Methanolysis of Fucoidan. II. The Presence of Sugars Other Than L-Fucose

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Fucoidan from *Macrocystis pyrifera* was depolymerized by methanolysis. After subsequent hydrolysis, L-fucose and, in small amounts, D-galactose and D-xylose were the only compounds isolated. A trace of mannose, but no uronic acids could be detected chromatographically. From unpurified as well as from purified samples of fucoidan, the proportion of D-galactose was about 6.2% of the L-fucose content. The proportion of D-xylose varied between 0.5 and 2.5%, depending on the degree and on the method of purification. From the data obtained, it is concluded that the major part of the fucoidan from *Macrocystis pyrifera* consists of a heteropolymer with L-fucose and D-galactose residues in a ratio of approximately 18:1. D-Xylose occurs in another polysaccharide, which seems to be present in smaller proportion. The presence of a pure fucan sulfate as a major constituent is unlikely. The mannose may be considered to be an impurity.

Fucoidan was described by Kylin[2a] as early as 1913 as a polysaccharide consisting mainly of fucose units. Later, the major constituent was found to be L-fucose and evidence for the presence of sulfate groups[2b] was obtained. Conchie and Percival[3] in 1950 explained details of the structure by methylating fucoidan and identifying the methylated sugars obtained after hydrolysis. They came to the conclusion that fucoidan is a fucan composed of α-(1→2)-linked L-fucose residues which are sulfated in the 4-position. Each fifth residue probably has a short branch in the form of another L-fucose unit. Percival and Ross[4] also identified and determined uronic acid, galactose, and xylose chromatographically from a highly purified sample of fucoidan from *Himanthalia lorea*. The quantities found were 3.3% of uronic acid, 4.1% of galactose, and 1.5% of xylose, besides 56.7% of L-fucose as the major compound. However, they considered galactose, xylose, and uronic acid to be impurities and not part of the fucoidan molecule.

In later years, the presence of small proportions of these sugars in other species of brown seaweeds has been established. Dillon, *et al.*,[5] detected galactose in *Ascophyllum nodosum*, and Dewar[6] identified D-galactose and D-xylose from *Fucus vesiculosus* as crystalline derivatives and obtained chromatographic evidence for the presence of small proportions of mannose and glucose. Mannose had been identified before by Manske[7] in the same seaweed. In 1957, Takemori[8] reported the isolation of five polysaccharide sulfates, with L-fucose as the major constituent, from three different species of brown seaweed. One was a pure fucan sulfate; the others contained a few other sugars, including D-galactose and D-xylose. In an effort to prepare highly purified samples of fucoidan in order to study their influence on blood coagulation, Schuler and Springer[9] obtained a polysaccharide sulfate composed of L-fucose and another (unidentified) 6-deoxyhexose.

Because fucoidan has a great tendency to retain salts and other impurities, even after several reprecipitations (or purifications by other methods), it cannot be readily decided whether the sugars present in small proportions are impurities or part of the fucoidan molecule. Although only relatively few species of brown seaweeds have been investigated in this respect, it seems that galactose and xylose are generally associated with fucoidan, but the presence of the other sugars depends to a large degree on the species of alga.

Very little work has been done on fucoidan from *Macrocystis pyrifera*, a major brown seaweed which is used for preparing commercial quantities of alginic acid and alginates. Methanolysis of fucoidan from this source has been described in the preceding paper.[10] Under the conditions applied the polysaccharide is completely depolymerized, forming methyl glycosides and, in the case of acidic material, methyl esters. The solution of these derivatives can be readily freed from salts and other impurities without noticeable losses or chemical changes of the reaction products. After subsequent hydrolysis with sulfuric acid, most of the L-fucose can be crystallized and removed. The filtrate contains the rest of the L-fucose and all other constituents in the form of free sugars or acids. Chromatographic evidence of the presence of galactose and xylose (and, in a few samples, a trace of mannose) was obtained. There was no evidence of uronic acids. D-Galactose and D-xylose could be isolated and identified in crystalline form after separation on a cellulose column. The reaction was carried out with several samples of fucoidan which had been purified by different methods and to different degrees. L-Fucose, D-galactose, and

(1) Presented before the Division of Carbohydrate Chemistry, 142nd National Meeting of the American Chemical Society, Atlantic City, N. J., September, 1962.

(2) (a) H. Kylin, *Z. Physiol. Chem.*, **83**, 171 (1913); **94**, 337 (1915). (b) G. Lunde, E. Heen, and E. Öy, *ibid.*, **247**, 189 (1937); C. M. Bird and P. Haas, *Biochem. J.*, **25**, 403 (1931); W. L. Nelson and L. H. Cretcher, *J. Biol. Chem.*, **94**, 147 (1931).

(3) J. Conchie and E. G. V. Percival, *J. Chem. Soc.*, 827 (1950).

(4) E. G. V. Percival and A. G. Ross, *ibid.*, 717 (1950).

(5) T. Dillon, K. Kristensen, and C. O'hEochdha, *Proc. Roy. Irish Acad., Sect. B*, **55**, 189 (1953).

(6) E. T. Dewar, *Chem. Ind.* (London), 785 (1954).

(7) R. H. F. Manske, *J. Biol. Chem.*, **86**, 571 (1930).

(8) S. Takemori, *Hirosaki Igaku*, **8**, 749 (1957).

(9) W. Schuler and G. F. Springer, *Naturwissenschaften*, **44**, 265 (1957).

(10) R. G. Schweiger, *J. Org. Chem.*, **27**, 4267 (1962).

TABLE I

AMOUNTS OF SUGARS[a] OBTAINED BY HYDROLYSIS

Sample of fucoidan	D-Galactose	D-Xylose	Mannose
Unpurified	0.658	0.145	...
Unpurified	.613	.172	Trace
Reprecipitated twice	.607	.052	...
Reprecipitated four times (not filtered through cotton)	.647	.101	(Trace ?)
Purified with formaldehyde	.628	.226	...
Purified with filter aid	.621	.055	...
Treated twice with filter aid	.591	.110	Trace

[a] In g./10 g. of L-fucose.

D-xylose were determined quantitatively, and the results are shown in Table I.

It may be noted that the proportion of D-galactose relative to L-fucose does not vary essentially and is, within experimental error, the same for unpurified as for all purified samples. The proportion of D-xylose, however, varies greatly, depending on the degree and on the method of purification. Reprecipitations usually lower the D-xylose content, and treatment with formaldehyde increases it.

This, assuming that different polymers have different solubilities, indicates that the fucoidan samples tested contain more than one polysaccharide. Otherwise, the D-xylose content would vary only within experimental error. The principal constituent apparently is a sulfated galactofucan. The presence of a very small proportion of D-xylose (<0.5%) in this polymer is unlikely but cannot be positively excluded by the data obtained. The ratio of L-fucose to D-galactose is approximately 18:1 if it is assumed that the percentage loss during all reaction and purification steps is the same, or at least very similar, for both sugars.

The D-xylose originates from another polysaccharide which either is present in only small proportion or which contains (besides D-xylose) L-fucose and D-galactose in a ratio similar to that of the galactofucan above. Consequently, removal of part of the polymer from the mixture would not change the relative D-galactose content. For the same reason, the presence of a pure fucan sulfate as a major constituent can be excluded.

Mannose could be identified chromatographically in trace amounts in only two samples and must be considered to be an impurity.

Experimental

Chromatography.—For the paper-chromatographic examinations, Whatman No. 1 filter paper was used; for separation on a column, Whatman cellulose powder, standard grade, was used. Irrigant A was a volumetric mixture of ethyl acetate–acetic acid–formic acid–water (18:3:1:4); irrigant B, a mixture of butyl alcohol–ethanol–water (40:11:19), and irrigant C, 2-butanone saturated with water containing 1% of ammonia. Spray reagents applied were: I, aniline hydrogen phthalate[11]; II, ammoniacal silver nitrate[12]; and III, permanganate–periodate.[13]

Isolation and Purification of Fucoidan.—Fucoidan was isolated as described in the preceding paper[10] by slowly pouring the exudate of freshly harvested *Macrocystis pyrifera* through a sieve into 1 to 1.5 vol. of isopropyl alcohol with stirring. The soft, fibrous precipitate was removed and hardened in fresh isopropyl alcohol; it then was dried at 45° in the presence of an air stream, ground, and extracted in a Soxhlet extractor with 90% methanol for 48 hr. This material was used as unpurified fucoidan.

For purification, the following methods were applied using 300 g. of unpurified fucoidan each time. (a) The crude product was reprecipitated twice by dissolving it in 4 l. of water, filtering the solution through cotton, and slowly pouring the filtrate into 1.5–2.0 vol. of isopropyl alcohol with stirring. The resulting product was extracted in a Soxhlet extractor with 90% methanol and dried; yield, 90 g. (b) Crude fucoidan was reprecipitated four times as described above, but filtration through cotton was omitted; yield 50 g. (c) Fucoidan was dissolved in water and about 160 g. of filter aid[14] was stirred into it as described previously by Percival and Ross.[4] After refrigeration for 24 hr. it was centrifuged to remove the solids. The purified product was precipitated by adding the solution to isopropyl alcohol with stirring; then it was extracted with 90% methanol for 24 hr.; yield, 130 g. (d) Another portion of fucoidan was treated twice with filter aid; yield 66 g. (e) Crude fucoidan was dissolved in 3 l. of water, 90 ml. of 40% formaldehyde was added, and the mixture was evaporated to dryness *in vacuo* (see Black, *et al.*[15]). The glassy residue was kept at 50° for 1 hr., dissolved in water, and centrifuged to remove an insoluble residue. The purified product was isolated by pouring the solution into isopropyl alcohol; then it was extracted with 90% methanol for 24 hr.; yield, 110 g.

TABLE II

ANALYSES[a]

	Unpurified fucoidan	Purified fucoidan (a)	(b)	(c)	(d)	(e)
L-Fucose[b]	23.9	24.9	25.0	30.8	33.5	30.5
Sulfate	18.02	18.86		19.6	19.5	18.93
Ash	34.9	32.0		33.6	38.2	31.5
Ca (as CaO)				1.2	1.6	1.4

[a] All samples were dried for 1 hr. over calcium chloride at 80° *in vacuo*. [b] See Black, *et al.*[16]

Methanolysis of Fucoidan.—Unpurified fucoidan (75 g.) was suspended in 500 ml. of methanol which contained 24 g. of hydrogen chloride, and the mixture stirred in a Pfaudler pressure-reaction apparatus for 22 hr. at 60° (see the preceding paper[10]). The mixture was filtered through a fritted-glass funnel, the clear, brown filtrate stirred with 180 g. of lead carbonate for 4 hr., the salts removed, and the solution concentrated *in vacuo* to a thin sirup which was diluted with hot water. A solution of ammonium carbonate was added until formation of a white precipitate was complete, the mixture filtered, the filtrate passed through columns of anion- and cation-exchange resins until neutral, and the effluent treated with activated carbon and concentrated *in vacuo* to a smaller volume. It was refluxed with *N* sulfuric acid, the sulfuric acid was removed as barium sulfate, and the solution was concentrated to a sirup.

(11) S. M. Partridge, *Nature*, **164**, 443 (1949).
(12) S. M. Partridge, *ibid.*, **158**, 270 (1946).
(13) R. U. Lemieux and H. F. Bauer, *Anal. Chem.*, **20**, 920 (1954).
(14) The filter aid used was Perlite purchased from Harborlite Corp., Escondido, Calif.
(15) W. A. P. Black, E. T. Dewar, and F. N. Woodward, *J. Sci. Food Agr.*, **3**, 122 (1952).
(16) W. A. P. Black, W. J. Cornhill, E. T. Dewar, E. G. V. Percival, and A. G. Ross, *Soc. Chem. Ind.* (London), **69**, 317 (1950).

The sirup was diluted with methanol, ether was added, and the mixture was refrigerated for crystallization. Crystals occasionally were removed from the sides of the flask. After 8–12 days, the crystallizate was filtered off, washed with a cold mixture of ethanol and ether, and dried in a desiccator; yield, 7.6 g., $[\alpha]^{20}D$ −75.3° (*c* 1.3, water), m.p. 136–139°, undepressed when mixed with authentic L-fucose.

The noncrystallizing syrup was chromatographically examined, using irrigants A, B, and C and spray reagents I, II, and III. Only three spots could be detected; they were identical with those for fucose, galactose, and xylose.

The other samples of fucoidan were treated with methanolic hydrogen chloride under the same conditions. The yields of crystalline L-fucose usually were very similar. The noncrystallizing residues from all samples showed evidence of the presence of fucose, galactose, and xylose. In addition to those sugars, one experiment using unpurified fucoidan and another one using quadruply reprecipitated fucoidan gave evidence of the presence of a trace of mannose.

Identification of D-Galactose and D-Xylose.—The noncrystallizing residue was placed on a cellulose column, 55 × 650 mm., and irrigant A was applied. The first fraction contained only L-fucose; this was followed by fractions containing a mixture of L-fucose and D-xylose, then of D-xylose only, and finally of D-galactose. The fractions containing D-xylose and D-galactose were concentrated separately to sirups, diluted with water, and treated with some Amberlite IR-120-(H^+) cation-exchange resin and activated carbon. After filtration, they were concentrated *in vacuo* to a very small volume. Ethanol was added and both solutions were kept in the refrigerator for crystallization. The crystals were filtered off on a fritted-glass funnel, washed with a cold mixture of ethanol and water, and dried in a desiccator over calcium chloride.

The hexose had $[\alpha]^{20}D$ + 79.2 ± 1.3° (*c* 1.4, water), m.p. 161–163° and, after recrystallizing from ethanol–water, 165–166°, undepressed when admixed with authentic D-galactose.

The data for the pentose were $[\alpha]^{20}D$ +17.5 ± 2.3° (*c* 0.6, water), m.p. 140–142.5°, undepressed when admixed with authentic D-xylose.

Quantitative Determinations.—The fractions containing L-fucose were concentrated *in vacuo*. The sirup obtained was diluted with water, treated with some Amberlite IR-120-(H^+) and activated carbon, filtered, concentrated to a smaller volume, and adjusted to 100 ml. with water. L-Fucose was determined by oxidation with Fehling solution (see Lane and Eynon[17]). The table values were corrected for L-fucose, 0.74 g. of L-fucose having the reducing power of 1 g. of D-glucose.

The fractions containing D-galactose were concentrated to a sirup and, after dilution with water and treatment with activated carbon and Amberlite IR-120-(H^+), adjusted to 100 ml. with water. D-Galactose was determined by oxidation with Fehling solution. The reducing power of 0.855 g. of D-galactose was found to be equivalent to that of 1 g. of D-glucose, and the values were corrected on this basis.

All fractions containing D-xylose and a mixture of D-xylose and L-fucose were combined, concentrated to a sirup, diluted with water, treated with Amberlite IR-120-(H^+) and activated carbon, and adjusted to 100 ml. with water. L-Fucose was determined as previously described.[16] The amount of D-xylose was found by subtraction of the calculated reducing power of L-fucose from the total reducing power determined by oxidation with Fehling solution. These values were, however, reliable only if the quantities of D-xylose and L-fucose were of a similar order of magnitude. The yields of D-xylose given in Table I were obtained by a chromatographic method. Spots of five different, known mixtures of D-xylose and L-fucose were placed on a paper strip together with a spot of the sample to be determined. The paper was developed in irrigant A and sprayed with spray reagent I. The size of the red spots from D-xylose was determined by cutting out the spots and weighing them. The weight was plotted on a curve against the amount of D-xylose, and the value for the unknown yield of D-xylose was taken from this curve. For each sample, five chromatograms were prepared to obtain one average value. The accuracy of the method was tested by using known amounts of D-xylose and L-fucose instead of the unknown samples. The method was found to be reliable within a limit of error of about ±7%.

(17) J. H. Lane and L. Eynon, *Soc. Chem. Ind.* (London), **42**, 32T–37T (1923).

A New Type of Smiles Rearrangement of *N*,*N*-Dialkyl-*N'*-[2-(*o*-bromophenoxy)phenyl]-1,3-propanediamines to 2-[*N*-(3-Dialkylaminopropyl)-*o*-bromoanilino]phenols[1a]

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A new type of Smiles rearrangement of *N*,*N*-dialkyl-*N'*-[2-(*o*-bromophenoxy)phenyl]-1,3-propanediamines (III) to the isomeric 2-[*N*-(3-dialkylaminopropyl)-*o*-bromoanilino]phenols (IV) has been studied in some detail. Cyclization of the latter constitutes a new ring closure by which 10-(3-dialkylaminopropyl)phenoxazines have been obtained. Phenoxazine analogs of chlorpromazine, methoxypromazine, and prochlorperazine were prepared.

The first paper in this series[1b] described two new syntheses of 2-chloro-10-(3-dimethylaminopropyl)-phenoxazine (Ia, Table I). These methods were designated as the *o*-phenoxyaniline route (A_1) and the modified Turpin reaction (route B). We now wish to report a novel "Smiles type" rearrangement of *N*,*N*-dialkyl-*N'*-[2-(*o*-bromophenoxy)-phenyl]-1,3-propanediamines (III) to the isomeric 2-[*N*-(3-dialkylaminopropyl)-*o*-bromoanilino]-phenols (IV). These rearranged products (IV) also afforded the desired phenoxazines (I) on ring closure; this is designated the 2-(*o*-bromoanilino)-

(1) (a) Presented in part before the Division of Organic Chemistry at the 142nd National Meeting of the American Chemical Society, Atlantic City, N. J., September 9–14, 1962; (b) G. E. Bonvicino, L. H. Yogodzinski, and R. A. Hardy, Jr., *J. Org. Chem.*, **26**, 2797 (1961).