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Studies on the Chemical Constitution of Agar-agar. XXIII.[1] Isolation of D-Xylose, 6-*O*-Methyl-D-galactose, 4-*O*-Methyl-L-galactose and *O*-Methylpentose

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Commercial agar was subjected to complete hydrolysis with dilute sulfuric acid, and the products were carefully investigated. Besides D- and L-galactose, which have been known to exist, there were isolated small amounts of D-xylose, 6-*O*-methyl-D-galactose, 4-*O*-methyl-L-galactose, and *O*-methylpentose, all the sugars except the last one being obtained in crystalline forms. Among these products, 4-*O*-methyl-L-galactose is a new compound, so its structure has been fully investigated. As commercial agar is made from *Gelidium amansii*, occasionally mixed with several other species of seaweeds, the experiments were repeated for the agar prepared from *G. amansii* only. The same products as those from commercial agar were isolated except the *O*-methylpentose.

A series of papers[2] contributed from this Institute have shown that the agar prepared from *Gelidium amansii* is composed mainly of D-galactose and 3, 6-anhydro-L-galactose, and that these two sugars constitute the molecule of agarose,[3] which is defined as a main polysaccharide of the agar. Several minor components involving sulfuric acid,[4] pyruvic acid,[5] D-glucuronic acid,[6] and L-galactose[7] have also been proved to be present in the agar of *G. amansii* or the commercial agar made mainly therefrom. The present paper will report the isolation of additional sugars in small amounts from the hydrolysis products both of commercial agar and the agar of *G. amansii*.

Commercial agar was subjected to complete hydrolysis with dilute sulfuric acid. From the hydrolysates, D-galactose, and acidic products were removed by appropriate treatment, involving solvent fractionation, ion exchange, crystallization and fermentation.[8] When the resulting mixture of non-fermentable sugars were separated on columns of charcoal-Celite, small amounts of D-xylose, 6-*O*-methyl-D-galactose, and 4-*O*-methyl-L-galactose were isolated in crystalline forms in addition to L-galactose. *O*-Methyl-pentose, whose structure has not yet been assigned, was also isolated.

D-Xylose was identified as its crystalline sugar and crystalline phenylosazone. The sugar had been detected in the products of both the hydrolysis[9] and mercaptolysis[10] of commercial agar, but it had not been obtained in a crystalline form. 6-*O*-Methyl-D-galactose was identified again as its crystalline sugar and crystalline phenylosazone. It has been found that the sugar can be easily characterized by its crystalline 1-methylphenylhydrazone, which has been prepared for the first time in the present time. This sugar had been reported to exist in the agar prepared from *Ceramium boydenii*,[11] but as far as commercial agar and the agar of *G. amansii* are concerned, this paper is the first reported instance of its isolation. 4-*O*-Methyl-L-galactose is a new compound, although its enantiomorph has been reported by Hirst and Jones[12] and by Jeanloz.[13] A comparison of the properties of the sugar itself, as well as those of its anilide and phenylosazone, with those of the corresponding D-compounds has proved that they

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1) Part XXII: C. Araki and S. Hirase, This Bulletin, **33**, 597 (1960).

2) See review: C. Araki, "Proc. 4th Intern. Congr. Biochem.," Vol. 1, Pergamon Press, London, New York-Paris (1959), p. 15; "Progress in Org. Chem. (Yūkikagaku no Shinpo)," Vol. 13, ed. by M. Murakami, Kyoritsu Shuppan, Tokyo (1959), p. 221.

3) C. Araki, This Bulletin, **29**, 543 (1956).

4) C. Neuberg and H. Ohle, *Biochem. Z.*, **125**, 311 (1921).

5) S. Hirase, This Bulletin, **30**, 68 (1957); **30**, 75 (1957).

6) C. Araki, *Nippon Kagaku Kwaishi (J. Chem. Soc. Japan)*, **58**, 1214 (1937).

7) N. W. Pirie, *Biochem. J.*, **30**, 369 (1936); C. Araki, *Nippon Kagaku Kwaishi (J. Chem. Soc. Japan)*, **59**, 424 (1938).

8) C. Araki and K. Arai, "Methods in Carbohydrate Chemistry," Vol I, ed. by R. L. Whistler and M. L. Wolfrom, Academic Press, New York and London (1962), p. 122.

9) S. Hirase and C. Araki, *Memoirs Fac. Ind. Arts, Kyoto Tech. Univ., Sci. and Tech.*, **1**, 19 (1952).

10) C. Araki and S. Hirase, This Bulletin, **26**, 463 (1953).

11) S. Hirase and C. Araki, *ibid.*, **34**, 1048 (1961).

12) E. L. Hirst and J. K. N. Jones, *J. Chem. Soc.*, **1946**, 506.

13) R. W. Jeanloz, *J. Am. Chem. Soc.*, **76**, 5684 (1954).

are optical antipodes to each other.

Independent proof has also been provided for the structure of 4-*O*-methyl-L-galactose. Demethylation with hydrogen iodide[14] yielded L-galactose, indicating that the sugar is a derivative of L-galactose. The treatment of the sugar with boiling methanolic hydrogen chloride afforded crystalline methyl glycoside, to which the α-L-pyranoside structure has been assigned on the basis of its highly negative value of optical rotation. The methyl glycoside consumed approximately one mole of periodate. This result shows that the methoxyl group is residing either on C_2 or C_4. The former possibility is excluded by the fact that the sugar yielded phenylosazone without the loss of its methoxyl group. Consequently, the only possible structure for the sugar under discussion is 4-*O*-methyl-L-galactose.

As is well known, commercial agar is made from *G. amansii*, occasionally mixed with several other species of seaweed. Some possibility exists, therefore, that the sugars isolated above might have originated from the admixed seaweeds. This possibility, however, is excluded by the fact that, when the agar prepared from *G. amansii* alone was examined in a similar manner, D-xylose, 6-*O*-methyl-D-galactose, and 4-*O*-methyl-L-galactose were isolated again in crystalline forms.

The yields of the isolated sugars in both cases were so low that it is possible that they arose as secondary products from D-galactose during the fermentation process. However, none of them could be detected when D-galactose was subjected to fermentation under the same conditions.

Experimental

General Procedure. The evaporation of solutions were carried out under reduced pressure below 40°C. Unless otherwise stated, the paper chromatograms were developed with 1-butanol - acetic acid - water (4 : 1 : 2 by volume) and were sprayed with *o*-aminophenol in ethanol acidified with phosphoric acid.[15]

Hydrolysis of Commercial Agar. Commercial agar powder (200 g, moisture 19.5%) was hydrolyzed with N sulfuric acid (2 *l*) on a boiling-water bath at 100°C for 20 hr. Then, according to the procedure previously described by two of the present writers,[8] the acidic products were removed from the hydrolysates by precipitation as barium salts and by absorption on ion exchange resins, while D-galactose was removed both by crystallization and fermentation. Finally, a mixture of non-fermentable sugars was obtained as a syrup; yield, 17.2 g.

Chromatography of a Mixture of Non-fermentable Sugars. The mixture obtained above was separated on a charcoal-Celite column[16] (31×6.5 cm), which was eluted successively with water, 2.5% ethanol, 5.0% ethanol, and 7.5% ethanol unitl the respective effluents showed no more reducing power. The effluents were then separately evaporated to dryness. The yields are shown in Table 1. Fraction 1 is investigated in the present work, while the others will be treated in a subsequent work.

Table 1. Chromatography of a mixture of non-fermentable sugars

Fraction	Eluant*	Effluent, *l*	Yield, g
1	Water	10.4	9.4
2	2.5% E	9.0	2.8
3	5.0% E	9.0	1.7
4	7.5% E	9.0	0.7

* E : Ethanol

Rechromatography of Fraction 1. Fraction 1 in Table 1 gave L-galactose on crystallization from methanol; yield, 2.6 g; mp and mixed mp 167°C; $[\alpha]_D^{12}$ −125.0°(5 min) → −81.1°(24 hr) (*c* 1.0, water). The mother liquor was evaporated to a syrup (5.5 g) which showed spots corresponding to galactose, xylose and mono-*O*-methylgalactose on a paper chromatogram. This syrup was then combined with that obtained from another similar batch, and the resulting mixture (10.4 g) was subjected to re-chromatography on a charcoal-Celite column (5×34 cm); the six fractions shown in Table 2 were thus obtained.

Isolation of D-Xylose. Fraction 1a in Table 2 gave crystals of D-xylose when dissolved in methanol-1-propanol (1 : 1 by volume) and seeded with D-xylose; yield, 0.10 g; mp and mixed mp 144—145°C; $[\alpha]_D^{31}$ +31.4° (10 min)→+18.7° (24 hr) (*c* 0.96, water). The literature values: mp 145°C; $[\alpha]_D$ +18.8° (equilibrium value, water). Found: C, 39.91; H, 6.44%.

For further identification, the sugar was converted into its phenylosazone; mp and mixed mp 166—167°C, $[\alpha]_D^{25}$ −56.8° (8 min)→ −41.9° (24 hr) (*c* 0.74, pyridine-ethanol (2 : 3 by volume)). Found: N, 17.00%.

Isolation of 4-*O*-Methyl-L-galactose. Fraction 1c (2.7 g) in Table 2 was dissolved in methanol (7 m*l*), after which the solution was kept in a refrigerator for several days. The 4-*O*-methyl-L-galactose which crystallized was collected by filtration; yield, 0.33 g; mp 202—203°C; $[\alpha]_D^{13}$ −74.8° (27 min)→−85.1° (24 hr) (*c* 2.70, water). An additional crop was obtained from the filtrate; yield, 0.06 g; mp 201—202°C. The crude crystals were combined and recrystallized by dissolving them in a small amount of hot water and then adding three times as much methanol; mp 204—205°C; $[\alpha]_D^{15}$ −81.5° (15 min)→−103.9° (26 hr) (*c* 2.1, water); R_f, 0.31 (R_{gal}, 1.55). The values reported for its enatiomorph are: mp 207°C and $[\alpha]_D^{20}$ +62°→+92° (water);[12] mp 218—221°C and $[\alpha]_D^{24}$ +61°→$[\alpha]_D^{22}$ +83° (water.)[13]

Found: C, 43.16; H, 7.38; OCH_3, 15.57%. Calcd for $C_6H_{11}O_5(OCH_3)$: C, 43.29; H, 7.27; OCH_3, 15.98%.

4-*O*-Methyl-L-galactosazone. 4-*O*-Methyl-L-galactose (0.07 g) in water (4 m*l*) was treated with phenylhydrazine (0.20 g) and 50% acetic acid (0.2 g) at

14) C. Araki and Y. Hashi, *Nippon Kagaku Kwaishi (J. Chem. Soc. Japan)*, **60**, 783 (1939).

15) S. Hirase, C. Araki and S. Nakanishi, This Bulletin, **26**, 183 (1953).

16) R. L. Whistler and D. F. Durso, *J. Am. Chem. Soc.*, **72**, 677 (1950).

TABLE 2. RE-CHROMATOGRAPHY OF FRACTION (1)

Fraction	Eluant*	Effluent, *l*	Yield g	Paper chromatography** (R_f and color of spot)
1 a	Water	0.1	0.38	0.29 (blue)
1 b	Water	0.1	3.10	0.20 (brown); 0.29 (blue); 0.31 (brown)
1 c	Water	2.0	2.70	0.31 (brown); 0.34 (brown)
1 d	Water	2.7	0.75	0.31 (brown); 0.34 (brown); 0.40 (blue)
1 e	2.5% E	2.7	1.05	0.34 (brown); 0.40 (blue)
1 f	5.0% E	3.2	0.05	0.05 (brown)

* E : Ethanol

** After being isolated, the individual sugar showed a spot with the following R_f and color : D-xylose, 0.29 (blue); L-galactose, 0.20 (brown); 4-*O*-methyl-L-galactose, 0.31 (brown); 6-*O*-methyl-D-galactose, 0.34 (brown), and *O*-methylpentose, 0.40 (blue).

TABLE 3. CHROMATOGRAPHY OF NON-FERMENTABLE SUGARS FROM *G. AMANSII* AGAR

Fraction	Eluant*	Effluent, m*l*	Yield, g	Sugar isolated
1	Water	800	1.17	D-Xylose; L-galactose
2	Water	1500	0.48	4-*O*-Methyl-L-galactose
3	2.5% E	1500	0.42	6-*O*-Methyl-D-galactose
4	5.0% E	1500	0.49	

* E : Ethanol

80—85°C for 2.5 hr. The resulting yellow crystals of osazone were then filtered and washed successively with methanol and ether; yield, 0.03 g; mp 138—139°C. Recrystallization from methanol raised the melting point to 150°C. The value reported for its enantiomorph is 150°C.[12]

Found: C, 61.34; H, 6.65; N, 15.21; OCH_3, 7.88%. Calcd for $C_{19}H_{24}O_4N_4$: C, 61.27; H, 6.48; N, 15.05; OCH_3, 8.33%.

***N*-Phenyl-4-*O*-methyl-L-galactosylamine.** A mixture of 4-*O*-methyl-L-galactose (0.08 g) and aniline (0.13 g) in 95% ethanol (4 m*l*) was heated under reflux for 4 hr. The crystals which formed were filtered and washed with ethanol and then ether; yield, 0.10 g; mp 165—166°C. Recrystallization from ethanol gave the pure anilide; mp 167—168°C; $[\alpha]_D^{12}$ −3.8° (22 min)→−21.0° (24 hr) (*c* 1.05, methanol). The values reported for its enantiomorph are: mp 168°C,[12] mp 167—168°C[13] and $[\alpha]_D^{21}$ −84°→$[\alpha]_D^{22}$ −39° (methanol).[11]

Found: C, 57.68; H, 6.81; N, 5.18; OCH_3, 11.33%. Calcd for $C_{13}H_{19}O_5N$: C, 57.98; H, 7.11; N, 5.20; OCH_3, 11.52%.

Demethylation of 4-*O*-Methyl-L-galactose. A solution of 4-*O*-methyl-L-galactose (0.15 g) in water (2 m*l*) was saturated with dry hydrogen iodide at 0—1°C, the iodide being allowed to bubble in the solution for 15 min after saturation. The resulting dark solution was sealed in a glass tube and kept in a refrigerator for 20 hr. The reaction mixture was then treated in the usual way.[14] The product was obtained as a syrup (0.14 g) which showed two spots corresponding to galactose and the unchanged compound on a paper chromatogram. Pure L-galactose was obtained after chromatographic purification on a filter paper sheet, with 1-butanol-ethanol-water (4 : 1 : 2 by volume) as a mobile phase, and subsequent crystallization from methanol; yield, 0.04 g; mp and mixed mp 167°C; $[\alpha]_D^{31}$ −100.9° (9 min)→−79.3°(24 hr) (*c* 0.95, water). Found: C, 39.87; H, 6.72%.

Methyl 4-*O*-Methyl-α-L-galactopyranoside. 4-*O*-Methyl-L-galactose (0.10 g) was heated with 1% methanolic hydrogen chloride (7 m*l*) under reflux for 20 hr. The neutralization of the solution with silver carbonate, followed by filtration and subsequent evaporation, afforded a syrup (0.11 g) which, on standing, crystallized spontaneously. The crystals were triturated with ethyl acetate and filtered; yield, 0.06 g; mp 99—100°C. Recrystallization from ethyl acetate gave the pure methyl α-glycoside; mp 109—110°C; $[\alpha]_D^{15}$ −151.0° (*c* 0.90, water).

Found: OCH_3, 29.67%. Calcd for $C_6H_{10}O_4 \cdot (OCH_3)_2$: OCH_3, 29.8%.

Periodate Oxidation of Methyl 4-*O*-Methyl-α-L-galactopyranoside. To a solution of the methyl glycoside (0.0233 g or 0.112 mmol) in water (10 m*l*), there was added sodium bicarbonate (0.05 g), followed by 0.193 M sodium metaperiodate solution (10.0 m*l*). The mixture was diluted with water to exactly 50 m*l* and kept at room temperature. A 10 m*l* portion of the solution was withdrawn at intervals, and the residual oxidant in it was titrated in the usual way.[17] The methyl glycoside consumed 1.11 mol, 1.15 mol and 1.21 mol of periodate per mole after 3 hr, 5 hr and 20 hr respectively.

Isolation of 6-*O*-Methyl-D-galactose. Fraction 1e (0.70 g) in Table 2 gave 6-*O*-methyl-D-galactose when crystallized from methanol-acetone; yield, 0.37 g; mp 116—117°C. It was purified by recrystallization

17) E. L. Jackson, "Organic Reactions," Vol. 2, John Wiley & Sons, New York (1944), p. 341.

from ethanol; mp 128—129°C; $[\alpha]_D^{19}$ +117.8° (15 min) →+78.5° (24 hr, *c* 1.51, water); R_f 0.34 (R_{gal} 1.72). The literature values are: mp 128°C and $[\alpha]_{578}^{20}$ +114°→ +77° (water);[18] mp 122—124°C and $[\alpha]_D^{20}$ +135°→ +77.0° (water).[11] Admixture with an authentic sample[11] showed no depression of the melting point. Found: C, 43.28; H, 7.56; OCH_3, 15.81%.

6-*O*-Methyl-D-galactosazone. The sugar obtained above was converted into its phenylosazone in the usual way; mp 198—199°C; $[\alpha]_D^{23}$ +134.4° (*c* 1.14, pyridine). The literature values are: mp 204—206°C and $[\alpha]_{578}^{17}$ +135° (pyridine);[18] mp 201—202°C and $[\alpha]_D^{20}$ +140° (pyridine).[11] Found: C, 61.35; H, 2.20; N, 15.25; OCH_3, 7.99%.

6-*O*-Methyl-D-galactose (1'-Methyl-1'-phenyl)-hydrazone. To a solution of 6-*O*-methyl-D-galactose (0.07 g.) in water (7 ml), ethanol (7 ml) and then 1-methyl-1-phenylhydrazine (0.30 g) and 50% acetic acid (0.40 g) were added. The reaction mixture was kept at 37°C for 6 hr. After being cooled, the hydrazone was filtered and dried; yield, 0.08 g; mp 172°C. It was recrystallized from 50% aqueous methanol; mp 176°C; $[\alpha]_D^{13}$ +23.1° (9 min)→+15.4°(24 hr) (*c* 0.11, methanol).

Found: C, 56.53; H, 7.26; N, 9.40; OCH_3, 10.29%. Calcd for $C_{14}H_{22}O_5N_2$: C, 56.36; H, 7.43; N, 9.39; OCH_3, 10.44%.

Isolation of *C*-Methylpentose. When fraction 1d in Table 2 was dissolved in methanol and left in a refrigerator, 4-*O*-methyl-L-galactose was crystallized; yield, 0.03 g. The mother liquor, after being evaporated to a syrup (0.60 g), was separated on three sheets of Toyo filter paper No. 52 (60+60 cm), with 1-butanol - ethanol - water (4 : 1 : 2 by volume) as a mobile phase. Strips containing *O*-methylpentose were then cut from the chromatograms, and the sugar was recovered therefrom by extraction with water and subsequent evaporation to a syrup; yield, 0.15 g; $[\alpha]_D^{21}$ −21.8° (*c* 3.85, water). Found: OCH_3, 17.71%. This syrup showed a blue spot with R_f 0.40 (R_{gal} 2.00 or R_{xyl} 1.55) on a paper chromatogram. Both the chromatographic behavior and the methoxyl content suggested that the sugar is a monomethyl ether of pentose. Further examination is necessary for its complete identification, however,

Hydrolysis of the *G. Amansii* Agar and Identification of the Products. The agar (40.0 g, moisture 9.5%) prepared from *G. amansii* was hydrolyzed with N sulfuric acid (700 ml) in a boiling water bath for 20 hr. The reaction solution was then treated in a similar manner used in the case of commercial agar described above. A mixture of non-fermentable sugars was thus obtained as a syrup (3.9 g). Paper chromatographic examination showed that it was a mixture of xylose, galactose, *O*-methylgalactose, and small amounts of three more sugars. The mixture was separated on a charcoal-Celite (1 : 1) column (4×28 cm) in the same way as has been described earlier. The four fractions shown in Table 3 were obtained.

D-Xylose. From Fraction 1 in Table 3, L-galactose was removed by crystallization from methanol; yield, 0.53 g; mp and mixed mp 167°C; $[\alpha]_D^{14}$ −78.1° (24 hr, *c* 1.05, water). The mother liquor (0.47 g), $[\alpha]_D^{15}$ −16.0° (*c* 1.50, water), was then added to a cellulose column (2.5×36 cm) and eluted with 1-butanol saturated with water. The fractions containing xylose were combined and evaporated to a syrup (0.16 g) which, on crystallization from methanol - 1-propanol (1 : 1), afforded D-xylose; mp and mixed mp 144—145°C; $[\alpha]_D^{13}$ +85.9° (15 min)→+16.3° (24 hr, *c* 0.86, water).

4-O-Methyl-L-galactose. Fraction 2 in Table 3 showed a spot corresponding to 4-*O*-methylgalactose on a paper chromatogram. Crystallization from methanol gave 4-*O*-methyl-L-galactose; yield, 0.07 g; mp 198—200°C. This was then recrystallized from methanol; mp 204—205°C; $[\alpha]_D^{18}$ −115.5° (11 min)→−85.5° (24 hr, *c* 1.0, water). Found: OCH_3 15.72%. Admixture with the sample of 4-*O*-methyl-L-galactose isolated from commercial agar showed no depression of the melting point.

6-O-Methyl-D-galactose. Fraction 3 in Table 3, $[\alpha]_D$ +42.6° (*c* 0.94, water), showed three spots with R_f 0.095, 0.36 and 0.47 on a paper chromatogram, the second one being most intense. A 0.36 g portion of the fraction was added to a cellulose column (2.5× 36 cm) and eluted with 1-butanol saturated with water. A fraction corresponding to R_f 0.36 was thus recoverd as a syrup (0.18 g); on crystallization from ethanol, this syrup afforded 6-*O*-methyl-D-galactose; yield, 0.03 g; mp 116—118°C. It was purified by recrystallization from ethanol; mp 122—123°C; $[\alpha]_D^{19}$ +115.5° (10 min)→ +77.5° (24 hr, *c* 1.10, water). Found: OCH_3, 15.66%. Admixture with an authentic sample showed no depression of the melting point.

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18) K. Freudenberg and K. Smeykal, *Ber.*, **59**, 100 (1926).