zations, the derivative was cyclized by refluxing for 5 minutes 100 mg. in 3 ml. of 80% ethanol containing a drop of concd. hydrochloric acid. The second derivative of the methone, thus prepared, melted at 174.5–175.5° without crystallization. A mixed m.p. with an authentic sample of the second methone derivative of acetaldehyde showed no depression. The acetaldehyde was also recovered and identified in the form of its 2,4-dinitrophenylhydrazone.

The original residue which was left after the ethyl acetate distillation was dissolved in ethyl acetate (15 ml.) and the solution was extracted with 25 ml. of water. From the aqueous layer 50 mg. of the tetramethone of malondialdehyde was obtained, m.p. 243–244°. The total yield of the tetramethone of malondialdehyde was 310 mg. (30%).[19]

The aromatic fragment obtained from the ethyl acetate layer was oxidized with potassium permanganate (0.5 g.) and the resulting oily acid was converted into its amide in 42% over-all yield.[19] The m.p. of the pure sample was 96°, and a mixed m.p. with an authentic sample[4b] of ω-(2,3-dimethoxyphenyl)-capryl amide was 96–97°. In an alternate experiment, the resulting acid was characterized as its anilide, m.p. 91–92°. A mixed m.p. with an authentic sample of ω-(2,3-dimethoxyphenyl)-capryl anilide showed no depression.

Anal. Calcd. for $C_{22}H_{29}NO_3$: C, 74.33; H, 8.22; N, 3.94. Found: C, 74.18; H, 8.25; N, 4.09.

Maleic Anhydride Adduct of the Triolefin.—A solution of 1.5 g. (0.0044 mole) of the triolefin, 0.48 g. (0.0049 mole) of maleic anhydride and a trace of hydroquinone in 25 ml. of benzene was refluxed for 24 hours under a nitrogen atmosphere. The solvent was distilled off and the unreacted maleic anhydride was removed by sublimation under reduced pressure. The yellowish oily residue was dissolved in benzene (10 ml.) and ligroin was then added to precipitate the adduct. The solvent was removed by decantation and the gummy precipitate when stirred into petroleum ether soon solidified. The yield was 0.85 g. (43%). It was crystallized from benzene–ligroin. The product, however, did not melt sharply. The m.p. of the compound was 95–100°. It was therefore extracted (soxhlet) with ligroin for 5 hours to remove any soluble impurities, especially traces of unreacted triolefins.

Ozonolysis of the Adduct.—A solution of 0.75 g. (0.0017 mole) of the maleic anhydride adduct was cooled to −80°, and ozonized as previously described. The resulting ozonide was reduced catalytically over palladium-on-calcium carbonate. It absorbed 54% of the theoretical amount of hydrogen. The ethyl acetate solution of the reduced ozonide was extracted with 40 cc. of water. To the aqueous layer was added a freshly prepared solution of 0.4 g. of 2,4-dinitrophenylhydrazine. There was no evidence of any hydrazone formation and all attempts to isolate the hydrazone of acetaldehyde were unsuccessful. The ethyl acetate layer was concentrated on a steam-bath to a yellowish oily residue, which was dissolved in acetone (25 ml.) and oxidized with potassium permanganate (0.5 g.). The resulting acidic material, subsequently identified as ω-(2,3-dimethoxyphenyl)-caprylic acid, was isolated as previously described. It was identified by converting into the amide which after two recrystallizations from ligroin melted sharply at 95–96° (20 mg.). A mixed m.p. with an authentic sample of ω-(2,3-dimethoxyphenyl)-capryl amide showed no depression.

NEW YORK, N. Y.

[CONTRIBUTION FROM THE MARITIME REGIONAL LABORATORY, NATIONAL RESEARCH COUNCIL OF CANADA]

Degradative Studies on Fucoidin[1]

BY A. N. O'NEILL

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Fucoidin, prepared from *Fucus vesiculosus*, was subjected to mild acetolysis and the material recovered from the acetolysate was converted to a mixture of acetylated alditols. Chromatographic resolution of this mixture on Magnesol has led to the isolation in crystalline form of L-fucitol pentaacetate and the acetylated derivative of a reduced disaccharide. This disaccharide derivative has been converted to the crystalline free sugar alcohol. This has been defined by analysis and periodate oxidation as 2-α-L-fucopyranosyl-L-fucitol, thereby providing further evidence for the presence of 1,2-glycosidic linkages in fucoidin.

Fucoidin is the chief polysaccharide sulfate ester of the *Phaeophyceae* where it occurs together with alginic acid, laminarin and mannitol. It was first described by Kylin[2] who isolated the polysaccharide from various species of *Laminaria* and *Fucus* by extraction with dilute acetic acid and proved the presence of L-fucose in a hydrolysate by isolating the phenylosazone. Bird and Haas[3] obtained crude fucoidin by aqueous extraction of the fronds of *Laminaria digitata* followed by precipitation with ethanol. Purification yielded a product containing 30.9% ash and 30.3% sulfate. Since the total sulfate found on hydrolysis was approximately double that found in the ash, fucoidin was considered to be the salt of a carbohydrate half ester of sulfuric acid.

More recent structural studies have been carried out on this polysaccharide.[4-6] It was noted by Percival and Ross[5] that, even after drying at 40° and a pressure of 0.1 mm. for a considerable length of time, fucoidin still retained 9.4% water and 6% alcohol. After correction for adsorbed solvents their analysis was as follows: sulfate, 38.3%; metals, 8.2%; uronic acid, 3.3%; fucose, 57%; galactose, 4.1%; xylose, 1.5%. The calcium salt of a fucan monosulfate would give sulfate, 39.2%; calcium, 8.2%; fucose, 66.9%. Fucoidin was believed to be a fucan monosulfate and that constituents other than fucose resulted from impurities in their preparation.

Methylation studies[6] combined with a study of the stability of the sulfate residue to alkaline hydrolysis indicated that fucoidin could be represented by a chain of L-fucopyranose units joined by α-glycosidic linkages through carbon atoms one and two of adjacent units, each fucopyranose unit carrying a sulfate on carbon four. From the data on methylation it is obvious that there is a high proportion of other linkages present.

In the present investigation an attempt has been made to define this polysaccharide more accurately through a study of the products resulting on partial hydrolysis.

Fucoidin was prepared from fresh *Fucus vesiculosus* by the method of Black, Dewar and Wood-

(1) Issued as N.R.C. No. 3384.
(2) H. Kylin, *Z. physiol. Chem.*, **83**, 171 (1913); **94**, 357 (1915).
(3) G. M. Bird and P. Haas, *Biochem. J.*, **25**, 403 (1931).
(4) G. Lunde, E. Heen and E. Oy, *Z. physiol. Chem.*, **247**, 189 (1937).
(5) E. G. V. Percival and A. G. Ross, *J. Chem. Soc.*, 717 (1950).
(6) J. Conchie and E. G. V. Percival, *ibid.*, 827 (1950).

ward[7] and subjected to a mild acetolysis similar to that employed successfully with cellulose.[8] The material isolated from the acetolysate was deacetylated, reduced catalytically to the corresponding mixture of sugar alcohols and reacetylated. The reduction was done to eliminate the anomers and provide a less complex mixture which would be more easily separated into crystalline components.[9,10] The resulting mixture of acetylated alditols was resolved chromatographically on columns of Magnesol–Celite[11] employing benzene–*t*-butyl alcohol as developer. The main product was 1,2,3,4,5-penta-O-acetyl-L-fucitol whose identification was effected by melting point, mixed melting point, specific rotation and X-ray diffraction. From the more highly adsorbed material a second crystalline compound was isolated whose molecular weight and analytical data indicated it to be a fully acetylated fucobiitol. This compound was isomorphous, crystallizing both as needles (m.p. 119–120°, $[\alpha]^{24}D$ −81.5° in chloroform) and as small prisms (m.p. 99–102°).

Deacetylation of the crystalline acetate with sodium methoxide yielded the crystalline alditol (m.p. 190–192°, $[\alpha]^{23}D$ −118° in water) which on acid hydrolysis gave one mole each of both L-fucose and L-fucitol per mole of compound.

The crystalline alditol was subjected to oxidation with sodium metaperiodate. Periodate was determined by the arsenite method, acetaldehyde by the method of Nicolet and Shinn,[12] and formic acid as acidity to phenolphthalein[13] after destruction of the excess periodate with ethylene glycol. The acetaldehyde was identified as its dimedone derivative and as its 2,4-dinitrophenylhydrazone. These experiments (Table I) show that the crystalline fucobiitol consumes four moles of periodate and produces essentially two moles of formic acid, one mole of acetaldehyde and no formaldehyde.

TABLE I

OXIDATION OF FUCOBIITOL IN 0.0025 *M* SOLUTION WITH 0.04 *M* SODIUM METAPERIODATE AT 25°

Time, hr.	Moles per mole of substance: Oxidant consumed[a]	Acetaldehyde produced	Formic acid produced
0.5	3.6		
1.0	3.7		1.72
2.0	3.8		
4.0	3.9		1.80
12.0	4.05		1.83
24.0	4.05 (4.00)[b]	1.01 (1.05)	(1.92)
45.0	(4.00)		(1.95)

[a] In the dark. [b] The figures in parentheses represent the results of a duplicate experiment.

These results adequately characterize the crystalline fucobiitol as 2-O-α-L-fucopyranosyl-L-fucitol, the α-linkage being inferred from the high negative rotation of the compound. The isolation of this compound from reduced fucoidin acetolysates provides definitive evidence, on a crystalline basis, for the existence of 1,2-linkages between L-fucopyranose units in this polysaccharide.

(7) W. A. P. Black, E. T. Dewar and F. N. Woodward, *J. Sci. Food Agr.*, **3**, 122 (1952).

(8) E. E. Dickey and M. L. Wolfrom, THIS JOURNAL, **71**, 825 (1949).

(9) R. A. Boissonnas, *Helv. Chim. Acta*, **30**, 1689 (1947).

(10) M. L. Wolfrom, A. Thompson, A. N. O'Neill and T. T. Galkowski, THIS JOURNAL, **74**, 1062 (1952).

(11) W. H. McNeely, W. W. Binkley and M. L. Wolfrom, *ibid.*, **67**, 527 (1945).

(12) B. H. Nicolet and L. A. Shinn, *ibid.*, **63**, 1456 (1941).

(13) Allene Jeanes and C. A. Wilham, *ibid.*, **72**, 2655 (1950).

Experimental

All melting points are corrected.

Preparation of Fucoidin.—*F. vesiculosus* (200 g.), dried and ground to 60 mesh, was extracted three times with 2000 ml. of 0.17 *N* hydrochloric acid at 65° and *p*H 2.2, the *p*H being adjusted before each extraction. The duration of each extraction was 1 hr. and mechanical stirring was used. After each extraction the material was centrifuged and the combined centrifugates were neutralized with sodium hydroxide and evaporated to dryness under reduced pressure at 50°. The dried extract was dissolved in 1250 ml. of water and 540 ml. of ethanol was added (30% by vol.). The resulting precipitate was removed by centrifugation and to the centrifugate ethanol (1340 ml.) was added (60% by vol.). The resulting crude fucoidin was removed by centrifugation, dissolved in 500 ml. of water containing 17.5 ml. of 40% formaldehyde and evaporated to dryness under reduced pressure at 50°. The glassy material so formed was extracted with 700 ml. of hot water and the insoluble residue removed by centrifugation at 15,000 r.p.m. Inorganic ions and alginate were removed by shaking the clear solution with a mixture of exchange resins, Amberlites IR-100 and IR-4b, and the resulting cloudiness was removed along with the resin by filtration. The filtrate was neutralized with sodium carbonate, treated with 1.5 g. of sodium chloride and ethanol added to a concentration of 70%. The fucoidin was recovered by centrifugation. Three additional reprecipitations at the same concentration of ethanol yielded on washing with absolute ethanol and ether and drying *in vacuo* 25.8 g. of an almost white powder. For analysis the fucoidin was further dried *in vacuo* over phosphorus pentoxide at 50° for 18 hr. Material dried in this manner gave: ash 26.1%; sulfate 26.5%; fucose[14] 44.6%; galactose a trace; $[\alpha]^{23}D$ −125° (*c* 0.5, water). Osmotic pressure measurements carried out in 0.1 *M* sodium chloride at 25° using a cellophane membrane, grade PT600, indicated a molecular weight of 133,000 ± 20,000.

Electrophoretic examination of this fucoidin in 1.5% concentration in an acetate buffer of ionic strength 0.1 and *p*H 5.0 indicated two components. The mobilities were 2.3×10^{-4} and 2.5×10^{-4} cm.²/v. sec. Attempts to separate these by other means have not been successful.

Acetolysis of Fucoidin.—Fucoidin (20 g. dried for 48 hr. at 50° *in vacuo* over phosphorus pentoxide) was added slowly, with shaking, to a chilled (4°) mixture of acetic anhydride (96 ml.), glacial acetic acid (64 ml.) and concentrated sulfuric acid (10 ml.). The mixture was allowed to stand at 4° for 30 min. and then shaken mechanically for 17 hr. at room temperature, when all the fucoidin had passed into solution. The clear solution was poured with stirring into 1200 ml. of ice and water, neutralized with sodium bicarbonate and allowed to stand overnight. The precipitated acetolysate material was removed by decantation and dissolved in chloroform. The supernatant liquid was extracted five times with a total of 1000 ml. of chloroform. The combined extracts were dried over anhydrous sodium sulfate and concentrated to a thick sirup by distillation under reduced pressure. The sirup was further dried in a vacuum desiccator; yield 17.2 g.

Deacetylation of the Acetolysate.—The above acetolysate material (17.2 g.) was dissolved in 172 ml. of absolute methanol and treated with 52 ml. of a solution of sodium methoxide (0.50 g. of sodium in 100 ml. of absolute methanol). The mixture was held at 5° with occasional shaking for 18 hr. Sufficient water was added to dissolve the precipitate which had formed and the solution was neutralized with dilute acetic acid. It was concentrated by distillation under reduced pressure to 175 ml.

Preparation of the Mixture of Acetylated Alditols.—The above solution (175 ml.) was reduced with approximately 10 g. of Raney nickel catalyst at 80° and 2000 p.s.i. of hydrogen for 18 hr. The catalyst was removed by filtration and the sirup obtained on solvent removal was dried by repeated distillation of added ethanol under reduced pres-

(14) A. E. Flood, E. L. Hirst and J. K. N. Jones, *J. Chem. Soc.*, 1679 (1948).

sure and finally in a desiccator under reduced pressure; yield 8.4 g.

This colorless amorphous material was acetylated with 5 g. of fused sodium acetate and 100 ml. of acetic anhydride by initiating the reaction at 120° and allowing it to proceed for one hour at 100°. The mixture was cooled, poured with stirring into 900 ml. of ice and water and allowed to stand with occasional stirring for 6 hr. The sirupy mixture was extracted with five 200-ml. portions of chloroform and the extract was washed with cold water until neutral, dried over anhydrous sodium sulfate and finally concentrated by distillation under reduced pressure to a thick sirup which was further dried to an amorphous solid in a vacuum desiccator; yield 13.5 g.

Chromatographic Resolution.—An amount of 6.5 g. of the above mixture of acetylated sugar alcohols was dissolved in 150 ml. of benzene and added at the top of a 265 × 74 mm. (diam.)[15] column of Magnesol–Celite (5:1 by wt.). The chromatogram was developed with 3500 ml. of benzene–*t*-butyl alcohol (100:1 by vol.). An alkaline permanganate streak on the extruded column disclosed four main zones, one at the bottom (A—some of this material had passed into the effluent), a second about the center (B) and two in the top half of the column (C). These were sectioned and eluted with acetone. Solvent removal from the acetone eluate of A left crystalline material. This was combined with that obtained from the effluent on solvent removal and recrystallized from ethanol. It was identified as L-fucitol pentaacetate; yield 3.2 g., m.p. 128–129° unchanged on admixture with authentic L-fucitol pentaacetate synthesized from known L-fucose, $[\alpha]^{23}D$ +21.3° (*c* 2.3, chloroform). These values are in agreement with those reported in the literature for L-fucitol pentaacetate.

The material obtained from the acetone eluate of B crystallized and was recrystallized from ethanol. It was shown to be identical with the material from A by mixed melting point, specific rotation and X-ray diffraction; yield 280 mg.

The sirupy material obtained from C of two such columns was combined and rechromatographed in the same manner by development with 7000 ml. of benzene–*t*-butyl alcohol (100:1 by vol.). Two zones were located on the extruded column and were eluted with acetone. The sirup obtained from the lower zone crystallized from ethanol and was shown by melting point (113–157°) to be exceedingly impure; yield 409 mg. This material was rechromatographed but no further separation was obtained. It was then separated by fractional crystallization from ethanol and the acetylated fucobiitol obtained pure. A small amount of a second crystalline substance also was obtained, but this has not yet been characterized. Acetylated fucobiitol; yield 225 mg., m.p. 119–120°, $[\alpha]D^{24}$ −81.5° (*c* 1.08, chloroform).

Anal. Calcd. for $(C_{12}H_{17}O_9)(CH_3CO)_7$: C, 51.48; H, 6.32; ($CH_3CO$), 11.54 ml. of 0.1 *N* NaOH per 100 mg.; mol. wt., 606. Found: C, 51.42; H, 6.38; (CH_3CO), 11.50 ml.; mol. wt. (Rast), 613.

If left in contact with alcohol for a few days this compound undergoes a change in crystal structure from needles with the above melting point to small prisms which melt at 99–102°.

Preparation of the Crystalline Alditol.—An amount of 255 mg. of the crystalline acetate (m.p. 119–120°) dissolved in absolute methanol (2.6 ml.) was treated with 0.8 ml. of sodium methoxide solution (0.5 g. of sodium in 100 ml. of absolute methanol) for 16 hr. at 5°. The solution was neutralized with dilute acetic acid, diluted with water and deionized by passage through a mixed-bed column of Amberlites IR-120 and IR-4b.[16] Concentration of the solution to dryness by distillation under reduced pressure left a white amorphous solid which was crystallized from ethanol and recrystallized from the same solvent; yield 132 mg., m.p., 190–192°, $[\alpha]^{23}D$ −118° (*c* 0.5, water). Periodate oxidation data on this compound are recorded in Table I.

Hydrolysis.—The above crystalline alditol (15 mg.) was hydrolyzed with 3 ml. of 0.4 *N* hydrochloric acid at 60°. The reaction was followed polarimetrically. The final specific rotation was $[\alpha]^{24}D$ −36.5° which corresponds to an equimolar mixture of L-fucose and L-fucitol. The solution was deionized by passing through a mixed-bed column of Amberlites IR-120 and IR-4b,[16] and concentrated to dryness under reduced pressure. The components were separated by fractional crystallization from ethanol. In this manner there were isolated L-fucitol (4 mg.), m.p. and mixed m.p. with authentic L-fucitol 151–153°, and L-fucose (5 mg.) m.p. 144–145° unchanged on admixture with an authentic specimen.

(15) Adsorbent dimensions.

(16) Products of the Resinous Products Division of Rohm and Haas Co., Philadelphia, Penna.

HALIFAX, N. S., CANADA

[CONTRIBUTION FROM THE SHELL DEVELOPMENT COMPANY]

Peptide Derivatives Containing Two Trifunctional Amino Acids

BY R. F. FISCHER AND R. R. WHETSTONE[1]

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A number of derivatives of histidyltyrosine, histidylserine, histidylglycine, seryltyrosine, tyrosylserine, tyrosylhistidine and serylglycylhistidine have been prepared by adaptation of the Curtius azide route. In the course of the work methods for the preparation of L-histidyl peptides employing carbobenzoxy-L-histidine azide or carbobenzoxyglycyl-L-histidine azide were developed.

There have been many peptides prepared containing several of the simpler amino acids, and a moderate number which have included one of the more complex trifunctional amino acids, but relatively few which include more than one of the trifunctional amino acids. This is especially true for peptides of L-serine and L-histidine, and when this work was undertaken no general method for synthesis of L-histidyl compounds had been described in the literature.

Recently, however, Holley and Sondheimer[2] have reported a synthesis of L-histidyl peptides which is identical in most respects with one employed by us as part of our synthesis program. We are able to confirm the authors' general statements and their physical constants for carbobenzoxy-L-histidine hydrazide and carbobenzoxy-L-histidylglycine hydrazide (Ib). For our purposes we found it more convenient to use carbobenzoxyglycyl-L-histidine hydrazide, since this compound could be prepared in 70% over-all yield. The coupling of the glycyl and histidine groups was accomplished *via* an azide reaction, which avoided the use of acyl halides.

In the normal azide procedure, hydrazides are converted to azides in excess aqueous mineral acid (often with acetic acid present also), whereupon the azides precipitate and are allowed to react with the

(1) Present address: Shell Development Co., Denver, Colo.

(2) R. W. Holley and E. Sondheimer, THIS JOURNAL, **76**, 1326 (1954).