CHARACTERIZATION OF A 4-AMINO-4:6-DIDEOXY ALDOHEXOSE FROM CHROMOBACTERIUM VIOLACEUM*

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A new deoxy amino sugar has been isolated from lipopolysaccharide obtained from Chromobacterium violaceum NCTC 7917 (Smith and Wheat, 1962; Smith, et al., 1962). Another deoxy compound, D-fucosamine (2-amino-2:6-dideoxy-D-galactose) had been previously isolated from this organism by Crumpton and Davies (1958). In contrast to fucosamine, however, the new amino sugar did not react in assays for 2-amino-sugars (Smith, et al., 1962). The new compound has now been obtained in crystalline form making it possible to start structural analyses. Evidence is presented here which indicates the compound to be a 4-amino-4:6-dideoxy-aldohexose.

A preliminary report of this work has been given (Wheat and Rollins, 1962).

EXPERIMENTAL

C. violaceum NCTC 7917 was grown and harvested as previously described (Smith, et al, 1962). "Degraded" polysaccharide, prepared according to Crumpton and Davies (1958), was hydrolyzed 1 hour in 2 N HCl and the new amino sugar isolated and separated from fucosamine by chromatographing twice on long (2 cm x 70 cm) columns of Dowex 50-H essentially as previously described (Smith, et al., 1962 ; Wheat and Rollins, 1962). Following removal of HCl, the compound was dissolved in water and crystallized by addition of a large excess of ethanol (to prevent separation as an oil) followed by diethyl ether. Yield of recrystallized material was about 7% dry weight from degraded polysaccharide.

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The crystalline material migrated as a single compound on paper chromatograms with color reactions and mobilities as previously reported (Smith, et al, 1962). In addition, the following peculiar properties were observed. Upon heating the compound, spotted on paper or paper chromatograms (usually after acidic solvents), a light yellow spot was seen in daylight which fluoresced yellow to yellow-green under ultraviolet light. If chromatograms were sprayed with ninhydrin before heating, a non-fluorescent yellow to orange chromophore appeared (similar to that given by proline and hydroxy proline) which sometimes faded to blue. Material of this nature had been previously recognized as a possible proline derivative on ninhydrin treated chromatograms of cell-wall and lipopolysaccharide hydrolyzates of C. violaceum (R. Barnes and R. Wheat, unpublished), and similar material had been isolated from C. violaceum as a nucleotide conjugate, which appears to be a thymidine derivative (Wheat and Rollins, unpublished).[2]

Elementary analyses[3] (C 35.94% , H 7.37% , N 6.93% , Cl 17.74%) and molecular weight, determined as 191 (vapor pressure osmometer)[3], agreed with the formula $C_6 H_{14} O_4 NCl$ of calculated molecular weight 199.5, which is compatible with the assumption that the compound is a 6-deoxyhexosamine. Mutarotation was observed, initial - 9°, $[\alpha]_D^{27}$ + 21° (c=1, 22 hrs), indicating it to be the β-anomer, presumably pyranose. The compound yielded about 0.2 mole glucose equivalent in the Park-Johnson (1949) assay, and yielded no color (below 10 μmoles) in the Rondle-Morgan (1955) and Levvy-McAllan (1959) assays. Negative results were also obtained at the 1 μmole level in the malondialdehydethiobarbituric acid reaction (Warren, 1959), as well as ketose, methyl pentose, anthrone and Molisch tests (Ashwell, 1959). A terminal aldehyde group was indicated by recovery of 0.82 moles aldose function per mole by hypoiodite oxidation (MacLeod and Robison, 1929) in 0.1 M carbonate:bicarbonate buffer. Formaldehyde, measured with chromotropic acid

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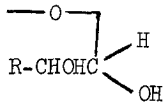
[2] Dr. Guy Barry (personal communication) has also isolated a compound from a polysaccharide of bacterial origin which yields a similar chromophore on ninhydrin treated chromatograms of 6 N HCl hydrolyzates ($R_{proline}$ 1.25), but differs in that a chromogen is not produced by 2 N HCl hydrolysis.

[3] Galbraith Laboratories, Knoxville, Tennessee.

(Frisell and McKenzie, 1958) was produced by periodate oxidation after, but not before, reduction at pH 8.0 by $NaBH_4$. Similar results were obtained with N-acetylat material. These data together with the hypoiodite and optical data suggest the structure  for C_1 and C_2.

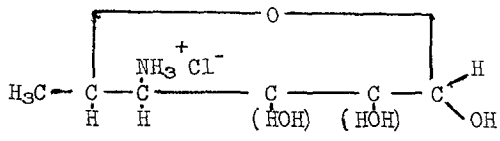
The presence of a 6-deoxy group had been previously suggested by the qualitative detection of acetaldehyde following periodate oxidation (Smith, et al. 1962). Quantitative measurement of acetaldehyde produced by periodate oxidation of the crystalline compound at pH 5 as previously described (Smith, et al., 1962) indicated a recovery of 0.6 to 0.7 moles of acetaldehyde per mole. Furthermore, it was observed that N-acetylation blocked acetaldehyde formation by periodate, indicating the amino group to be at position 4 or 5 on the carbon chain. To determine whether the amino group was at C_4 or C_5, both N-acetylated and $NaBH_4$ reduced-N-acetylated unknown and fucosamine were oxidized sequentially with periodate (pH 5.0), hypoiodite (pH 10.4) and after acidifying for removal of halogen by $CHCl_3$ extraction, heated in sealed tubes for two hours in 2N acid to to remove the N-acetyl group. Amino compounds were isolated on Dowex 50-H^+, eluted with ethanol-ammonia (2:1), dried in vacuo, dissolved in a minimum of water and chromatographed on paper with alanine, serine, L-(n)-threonine and D-L-allothreonine as markers using the solvent systems (A) n-butanol:HOAc:H_2O (5:1:2), (B) 73% phenol:H_2O, (C) t-butanol:methylethylketone:H_2O:diethylamine (44:4:2:0.4), and (D) n-butanol:methylethylketone:H_2O:NH_4OH (5:3:1:1). Fucosamine controls yielded serine only from reduced N-acetyl fucosamine, indicating the general validity of the procedure. The unknown amino sugar yielded allothreonine but no threonine, using solvents (C) and (D). Since no threonine was seen, it is assumed that racemization did not occur during isolation, since either D-(n)- or L-(n)-threonine would have been formed if either of the two asymmetric groups had been racemized. Mobility values, related to serine are shown in Table I. Starting material was not recovered, and additional minor spots present, one of which exhibited an R_{serine} of 1.0 to 1.1 in solvent (A) were also seen when allo-

TABLE I

R_{serine} Values

Solvent	Oxidation Product of Fucosamine	Unknown	Marker L-n-threonine	DL allo-threonine	alanine
A	1.0	1.37	1.37	1.37	1.67
B	---	1.42	1.41	1.41	1.75
C	---	1.30	1.57	1.32	1.0
D	---	1.92	3.0	1.82	1.09

threonine or threonine were N-acetylated, treated with periodate, etc. The amino sugar product, chromatographed on Dowex-50-H^+, was eluted with 0.3 N HCl in the position expected for known threonine or allothreonine. Furthermore a positive test for acetaldehyde was obtained when the product was treated with periodate indicating it to contain a threonine derivative. An attempt was made to determine the configuration of the allothreonine by exposure to L- and D-amino acid oxidases (Greenstein and Winitz, 1961) in the presence of a peroxidase-dianisidine chromoger forming system[4] to detect H_2O_2 produced. Positive results were obtained with purified L-amino acid oxidase[4], but appeared to be negative with crude hog kidney D-amino acid oxidase. Configurations at carbons 2 and 3 are as yet unknown. However, a tentative structure compatible with the data so far obtained is:



α-4-amino-4:6-dideoxy-(L)-aldo (pyrano) hexose.

[4] Worthington Biochemical Corporation, Freehold, New Jersey.

Possible related hexose configurations would be limited to the 6-deoxy derivatives of mannose, glucose, allose and altrose. Known naturally occurring 6-deoxy sugars include 6-deoxy-L-mannose (L-rhamnose), 6-deoxy-D-glucose (D-quinovose), 6-deoxy-3-O-methyl-L-glucose (L-thevetose) (cf. Pigman, 1957), and 6-deoxy-D-mannose (D-rhamnose, Markovitz, 1961). It is of interest to note that deamination of the 4-amino compound could yield a 4-keto, 6-deoxy-sugar: such compounds are known to be intermediates, as nucleoside diphosphate derivatives, in the synthesis of L-fucose and L-rhamnose (Ginsburg, 1960; Glaser and Kornfeld, 1961).

Sharon and Jeanloz (1960) have reported the isolation of a 4-acetamido-2 amino-2,4,6-tridoxyhexose from acid hydrolyzates of a polysaccharide obtained from Bacillus subtilis, and although several 3- and 6-amino sugars are known to occur in various antibiotics (Foster and Horton, 1960; Chain, 1958), the characterization of a 4-amino hexose obtained from polysaccharides or other sources has not, to our knowledge, been previously reported. Possibly similar N-acetyl amino sugars, isolated previously from Escherichia coli as thymidine diphosphate-compounds (Strominger and Scott, 1959; Okazaki, et al., 1960) have been recently reported (Okazaki, et al., 1962): crystalline material was not obtained and although elements analysis, empirical formula and molecular weight were not established, functional group similarities are evident from (a) the colorimetric, chromatographic, infra red, and periodate oxidation evidence reported which indicate a non C-2 amino group and (b), the identification of threonine after periodate and bromine oxidation followed by deacetylation. It is thus probable that 3-, 4- and 5-amino sugars will be found to occur in other microbial, plant and animal tissues. This family of compounds would be expected to be categorized as cationic reducing compounds which yield negative Elson-Morgan and Morgan-Elson tests for 2-amino sugars.

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