THE CHEMISTRY OF BLEOMYCIN. VIII*

THE STRUCTURE OF THE SUGAR MOIETY OF BLEOMYCIN A_2**

Sir:

Previously, we reported that L-gulose and 3-O-carbamoyl-D-mannose are components of bleomycin A_2 [1]. In this communication, we report that the sugar moiety of bleomycin A_2 is present as a disaccharide, 2-O-(3-O-carbamoyl-α-D-mannopyranosyl)-L-gulose, which is linked to the hydroxyl group of L-*erythro*-β-hydroxyhistidine[2] of bleomycin A_2 through an α-glycosidic linkage.

The molar ratio of gulose and carbamoyl-mannose in each bleomycin was examined by gas chromatography. The trimethylsilyl derivative of the methanolyzates[1] of the individual bleomycins was chromatographed at 170~180°C on a column of Chromosorb W.AW coated with SE-30.[3] The result indicated that the bleomycins each contain one mole of gulose and 3-O-carbamoyl-mannose.

The next step was to find if these sugars exist as a disaccharide or are attached separately to the peptide part of bleomycin. We found that a disaccharide was liberated by mild acid hydrolysis of bleomycin A_2: 0.3 N H_2SO_4 at 80~ 81°C for 6 hours. The disaccharide was isolated by cellulose column chromatography of the neutral fraction of the hydrolyzate, $[\alpha]_D^{20}$ +54° (*c* 1.7, H_2O), ν^{KBr} 1620, 1705 cm^{-1} (carbamoyl).

Reduction of the disaccharide with $NaBH_4$ followed by methanolysis afforded D-sorbitol and methyl 3-O-carbamoyl-α-D-mannopyranoside. This shows that the carbamoyl-mannose is linked with gulose by a glycosidic linkage and gulose is attached to the peptide part through C-1, because bleomycin is non-reducing. The point of attachment of carbamoyl-mannose was determined by analysis of the NMR spectrum of the peracetylated disaccharide. The peracetylated disaccharide was obtained by treatment with acetic anhydride and pyridine, and the major product (β-anomer) was used for the analysis, $[\alpha]_D^{20}$ +31° (*c* 2, $CHCl_3$), *m/e* 620 (M-59), 578, 556, 332. The NMR spectrum was taken in deuterochloroform solution with internal TMS reference. The methine (except C-1 proton of gulose moiety) and methylene protons, on which acetoxyl groups are present, appeared at δ 4.8~5.5 (ppm) and δ 4.0~4.5, respectively. The C-1 proton of the gulose moiety appeared at δ 5.90 (J= 8.5 Hz). The C-2 and C-3 protons of gulose could be assigned to a double doublet signal at δ 4.00 (J=8.5 and 3.6 Hz) and a triplet signal at δ 5.45 (J=3.6 Hz), respectively, by double and triple resonance techniques. The high field chemical shift (δ 4.00) of the C-2 proton of gulose, compared to the other methine protons (δ 4.8~5.5), on which acetoxyl groups are present, suggested that the C-2 hydroxyl group of gulose was involved in the glycosidic linkage. The configuration of the glycosidic linkage of carbamoyl-mannose was determined to be α by application of HUDSON's rule.[4] The calculated molecular rotation for peracetyl 2-O-(3-O-carbamoyl-$\underline{\alpha}$-D-mannopyranosyl)-β-L-gulopyranose was +224° and the observed value was +236°, while the calculated value for the $\underline{\beta}$-glycoside was −128°.

The glycosidic linkage to the hydroxyl group of threonine in glycoproteins has been studied by the following method: the glycoprotein is treated with dilute alkali under mild conditions to split the sugar residue by β-elimination and, concomitantly, the threonine moiety is transformed to dehydrobutyrine, and the dehydrobutyrine is then hydrogenated to yield butyrine which is stable to further acid hydrolysis.[5,6]

Bleomycin was studied in the same way. Bleomycin A_2 was dissolved in 0.1 N NaOH and the solution was kept at room temperature. The UV absorption at 290 nm of the reaction mixture increased gradually and reached a maximum at 5 days. After 7 days, the reaction mixture was passed through an Amberlite CG-50 (H form) column. The effluent fraction was purified after acetylation. The NMR and mass spectra indicated the presence of peracetylated 2-O-(α-D-

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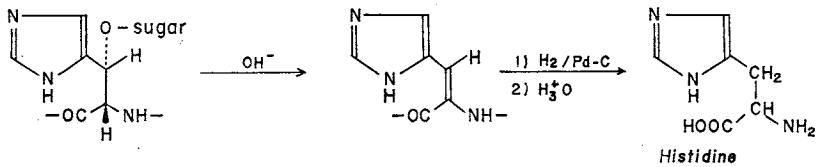
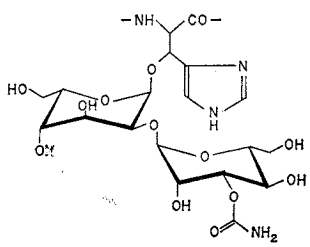


mannopyranosyl)-L-gulose in the effluent. The adsorbed peptide on the column was eluted with 0.04 N HCl and methanol (1 : 1) after washing with 0.02% acetic acid solution. The NMR spectrum showed a newly formed olefinic proton at δ 8.52 (external TMS reference in D_2O).

The peptide was hydrogenated with palladium on carbon and then hydrolyzed with 6 N HCl at 105°C for 18 hours. Paper electrophoresis showed that the hydrolyzate contained all the amine components of bleomycin A_2 except β-hydroxyhistidine. Instead, histidine was present in the hydrolyzate. The hydrolyzate of the unhydrogenated peptide contained neither β-hydroxyhistidine nor histidine. As a control experiment, tetrapeptide A[7], a sugar-free β-hydroxyhistidine containing peptide obtained by partial acid hydrolysis of bleomycin A_2, was treated with dilute alkali in the same way as described above and then hydrolyzed. The hydrolyzate contained one mole of β-hydroxyhistidine.

These results suggest that the sugar moiety of bleomycin A_2 is glycosidically linked to the hydroxyl group of β-hydroxyhistidine moiety. With alkali treatment, the sugar is liberated by β-elimination to give a peptide containing dehydrohistidine, with an increase of UV absorption at 290 nm. Hydrogenation of the dehydrohistidine moiety, and hydrolysis yields histidine.

The anomeric configuration of the disaccharide was dertermined by analysis of the NMR spectrum of bleomycin A_2. The C-2 proton of gulose is axial. So, if the anomeric proton is axial (L-β-anomer), a large coupling constant (*ca.* 8~9 Hz) would be expected, while if the anomeric proton is equatorial (L-α-anomer), a broad doublet (J=*ca.* 3~4 Hz), caused by long range coupling with C-3 and C-4 protons, should be observed[1].



The chemical shift of the anomeric proton is expected to be in the region between δ 5.0 and 6.0 (external TMS reference in D_2O). In this region of the spectrum of bleomycin A_2, there are signals from five protons: δ 5.91 (doublet, J=6.5 Hz), 5.73 (broad doublet, J=*ca.* 3.5 Hz), 5.52 (doublet, J=6.5 Hz), 5.49 (doublet, J=1.5 Hz) and 5.20 (double doublets, J=9 and 3 Hz).

The chemical shift of the proton at δ 5.91 was sensitive to pH change between pH 4 and 6. It can be assigned to the β-CH proton of β-hydroxyhistidine. This proton was coupled with a proton at δ 5.52 (J=6.5 Hz). Thus, the latter signal is assigned to the α-CH proton of β-hydroxyhistidine.

The other three signals should be from the two anomeric protons of gulose and 3-O-carbamoyl-mannose and the C-3 proton of 3-O-carbamoyl-mannose. The coupling constant of the anomeric proton of α-D-mannopyranoside is characteristically small[1], and thus the signal at δ 5.49 (J=1.5 Hz) is assigned to the anomeric proton of 3-O-carbamoyl-mannose. The C-3 proton of 3-O-carbamoyl-mannose is axial, while the C-4 proton is axial and the C-2 proton is equatorial. Then, the signal at δ 5.20 (double doublets, J=9 and 3 Hz) can be assigned to the mannose C-3 proton. The remaining signal at δ 5.73 can be assigned to the anomeric proton of gulose. It is a broad doublet (J=*ca.* 3.5 Hz) indicative for equatorial conformation of the anomeric proton.

Then, the sugar moiety of bleomycin A_2 is determined to be 2-O-(3-O-carbamoyl-α-D-mannopyranosyl)-L-gulopyranose, which is connected to the hydroxyl group of L-*erythro*-β-hydroxyhistidine by an α-glycosidic linkage.

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