BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 40 194—196 (1967)

Studies on Amino-hexoses. XI. A Synthetic Substrate of Egg White Lysozyme: Phenyl 6-*O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-2-acetamido-3-*O*-(D-1′-carboxyethyl)-2-deoxy-β-D-glucopyranoside*[1]

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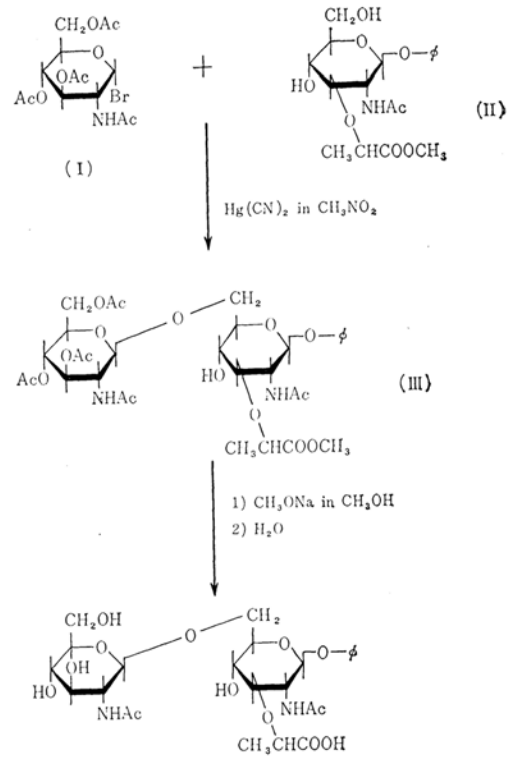
(Received June 6, 1966)

In the course of the study of the substrate specificity of egg white lysozyme, a phenyl glycoside of a disaccharide, *N*-acetyl-β-D-glucosaminyl(1→6)*N*-acetyl-muramic acid, was synthesized as a model substrate of the enzyme. Evidence for the 1→6 linkage between two component sugars was obtained by chemical and NMR studies. As a reference standard for a chromatographic investigation, 4-*O*-methyl muramic acid was prepared. The lysozyme action on the model substrate, if any, was found to be extremely weak.

The bacteriolytic action of egg white lysozyme was attributed to the hydrolysis of bacterial-cell-mucopolysaccharide,[1] as the study of the chemical structure of the cell-wall substance advanced. It was recognized that the lysozyme split the glycosidic bonds of *N*-acetyl muramic acid in the mucopolysaccharide of bacterial cell walls and that the smallest fragment released was a disaccharide which had the structure: *N*-acetyl-β-D-glucosaminyl(1→6)*N*-acetyl-muramic acid.[2] In more recent studies, however, it has been claimed[3] that the glycosidic linkage between two component sugars was 1→4 instead of 1→6.

In the course of our study of the substrate specificity of egg white lysozyme, it was found that phenyl *N*-acetyl-α- and β-muramide and their methyl esters[4] and *p*-acetaminophenyl *N*-acetyl-β-muramide[5] and its methyl ester were all immune to lysozyme. It seemed, then, to be of interest to examine the lysozyme action upon the phenyl glycoside of the disaccharide, *N*-acetyl-β-D-glucosaminyl(1→6)*N*-acetyl-muramic acid. The course of the synthesis of this compound was as follows: freshly-prepared acetobromoglucosamine in a chloroform solution[6] was condensed with the methyl ester of phenyl *N*-acetyl-β-muramide,[4] which had free hydroxyl groups at both 4 and 6 positions. As it was known that the hydroxyl at the 6 position in glucopyranose was more reactive than that at the 4 position[7], it was expected that the main condensation product would have the 1→6 glycosidic linkage. A Koenigs-Knorr reaction using mercuric cyanide in dry nitromethane[8]



*1 Presented in part at the 17th Annual Meeting of the Chemical Society of Japan, Tokyo, 1964.

1) L. R. Berger and R. S. Weiser, *Biochim. Biophys. Acta*, **26**, 517 (1957); M. R. J. Salton and J. M. Ghuysen, *ibid.*, **36**, 552 (1959).

2) M. R. J. Salton and J. M. Ghuysen, *ibid.*, **45**, 355 (1960); H. R. Perkins, *Biochem. J.*, **74**, 182 (1960).

3) R. W. Jeanloz, N. Sharon and H. M. Flowers, *Biochem. Biophys. Research Communs.*, **13**, 20 (1963); N. Sharon, T. Osawa, H. M. Flowers and R. W. Jeanloz, *J. Biol. Chem.*, **241**, 223 (1966).

4) K. Yamamoto, M. Fujinaga and Y. Matsushima, This Bulletin, **36**, 1275 (1963).

5) S. Isemura and Y. Matsushima, This Bulletin, in press.

6) Y. Inoue, K. Onodera, S. Kitaoka and H. Ochiai, *J. Am. Chem. Soc.*, **79**, 4218 (1957).

7) J. M. Sugihara, "Advance Carbohydrate Chem.," **8**, 1 (1953).

8) H. M. Flowers and R. W. Jeanloz, *J. Org. Chem.*, **28**, 1564 (1963).

gave the highest yield. The scheme of the synthesis is shown in Fig. 1. In order to examine the position of the linkage between two component sugars, acid hydrolysates of the *O*-methylated derivative of III were run on a thin-layer chromatogram. As the authentic 4-*O*-methyl-muramic acid was not yet known, the acid hydrolysates of the *O*-methylated ethyl 6-*O*-trityl-muramide methyl ester were used as the reference. The acid hydrolysates of the *O*-methylated derivative of III revealed two spots, corresponding to 4-*O*-methyl-muramic acid and to D-glucosamine. Also, the proton magnetic spectrum supported the $1 \rightarrow 6$ linkage in III, showing a doublet attributable to the secondary hydroxyl at 5.7 ppm of δ-value.[9]

As is shown in the Experimental section, egg white lysozyme exerted a recognizable but extremely low effect upon the synthetic substrate, compound IV.

Experimental

Optical rotation was determined on a Rudolph photoelectric polarimeter, Model 200S-80Q. The molecular weight was determined on a Mechrolab Vapor Pressure Osmometer, Model 301A. A proton magnetic resonance spectrum was produced using a Varian A-60 spectrometer, with tetramethylsilane as the external standard. Thin-layer chromatography was carried out by the ascending method on silica gel G (Merck). The phenyl glycosides were detected with an anisaldehyde-sulfuric acid reagent, and the free amino sugars, with a ninhydrin reagent. The enzyme specimen was thrice-recrystallized egg white lysozyme purchased from the Sigma Chemicals Co.

Phenyl 6-*O*-(2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-2-acetamido-3-*O*-[D-1'-(methyl carboxylate)ethyl]-2-deoxy-β-D-glucopyranoside (III). To a solution of 1.7 g of phenyl 2-acetamido-3-*O*-[D-1'-(methyl carboxylate)ethyl]-2-deoxy-β-D-glucopyranoside (II)[4] in 200 m*l* of dry nitromethane, there were added 2.1 g of mercuric cyanide and 2.1 g of Drielite.[10] 2-Acetamido-3, 4, 6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl bromide (I),[6] freshly prepared from 5 g of acetyl 2-acetamido-3, 4, 6-tri-*O*-acetyl-2-deoxy-D-glucopyranoside in 20 m*l* of chloroform, was then dropped into the above solution while it was being stirred mechanically. After the reaction had been continued for 17 hr at room temperature, to the reaction mixture there was added 1.3 g of mercuric cyanide, 1.3 g of Drielite, and the same amount of I; this mixture was allowed to stand for a further 20 hr. An insoluble substance was then removed and the clear filtrate was evaporated in vacuo to a heavy syrup; this syrup was dissolved in 300 m*l* of chloroform, and washed three times with water, and the chloroform layer was dried with anhydrous magnesium sulfate. From the water washings, a part of the starting compound, II, which had escaped the reaction was recovered. The chloroform layer was then evaporated in vacuo to a syrup which was crystallized in acetone. Recrystallization from ethyl acetate gave 40 mg (1.3%) of colorless crystals which melted at 261—262°C with decomposition. $[\alpha]_D^{20}$ −7.30 (*c* 0.753, pyridine).

Found: C, 53.85; H, 6.45; N, 3.86%. Calcd for $C_{32}H_{44}O_{16}N_2$: C, 53.93; H, 6.22; N, 3.93%.

A molecular weight determination in a chloroform solution gave a value of 780 against the theoretical value of 713. The thin layer chromatogram of III with the acetone : ethyl acetate = 1 : 1 solvent system, revealed only one spot, one with the R_f value of 0.37.

Phenyl 6-*O*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-2-acetamido-3-*O*-(D-1'-carboxyethyl)-2-deoxy-β-D-glucopyranoside (IV). Fifty milligrams of III were dissolved in 30 m*l* of anhydrous methanol which contained an amount of sodium methoxide corresponding to 10 mg of metal sodium. The solution was kept overnight at room temperature while it was being stirred, and then 30 m*l* of water was mixed into the solution. After it had stood for 2 hr at room temperature, the solution was passed through a column of Dowex-50 (H^+ form) in order to remove the sodium ions. The elute was evaporated in vacuo to dryness; the crystallization of the residue in ethyl ether gave 30 mg (75%) of colorless crystals which melted at 235—237°C with decomposition. $[\alpha]_D^{17}$ −32.3 (*c* 0.266, water).

Found: C, 52.28; H, 6.41; N, 4.74%. Calcd for $C_{25}H_{36}O_{13}N_2$: C, 52.44; H, 6.34; N, 4.89%.

A thin-layer chromatogram of the compound IV with the acetone: formic acid : water = 8 : 2 : 1 solvent system revealed only one spot. The hydrolysates of IV in 2 N hydrochloric acid at 100°C for 3 hr revealed, on a thin-layer chromatogram with the same solvent system, two spots with the R_f values of 0.51 and 0.73, spots corresponding to glucosamine and muramic acid respectively.

Ethyl 2-Acetamido-3-*O*-[D-1'-(methyl carboxylate)ethyl]-6-*O*-trityl-2-deoxy-($\alpha+\beta$)-D-glucopyranoside (V).—A solution of 2 g of ethyl *N*-acetyl-($\alpha+\beta$)-muramide[11] and 2 g of trityl chloride in pyridine was stirred for 20 hr at room temperature. The reaction mixture was poured into ice water while being vigorously stirred, and the precipitates were collected and dried. The colorless powder thus obtained was washed with petroleum ether and recrystallized from toluene. The yield was 2.6 g (76%). The crystals were contaminated with a small amount of triphenyl carbinol and melted at 94—96°C.

Found: C, 70.08; H, 6.89; N, 2.25%. Calcd for $C_{33}H_{39}O_8N$: C, 68.61; H, 6.80; N, 2.43%.

The elemental analyses did not give a good agreement with the theoretical values because of the contamination, but the 4-*O*-acetylated compound gave good analytical values, as is shown below.

Ethyl 2-Acetamido-3-*O*-[D-1'-(methyl carboxylate)ethyl]-4-*O*-acetyl-6-*O*-trityl-2-deoxy-($\alpha+\beta$)-D-glucopyranoside. A solution of 1 g of V and 10 m*l* of acetic anhydride in 20 m*l* of pyridine was kept at room temperature for 20 hr; then the reaction mixture was evaporated in vacuo to dryness. The recrystallization from methanol of the residue thus obtained

9) O. L. Chapman and R. W. King, *J. Am. Chem. Soc.*, **86**, 1256 (1964).

10) Anhydrous calcium sulfate purchased from the Hammond Drielite Co.

11) Y. Matsushima and J. T. Park, *J. Org. Chem.*, **27**, 3581 (1962).

gave 0.67 g (63%) of colorless crystals which melted at 206—207°C.

Found: C, 67.78; H, 6.60; N, 2.25%. Calcd for $C_{35}H_{41}O_9N$: C, 67.83; H, 6.67; N, 2.26%.

Ethyl 2-Acetamido-3-*O*-[D-1'-(methyl carboxylate)ethyl]-4-*O*-methyl-6-*O*-trityl-2-deoxy-(α+β)-D-glucopyranoside (VI). A mixture of 0.3 g of V, 0.2 g of silver oxide, and 25 g of methyl iodide was refluxed for 20 hours. The insoluble substance was removed, and the solution was evaporated *in vacuo*. The residue was again treated with silver oxide and methyl iodide under the same conditions. The filtrates from the insoluble substance gave, after evaporation and recrystallization from cyclohexane, 0.17 g of colorless crystals which melted at 217—218°C.

Found: C, 70.11; H, 6.64; N, 2.54%. Calcd for $C_{34}H_{41}O_8N$: C, 69.01; H, 6.98; N, 2.37%.

Evidence for the 1→6 Glycosidic Linkage in the Compound III. *Methylation Study.* A mixture of 10 mg of III, 25 g of methyl iodide, and 100 mg of freshly-prepared silver oxide was refluxed for 20 hr. The solution was separated from the insoluble substance, and the residue obtained by evaporating the solution was hydrolyzed in 4 N hydrochloric acid for 6 hr at 100°C. Thin-layer chromatography of the hydrolysates with the isopropanol : acetone : formic acid= 6 : 3 : 1.5 solvent system, revealed two spots with R_f values of 0.67 and 0.21, spots corresponding to 4-*O*-methyl-muramic acid and glucosamine respectively. As the reference standard of 4-*O*-methylmuramic acid in the thin-layer chromatogram, the compound VI was hydrolyzed with 2 N hydrochloric acid at 100°C for 3 hr; the hydrolysates freed from the insoluble substance were thus spotted.

Proton Magnetic Resonance Study. The hydroxyl proton magnetic resonance pattern of the compound III was obtained using 40 mg of the specimen dissolved in 0.7 m*l* of dimethyl sulfoxide. The hydroxyl proton signal was assigned on the basis of the disappearance of the signal because of hydrogen-deuterium exchange reaction. Among the proton resonance signals observed, only a doublet (δ, 5.70 ppm; J, 5.0 cps) disappeared after the addition of deuterium oxide. This indicates the existence in the compound III of a secondary hydroxyl instead of a primary one.

Lysozyme Activity on Compound IV. The activity of the enzyme was assayed by measuring the increment of the reducing power in the reaction mixture using the ferri-ferrocyanide method.[12] The results are shown in Table 1.

TABLE 1. ABSORBANCY AT 700 mμ OF THE INCUBATION MIXTURE

	Substrate	5 min	20 hr	42 hr
Exp. 1	Compound IV[a]	0.005	0.040	0.040
	Cell Wall[b]	0.910	1.16	1.26

The substrates were incubated with 0.085 mg of the enzyme in 2.5 m*l* of 0.1 M ammonium acetate (pH 7.0) at 37°C. a) 11.4 mg b) 1.5 mg, isolated from Micrococcus lysodeikticus.

	Substrate	pH	15 min	20 hr
Exp. 2	Compound IV[c]	7.0	0.025	0.060
	Compound IV[c]	6.0	0.040	0.050
	Cell Wall[d]	7.0	1.20	1.80

The substrates were incubated with 0.030 mg of the enzyme in 2.5 m*l* of 0.1 M veronal-acetate at 37°C. c) 4.0 mg d) 3.5 mg, isolated from Micrococcus lysodeikticus.

This investigation was supported by U. S. Public Health Service Grant AI-04586-03. The authors' thanks are also due to Dr. James T. Park for his gifts of Micrococcus lysodeikticus cells and *N*-acetylglucosamine.

12) J. T. Park and M. J. Johnson, *J. Biol. Chem.*, **181**, 149 (1949).