and Waters [1931] quote M.P. 106° and $[\alpha]_{5461}^{25°}$ + 176·05° in benzene, while Charlton *et al.* [1931] quote M.P. 107° and $[\alpha]_D^{18°}$ + 146·5° in benzene.

Summary.

β-Bornyl-*d*-glucuronide (borneolglucuronic acid) $C_{16}H_{26}O_7, 1·5H_2O$, isolated as the zinc salt from the urine of human beings and dogs fed with borneol, gives crystalline 2:3:4-trimethyl-*β*-bornyl-*d*-glucuronide methyl ester by methylation with silver oxide and methyl iodide. This ester is converted into a mixture

of α- and β-2:3:4-trimethylmethyl-*d*-glucuronide methyl esters by the action of 0·2*N* sulphuric acid in methyl alcohol at 100° under pressure. Oxidation of the fully methylated glucuronic acid with nitric acid yields *d*-dimethoxysuccinic acid, *i*-xylotrimethoxyglutaric acid and 2:3:4-trimethyl-δ-saccharolactone. The first two of these were identified as the crystalline diamides, while the saccharolactone was identified as the crystalline methyl ester. The isolation of *i*-xylotrimethoxyglutaric acid and 2:3:4-trimethyl-δ-saccharolactone establishes a pyranoid structure for the glucuronic acid residue of bornylglucuronide, a typical conjugated glucuronic acid, synthesised in the animal body.

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