Studies on the Chemical Constitution of Agar-agar. XXII.[1] *Partial Methanolysis of Methylated Agarose of Gelidium Amansii*

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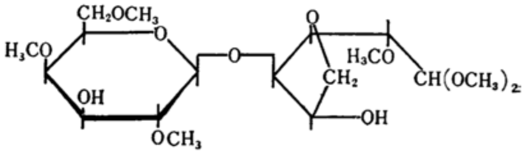
(Received October 8, 1959)

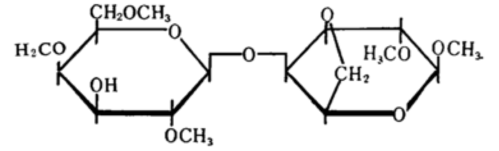
Agarose, a chief polysaccharide of agar, has been suggested by Araki[2], one of the present authors, to be a chain composed of alternating β-D-galactopyranose and 3,6-anhydro-α-L-galactopyranose residues. The preceding paper[1] concerned with the complete methanolysis of methylated agarose of *Gelidium amansii* provided a strong evidence to verify the above mentioned structure of agarose. A further support has now been obtained herein by partial methanolysis of the methylated polysaccharide.

The partial methanolysis was conducted with 0.5% methanolic hydrogen chloride for four hours, during which time 74% of the methylated polysaccharide was concerned in reaction. After treatment with barium hydroxide, the products were separated into neutral and acid components by fractionation with ether. The acidic component in the barium salt was obtained in too small a yield to be of structual significance. Further separation of the neutral components on a charcoal-Celite column led to the isolation of *O*-2, 4, 6-tri-*O*-methyl-β-D-galactopyranosyl-(1→4)-2-*O*-methyl-3, 6-anhydro-L-galactose dimethylacetal (I) and methyl *O*-2, 4, 6-tri-*O*-methyl-β-D-galactopyranosyl-(1→4)-2-*O*-methyl-3, 6-anhydro-β-L-galactopyranoside (II), both being derivatives of agarobiose (4-*O*-β-D-galactopyranosyl-3, 6-anhydro-L-galactose), which constitutes the repeating units of the agarose molecule. The total yield of compounds I and II amounted to 82% of the neutral products by weight.

The dimethylacetal (I) was eluted from the chromatographic column with 10～20% ethanol in water at 26～27°C and isolated in a crystalline form with the empirical formula $C_{12}H_{16}O_5 \cdot (OCH_3)_6$. Further, methylation with methyl iodide and silver oxide afforded hexa-*O*-methylagarobiose dimethylacetal, which on methanolysis yielded methyl 2, 3, 4, 6-tetra-*O*-methyl-α, β-D-galactoside and 2, 5-di-*O*-methyl-3, 6-anhydro-L-galactose dimethylacetal, identifying thereby compound I as a derivative of agarobiose. Hydrolysis with 0.02 N oxalic acid solution removed two methoxyl groups from the molecule to yield tetra-*O*-methylagarobiose, indicating that the compound I was a dimethylacetal. On methanolysis the compound I produced a mixture of methyl 2, 4, 6-tri-*O*-methyl-α, β-D-galactosides, methyl 2-*O*-methyl-3, 6-anhydro-β-L-galactopyranoside and 2-*O*-methyl-3, 6-anhydro-L-galactose dimethylacetal, the separation of the mixture being effected by selective hydrolysis of the anhydrosugar derivatives followed by fractionation with *n*-hexane. This established the positions of the four methoxyl groups connected in ether linkages with the rest of the molecule of compound I.



(I)



(II)

The methyl glycoside (II) was eluted from the charcoal column with 50% ethanol in water at 26～27°C and isolated in a crystalline form with the empirical formula $C_{12}H_{15}O_5(OCH_3)_5$. When treated with boiling methanol containing 3% of hydrogen chloride, it was converted with ease into the fore-going dimethylacetal (I). Hydrolysis of compound II with 0.02 N oxalic acid solution resulted in the formation of the reducing sugar identical with that obtainable from the dimethylacetal (I). These facts have enabled to be assigned structure II to the compound under problem, the β-configuration of the glycosidic methoxyl group being suggested on the basis of the positive value of optical rotation. Moreover, further methylation of compound II with methyl iodide and silver oxide afforded crystalline methyl penta-*O*-methyl-β-agarobioside, which agreed in all respects

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[1] Part XXI: C. Araki and S. Hirase, This Bulletin, 33, 291 (1960).

[2] C. Araki, This Bulletin, 29, 543 (1956); *Memoirs Fac. Ind. Arts, Kyoto Tech. Univ.*, 5, 21 (1956).

with the methylated disaccharide derivative previously isolated by Araki[3], one of the present authors, by re-methylation of the methanolysis products of methylated agarose of *G. amansii.*

The isolation of compounds I and II in a good total yield emphasizes that agarobiose units are repeated through their 1 and 3′ positions in the molecule of agarose of *G. amansii* as previously suggested by Araki.

Experimental

Evaporation and concentration of solutions were carried out under reduced pressure below 40°C. Melting points are uncorrected.

Methanolysis of Methylated Agarose.—Methylated agarose (10 g.) was suspended in methanol (150 ml.) containing 0.5% of hydrogen chloride and the suspension was heated under reflux for four hours, at which time undissolved material was removed by filtration. The filtrate was neutralized with silver carbonate, refiltered and evaporated to afford a sirup, which was then treated with 0.2 N barium hydroxide solution (100 ml.) at 60°C for two hours to saponify the methyl ester of the acidic component. The excess of the barium hydroxide was removed by neutralization with carbon dioxide and subsequent filtration, and the filtrate was evaporated to a sirup (7.4 g.).

Separation of Neutral and Acidic Components.—When the sirup obtained above was dissolved in ether (150 ml.), the acidic component was precipitated as a barium salt form. It was filtered off, washed with ether and dried in vacuo; yield, 0.18 g., $[\alpha]_D^{27}$ $-26.4°$ in water (*c* 0.72). Evaporation of the filtrate afforded a mixture of neutral components as a colorless sirup; yield, 7.2 g., $[\alpha]_D^{27}$ $-1.21°$ in water (*c* 0.83).

Chromatographic Separation of Mixture of Neutral Components.—The mixture of the neutral components (7.2 g.) obtained above was chromatographed at 25~27°C on a column (5.2×20 cm.) packed with charcoal (60 g.) and Celite (40 g.), successively higher concentrations of ethanol in water being used as developing solvents. The effluents were collected in separate receivers and evaporated to dryness to recover the contents, which were then weighed and the specific optical rotation, the methoxyl content and the molecular weight were measured. The results are shown in Table I.

TABLE I. CHROMATOGRAPHIC SEPARATION OF THE MIXTURE OF NEUTRAL COMPONENTS

Fraction	Developer %*	l.	Yield g.	$[\alpha]_D^{27}$ (°) H_2O	OCH_3 %	M.W.**
I	5	5	0.3	− 0.6	42.1	211
II	7.5	5	0.5	+97.8	48.3	225
III	10	5	1.6	−29.3	43.3	423
IV	15	5	1.8	−30.8	43.1	423
V	20	5	0.8	−30.9	43.2	425
VI	30	6	0.4	−11.0	40.8	410
VII	50	3	1.1	+38.4	39.6	398
VIII	70	2	0.1	+39.0	39.5	395

* Concentration of ethanol in water.

** Molecular weight was determined by Rast's method.

Fractions I and II were obtained as colorless sirups, but on standing the latter fraction crystallized partly. Papar chromatographic examination, using cyclohexanol saturated with water as a mobile phase[1] and *O*-aminophenol in aqueous ethanol acidified with phosphoric acid as a spraying reagent[4], indicated the presence of 2-*O*-methyl-3,6-anhydro-L-galactose dimethylacetal (R_f 0.67) and methyl 2-*O*-methyl-3,6-anhydro-β-L-galactopyranoside (R_f 0.77) in fraction I and of the latter compound in fraction II. When these were hydrolyzed with 1 N sulfuric acid for five hours in a boiling water bath and subsequently examined on paper chromatograms with *n*-butanol-ethanol-water (4:1:2) as a mobile phase and aniline hydrogen phthalate in moist *n*-butanol as a spraying reagent, 2,4,6-tri-*O*-methyl-D-galactose was detected in the hydrolyzates of both fractions I and II, the anhydrosugar derivatives being decomposed during the hydrolysis. It was therefore considered that fraction I was a mixture of 2-*O*-methyl-3,6-anhydro-L-galactose dimethylacetal, methyl 2-*O*-methyl-3,6-anhydro-L-galactopyranoside and methyl 2,4,6-tri-*O*-methyl-D-galactoside and that fraction II was a mixture of the latter two.

On the other hand, fractions III—V were proved to be entirely the dimethylacetal (I), and fractions VII and VIII were also proved to be entirely the methyl glycoside (II) as described below. Fraction VI seemed to be a mixture of compounds I and II.

Tetra-*O*-methylagarobiose Dimethylacetal (I).—All fractions III—V in Table I crystallized completely on standing and melted at 84~86°C. These were combined and recrystallized twice from ether containing a small amount of petroleum ether, affording the dimethylacetal (I) in prisms; m. p. 88~89°C, $[\alpha]_D^{10}$ $-25.2°$ in water (*c* 1.19), $[\alpha]_D^{10}$ $-41.9°$ in methanol (*c* 1.05) and $[\alpha]_D^{10}$ $-46.7°$ in chloroform (*c* 1.07). The crude yields of the dimethylacetal thus amounted to 58% of the sample applied.

Found: C, 50.65; H, 8.06; OCH_3, 43.45; mol. wt., 425. Calcd. for $C_{12}H_{16}O_5(OCH_3)_6$: C, 50.69; H, 8.04; OCH_3, 43.67%; mol. wt., 426.5.

Methanolysis of Dimethylacetal (I).—The dimethylacetal (I) (1.5 g.) obtained above was dissolved in 3% methanolic hydrogen chloride (30 ml.) and heated under reflux for two hours, at which time the optical rotation of the solution reached a constant value ($[\alpha]_D$ $-29° \rightarrow +42°$). The solution was neutralized with silver carbonate, filtered and evaporated to dryness, when a mixture of methyl 2-*O*-methyl-3,6-anhydro-β-L-galactopyranoside, 2-*O*-methyl-3,6-anhydro-L-galactose dimethylacetal and methyl 2,4,6-tri-*O*-methyl-D-galactoside was obtained as a sirup (1.3 g.); $[\alpha]_D^{25}$ $+51.0°$ in water (*c* 1.0).

a) Identification of methyl 2,4,6-tri-O-methyl-α,β-D-galactosides.—The sirup obtained above was

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3) C. Araki, *J. Chem. Soc. Japan, (Nippon Kwagaku Kwaishi)*, **26**, 733 (1941).

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

5) S. M. Partridge, *Nature*, **164**, 443 (1949).

heated with 0.02 N oxalic acid solution for two hours in a boiling water bath to hydrolyze selectively the derivatives of 2-*O*-methyl-3,6-anhydro-L-galactose. The resulting product was isolated as a sirup in the usual manner and then extracted seven times each with 15 ml. of *n*-hexane at 50°C. The extracts were combined and evaporated to dryness, an anomeric mixture of methyl 2,4,6-tri-*O*-methyl-D-galactosides being obtained as crystals; yield, 0.50 g., m. p. 44～60°C, $[\alpha]_D^{27}$ $+81.6°$ in water (*c*. 0.38), OCH_3 found: 51.67% (calcd. for $C_6H_8O_2(OCH_3)_4$: 52.52%). The methyl glycoside was identified by conversion in the usual manner into the corresponding reducing sugar⁽⁶⁾, m. p. and mixed m. p. 109～110°C, $[\alpha]_D^{26}$ $+116° \rightarrow +90.1°$ in water (*c* 0.81), and its aniline derivative, m. p. and mixed m. p. 174°C, $[\alpha]_D^{26}$ $-103°$ (initial) in acetone (*c* 0.40).

b) Identification of 2-O-methyl-3,6-anhydro-L-galactose.—The residual sirup, which had been separated from the methyl tri-*O*-methyl-D-galactoside by extraction with *n*-hexane as mentioned above was dissolved in acetone (5 ml.) and filtered. Evaporation of the filtrate afforded 2-*O*-methyl-3,6-anhydro-L-galactose⁽¹⁾ (0.65 g.), which seemed to be still contaminated with appreciable quantities of the methyl tri-*O*- methyl-D-galactoside as indicated by its positive value of the optical rotation $[\alpha]_D^{27}$ $+24.1°$ in water (*c* 0.83) and its high methoxyl value 28.43% (calcd. for $C_6H_9O_4(OCH_3)$: 17.59%). But, without further purification, the sugar was oxidized with bromine water in the usual manner, and the resulting 2-*O*-methyl-3,6-anhydro-L-galactonic acid was crystallized from ethyl acetate; yield, 0.41 g., m. p. and mixed m. p. 141～142°, $[\alpha]_D^{25}$ $-70.8°$ in water (*c* 0.72). One of the present authors has reported m. p. 142°C and $[\alpha]_D^{12}$ $-70.0°$ in water for 2-*O*-methyl-3,6-anhydro-L-galactonic acid⁽⁷⁾.

Hexa-*O*-methylagarobiose Dimethylacetal from I.—The dimethylacetal (I) (1.0 g.) was methylated twice with methyl iodide (15 g.) and silver oxide (10 g.) in the usual manner, giving hexa-*O*-methylagarobiose dimethylacetal as a colorless sirup; yield, 1.0 g., $[\alpha]_D^{10}$ $-21.6°$ in water (*c* 1.11), $[\alpha]_D^{10}$ $-37.3°$ in methanol (*c* 1.10), $[\alpha]_D^{10}$ $-40.5°$ in chloroform (*c* 1.11), n_D^{25} 1.4611, OCH_3 found: 54.18% (calcd. for $C_{12}H_{16}O_5(OCH_3)_6$: 54.61%). One of the present authors has reported $[\alpha]_D^{23}$ $-11.09°$ in water, $[\alpha]_D^{23}$ $-29.46°$ in chloroform and n_D^{25} 1.4632 for hexa-*O*-methylagarobiose dimethylacetal⁽⁸⁾.

Methanolysis of Hexa-*O*-methylagarobiose Dimethylacetal.—Hexa-*O*-methylagarobiose dimethylacetal (0.80 g.) obtained above was subjected to methanolysis with 3% methanolic hydrogen chloride (20 ml.) for twenty five hours. The hydrogen chloride was removed by neutralization with silver carbonate and filtration, and the filtrate on evaporation afforded a mixture of methyl 2,3,4,6-tetra-*O*-methyl-*α*,*β*-D-galactosides and 2,5-di-*O*-methyl-3,6-anhydro-L-galactose dimethylacetal; yield, 0.85 g., $[\alpha]_D^{10}$ $+48.0°$ in water (*c* 1.00).

The mixture was separated at 10～13°C on a column (3.7×11 cm.) packed with charcoal and Celite into four fractions shown in Table II. Fraction I was identified as 2,5-di-*O*-methyl-3,6-anhydro-L-galactose dimethylacetal and fractions III and IV were done as methyl 2,3,4,6-tetra-*O*-methyl-*α*,*β*-D-galactosides as described below; while, fraction II seemed to be a mixture of the two components.

TABLE II. SEPARATION OF THE PRODUCTS OF METHANOLYSIS OF HEXA-*O*-METHYLAGAROBIOSE DIMETHYLACETAL

Fraction	Developer %*	Developer l.	Yield g.	$[\alpha]_D^{13}$ (°)H_2O	n_D^{25}	OCH_3 %
I	7.5	2.0	0.20	− 30.9	1.4547	52.31
II	10	2.0	0.18	+ 36.0	1.4531	55.41
III	10	2.5	0.12	+180.0	1.4489	61.88
IV	12.5	2.0	0.06	+180.0	1.4488	61.85

* Concentration of ethanol in water.

a) Identification of 2,5-O-methyl-3,6-anhydro-L-galactose Dimethylacetal.—The properties of fraction I in Table II agree with those of 2,5-di-*O*-methyl-3,6-anhydro-L-galactose dimethylacetal, which have been recorded by the present authors as: $[\alpha]_D^{17}$ $-6.90°$ ⁸⁾ or $[\alpha]_D^{19}$ $-6.67°$ ⁹⁾ in water and n_D^{25} 1.4533⁸⁾ or n_D^{20} 1.4563 ⁹⁾.

For confirmative identification, the dimethylacetal (0.15 g.) was converted into 2,5-di-*O*-methyl-3,6-anhydro-L-galactonic acid by hydrolyzing with 3% hydrobromic acid for five minutes in a boiling water bath and then oxidizing with bromine in the usual manner. The resulting carboxylic acid (0.16 g.) was purified by recrystallization from ethyl acetate; m. p. and mixed m. p. 160～161°C, $[\alpha]_D^{12}$ $-62.2°$ in water (*c* 0.90). The present authors⁸·⁹⁾ have reported m. p. 161°C, $[\alpha]_D^{23}$ $-54.32°$ in water and $[\alpha]_D^{21}$ $-61.9°$ in water for the compound.

Found: C, 46.45; H, 6.88; OCH_3, 30.38. Calcd. for $C_6H_8O_4(OCH_3)_2$: C, 46.60; H, 6.84; OCH_3, 30.12%.

b) Identification of Methyl 2,3,4,6-tetra-O-methyl-D-galactoside.—Fractions III and IV in Table II agree in properties with methyl 2,3,4,6-tetra-*O*-methyl-D-galactoside. The fractions were combined and hydrolyzed with 1 N sulfuric acid in the usual manner to give the corresponding reducing sugar, which was crystallized from *n*-hexane; m. p. and mixed m. p. 70～72°C, $[\alpha]_D^{12}$ $+144.4°$ (initial) → $+120°$ (final) in water (*c* 0.45).

Found: C, 50.41; H, 8.51; OCH_3, 52.45. Calcd. for $C_6H_8O_2(OCH_3)_4$: C, 50.83; H, 8.53; OCH_3, 52.52%.

Methyl Tetra-*O*-methyl-*β*-agarobioside (II).—Fractions VII and VIII in Table I crystallized completely and melted at 143～144°C. These were combined and recrystallized twice from ethyl acetate, affording the pure sample of compound II in prisms; m. p. 143～144°C, $[\alpha]_D^{10}$ $+41.7°$ in water (*c* 1.15), $[\alpha]_D^{15}$ $+23.6°$ in methanol (*c* 1.10) and $[\alpha]_D^{10}$ $+35.4°$ in chloroform (*c* 1.13).

Found: C, 51.67; H, 7.53; OCH_3, 39.51; mol.

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6) C. Araki, *J. Chem. Soc. Japan* (*Nippon Kwagaku Kwaishi*), **58**, 1362 (1937); E. G. V. Percival and J. C. Somerville, *J. Chem. Soc.* **1937**, 1615.

7) C. Araki, *J. Chem. Soc., Japan* (*Nippon Kwagaku Kwaishi*), **61**, 775 (1940).

8) C. Araki, ibid., **65**, 533, 627 (1944).

9) S. Hirase, This Bulletin, **30**, 75 (1957).

wt., 394. Calcd. for $C_{12}H_{15}O_5(OCH_3)_5$: C, 51.77; H, 7.67; OCH_3, 39.35%; mol. wt., 394.4.

Conversion of the Methyl Glycoside (II) into the Dimethylacetal (I).—When methyl tetra-*O*-methyl-β-agarobioside (0.20 g.) obtained above was heated in 3% methanolic hydrogen chloride (20 ml.), the specific rotation of the solution changed from +14° to −29° within five minutes, at the end of which time the hydrochloric acid was removed by neutralization with silver carbonate and filtration. Evaporation of the filtrate gave a sirupy mixture of the dimethylacetal (I) and the starting material; yield, 0.20 g., $[\alpha]_D^{10}$ −11.3° in water (*c* 0.53). The mixture was then resolved into the components on a column (2.7×11 cm.) of charcoal and Celite. The dimethylacetal was eluted from the column with 30% ethanol in water and crystallized from ether containing a few drops of petroleum ether; yield, 0.14 g., m. p. 88∼89°C, $[\alpha]_D^{10}$, −25.0° in water (*c* 0.80). Admixture with a sample of the dimethylacetal (I) described earlier showed no depression of the melting point. Subsequent elution of the column with 50% ethanol in water led to recovery of a small amount of the starting material.

Methyl Penta-*O*-methyl-β-agarobioside from II.—Methyl tetra-*O*-methyl-β-agarobioside (II) (0.30 g.) was methylated twice with methyl iodide (20 g.) and silver oxide (5 g.) in the usual manner. Extraction of the product with acetone followed by evaporation afforded methyl penta-*O*-methyl-β-agarobioside (0.30 g.), which was then recrystallized by dissolving in a small volume of ether and adding petroleum ether; m. p. 98∼100°C, $[\alpha]_D^{10}$ +48.1° in water (*c* 1.04). One of the present authors has reported m. p. 99∼100°C and $[\alpha]_D^{27}$ +48.32° in water for the methylated disaccharide derivative[3] previously isolated from methylated agarose. Admixture of the two showed no depression of the melting point.

Found: C, 52.85; H, 7.72; OCH_3, 45.68. Calcd. for $C_{12}H_{14}O_4(OCH_3)_6$: C, 52.93; H, 7.90; OCH_3, 45.59%.

Tetra-*O*-methylagarobiose.—*a) From the Dimethylacetal (I).*—The dimethylacetal (I) (0.20 g.) in 0.02 N aqueous oxalic acid solution (20 ml.) was heated in a boiling water bath for one and a half hours, during which time the specific rotation of the solution changed from an initial value −25° to a constant value +12°. The solution was neutralized with calcium carbonate, filtered and evaporated to a sirup, which was then extracted with acetone. Filtration and evaporation of the extract afforded tetra-*O*-methyl-agarobiose as glass; yield, 0.17 g., $[\alpha]_D^{13}$ −15.1° (constant) in water (*c* 0.86), OCH_3 found: 41.65% (calcd. for $C_{12}H_{16}O_6(OCH_3)_4$: 42.07%). It was soluble in water and organic solvents except ether and petroleum ether. It slowly reduced the Fehling solution at room temperature and restored the color of Schiffs reagent. Attempt to prepare a crystalline aniline derivative was unsuccessful.

b) From methyl glycoside (II).—The methyl glycoside (II) (0.20 g.) was hydrolyzed with 0.02 N oxalic acid solution in a boiling water bath for one and a half hours, during which time the specific rotation of the solution changed from an initial value +41° to a constant value −12°, the final value being in exact agreement with that observed for the hydrolysis of dimethylacetal (I). The eesulting tetra-*O*-methylagarobiose was isolated as glass in exactly the same manner as described above; yield, 0.19 g., $[\alpha]_D^{13}$ −15.6° (constant) in water (*c* 0.64), OCH_3 found 41.69%.

The authors wish to express their hearty thanks to Mr. K. Arai for micro-combustion and to Mr. T. Itô for assistance.

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