THE CARBOHYDRATE–PROTEIN LINKAGE IN GLYCOPROTEINS

PART I. THE SYNTHESES OF SOME MODEL SUBSTITUTED AMIDES AND AN L-SERYL-D-GLUCOSAMINIDE[1]

J. K. N. JONES, M. B. PERRY, B. SHELTON, AND D. J. WALTON[2]

ABSTRACT

The syntheses of *N*-L-seryl-D-glucosamine, *N*-glycyl-D-glucosamine, *N*-L-glutamyl-D-glucosamine, and 1-*O*-β-L-seryl-*N*-acetyl-D-glucosaminide are described. These D-glucosamine derivatives may result from the fragmentation of some glycoproteins. The chemical properties of these compounds indicate that *N*-peptides are relatively stable in acid solution but unstable in neutral and alkaline solution. The seryl glucosaminide is relatively stable in alkaline solution but is hydrolyzed by acid.

INTRODUCTION

The mode of the linkage of sugars to amino acids in glycoproteins and mucoproteins which contain small amounts of carbohydrate, is obscure. Examples of this group are collagen and reticulin. Reticulin (1) is found in fairly large amounts in some internal organs (e.g. kidney and lung), and contains 3–4% of neutral sugars and 0.2% of hexosamine. Collagen (2) contains 0.3–0.7% of glucose and galactose. Several collagens which have been investigated (3) have been shown to contain D-glucosamine. Histochemical work has demonstrated that in some diseases, such as rheumatoid arthritis, there appear to be increases in the carbohydrate content of the glycoprotein of the subcutaneous tissue, which is rich in collagen. It was therefore of interest to investigate the mode of linkage of carbohydrate to amino acid in glycoproteins and mucoproteins. Bovine subcutaneous tissue, which is rich in collagen and elastic fibers, when degraded by proteolytic enzymes yielded a complex mixture of peptides to which were attached sugar residues. It was clear that this subcutaneous tissue did not contain any appreciable quantities of high molecular weight polysaccharides (4). This suggested that carbohydrate residues of low molecular weight were covalently linked to the amino acid residues of the proteins. It was therefore decided to attempt the syntheses of carbohydrate–amino acid derivatives which might result from the degradation of glycoproteins. A survey of the literature indicates that little was known about the type of linkage in such a derivative, but that linkages through glycine, aspartic acid, serine, threonine, and amides have been implicated (5, 6, 7). Many glycoproteins contain D-glucosamine and the possible biological role of *N*-peptides of this amino sugar have been investigated. The syntheses of *N*-glycyl-D-glucosamine (8) and of *N*-L-alanyl-D-glucosamine (9) have been reported in which they were isolated either as the hydrochloride or as some alternative derivative, with the amino group protected. *N*-(L-Alanyl-L-alanyl)-D-glucosamine has been found to be stable (10), as have several other similar dipeptides, the syntheses of which have been reported. A peptide composed of D-glucosamine and L-glutamic acid has been synthesized (11) but this was reported to be unstable even when the amino group was protected. It was of particular interest to examine these *N*-peptides, rather than their more stable substituted derivatives to determine whether they might be expected to survive during the hydrolysis

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[2]*Present address: 18, Maiden Castle Road, Dorchester, Dorset, England.*

of glycoproteins. Accordingly, we have attempted the syntheses of several amino acids amide linked to D-glucosamine, in order to examine their properties. The *N*-peptide of L-serine was chosen as it had been reported by Masamune (12) to be present in gastric mucin together with glucosamine. Glycine has been reported by Gross (13) to constitute about 30–35% of all collagens investigated, and may therefore be linked to sugar residues. The *N*-peptide of L-glutamic acid was prepared in order to investigate whether the presence of a free carboxylic acid end group might confer stability to the derivative. L-Serine contains a primary hydroxyl group, and is therefore capable of being united glycosidically with a sugar residue: an L-seryl-*N*-acetyl-D-glucosaminide has been synthesized and its properties examined.

N-L-*Seryl*-D-*glucosamine*

N-(*N*-Carbobenzoxy-L-alanyl)-1,3,4,6-tetra-*O*-acetyl-β-D-glucosamine hydrochloride and the corresponding *N*-glycyl compound were synthesized by Bergmann and Zervas (8) by treating 1,3,4,6-tetra-*O*-acetyl-β-D-glucosamine with *N*-carbobenzoxy-L-alanyl chloride or with *N*-carbobenzoxyglycyl chloride. L-Serine, however, contains a primary hydroxyl group which must be protected before a reaction of this type can be used. Accordingly we turned our attention to the possibility of condensing a suitably substituted D-glucosamine derivative with *N*-carbobenzoxy-L-serine by the carbodiimide method (14). Unfortunately the L-serine derivative was not soluble in methylene chloride which is the solvent usually employed in this type of reaction. To overcome this difficulty the primary hydroxyl group of the L-serine derivative was protected by a tetrahydropyranyl group which led to the formation of two separable optical isomers which were soluble in methylene chloride. Racemization does not occur in the formation of the tetrahydropyranyl derivatives (15). Each isomer was then condensed with the suitably substituted 1-*O*-α-acetyl- and 1-*O*-β-acetyl-D-glucosamine derivatives, resulting in the formation of four isomers. The yields were poor when dicyclohexyl carbodiimide was used because of the difficulty in separating dicyclohexyl urea from the products. When diisopropyl carbodiimide was used as a condensation agent, better yields were obtained since the product was more readily separable from the diisopropyl urea by-product. When these isomers were warmed in dilute acetic acid solution, dihydropyran was eliminated and *N*-(*N*-carbobenzoxy-L-seryl)-1,3,4,6-tetra-*O*-acetyl-α- (and β-) D-glucosamine were produced, respectively. When the carbobenzoxy grouping was removed by reduction the resulting *N*-L-seryl-1,3,4,6-tetra-*O*-acetyl-α- (and β-) D-glucosamine decomposed to a brown tar within a few minutes. Presumably this was due to the basic character of the amino group of the amino acid moiety. Despite this instability it was possible to prepare the fully acetylated derivative. However, it was found that in the presence of hydrochloric acid the deacetylated product yielded a stable hydrochloride on removal of the carbobenzoxy grouping by hydrogenolysis.

N-Glycyl-D-*glucosamine*

Bergmann (8) synthesized the hydrochloride of this material but the parent compound has not been reported. *N*-(*N*-Carbobenzoxyglycyl)-D-glucosamine was obtained by the deacetylation of *N*-(*N*-carbobenzoxyglycyl)-1,3,4,6-tetra-*O*-acetyl-β-D-glucosamine. This compound was prepared by Bodanszky and Du Vigneaud's method (16) for condensing amino groups and carboxylic acid groups to form peptide linkages. The condensation is effected between the hydrogen halide of an amine and the *p*-nitrophenyl ester of a carboxylic acid. In this case the amine salt used was 1,3,4,6-tetra-*O*-acetyl-β-D-glucosamine hydrobromide, and the ester was glycyl *p*-nitrophenyl ester. The fully

substituted product was then deacetylated and reduced in the presence of hydrochloric acid to give *N*-(glycyl hydrochloride)-D-glucosamine. Hydrogenolysis in neutral solution caused both the acetylated and the deacetylated derivative to decompose rapidly.

N-L-Glutamyl-D-glucosamine

In the synthesis of this compound the method of Bodanszky and Du Vigneaud (16) was again used in an analogous manner to that used in the preparation of the *N*-glycyl-D-glucosamine. The amino acid was converted into *N*-carbobenzoxy-α-*p*-nitrophenyl ester-γ-L-glutamine by condensation of *N*-carbobenzoxy-L-glutamic acid with *p*-nitrophenol, using diisopropyl carbodiimide as a dehydrating agent, to form the diester. The diester was then treated with the calculated amount of methanolic ammonia solution to form the monoester–monoamide. It is reasoned from steric considerations that the replacement is effected in the less hindered γ-position. When the derivative had been formed and the terminal amide group removed, attempts to synthesize the free amine by hydrogenolysis of the carbobenzoxy group yielded very unstable products which could not be isolated. This was also true of the *N*-peptide after deacetylation. The hydrogen reduction was also carried out in the presence of hydrochloric acid, and in this case the stable *N*-peptide hydrochloride was formed.

1-O-β-L-Seryl-N-acetyl-D-glucosaminide

The glucosaminide was formed using a modified Koenigs–Knorr (17) type synthesis. 1-Chloro-1-deoxy-*N*-acetyl-3,4,6-tri-*O*-acetyl-D-glucosamine was prepared by the action of titanium tetrachloride on fully acetylated D-glucosamine in the manner described by Inouye and his co-workers (18). The acetohalogen sugar was allowed to react with *N*-carbobenzoxy-L-seryl methyl ester, using anhydrous alcohol-free chloroform as a solvent, and employing the Koenigs–Knorr conditions.

The initial yields of the product were low owing to steric hindrance, but when the reaction was allowed to proceed for 6 days with vigorous shaking, better yields were obtained. The product was not purified at this stage, but was de-*O*-acetylated with barium methylate. The deacetylated material was purified by chromatography, and was then subjected to hydrogenolysis, under neutral conditions, to remove the carbobenzoxy group. The over-all yield was small and the product at this stage, 1-*O*-β-(L-seryl methyl ester)-*N*-acetyl-D-glucosaminide, was stable and the corresponding hydrochloride was synthesized. The substituted D-glucosaminide was then saponified using sodium hydroxide solution to give the free acid, 1-*O*-β-L-seryl-*N*-acetyl-D-glucosaminide, which was found to be stable.

EXPERIMENTAL

All melting points reported are uncorrected. Solutions were concentrated by rotary evaporation under reduced pressure below 40°, unless otherwise stated.

Paper chromatography was performed by the descending method on Whatman No. 1 paper, using 1-butanol/ethanol/water (3:1:1 v/v) as the mobile phase. The following sprays were used to detect the compounds on the paper chromatograms: (A) 2% solution of *p*-anisidine hydrochloride in 1-butanol, (B) 1% solution of silver nitrate in acetone, followed by 2% ethanolic sodium hydroxide, and (C) 2% ninhydrin in 1-butanol. The rate of movement of the compounds on the chromatograms is given relative to that of D-glucosamine hydrochloride ($R_{GN.HCl}$), *N*-acetyl-D-glucosamine (R_{GNAc}), or pentaacetyl-D-glucosamine (R_{GNpAc}).

The infrared spectra of crystalline compounds were measured as 0.8% dispersions

in potassium bromide disks, and non-crystalline materials were examined as 6% solutions in chloroform, using a Perkin–Elmer Model 21 spectrophotometer.

Elemental analyses were carried out by the Schwarzkopf Microanalytical Laboratory, Woodside, New York.

*1. N-*L*-Seryl-*D*-glucosamine*

(a) Carbobenzoxychloride

The reagent was prepared by the method of Carter *et al.* (19). Benzyl alcohol (65 ml) was added to a solution of phosgene (69 g) in toluene (200 ml) at 0°. After 3 hours the excess benzyl alcohol and the toluene were removed by distillation under reduced pressure. The remaining solution (104 g) contained 76 g of carbobenzoxychloride.

*(b) N-Carbobenzoxy-*L*-serine*

The method of Moore *et al.* (14) was used in a slightly modified form. Between the additions of the reactants the alkaline solution was transferred to a tap funnel and vigorously shaken, then returned to the reaction vessel. The product (98% yield) had m.p. 116°.

*(c) N-Carbobenzoxy-*L*-seryl Methyl Ester*

N-Carbobenzoxy-L-serine (20 g) was dissolved in 2.5 *N* methanolic hydrogen chloride solution (200 ml). The solution was allowed to stand overnight and it was then deacidified with Duolite A4(OH^-) exchange resin. The solution was concentrated to a syrup (20 g; 94% yield) which was kept in a desiccator.

*(d) N-Carbobenzoxy-O-tetrahydropyranyl-*L*-seryl Methyl Ester*

The procedure of Iselin and Schwyzer (15) was followed. *N*-Carbobenzoxy-L-seryl methyl ester (12.6 g) was dissolved in purified 2,3-dihydro-4-pyran (6 g) and a solution of 2.2 *N* hydrogen chloride in dry ethyl acetate (0.7 ml) was added. The reaction mixture was allowed to stand for 3 hours at room temperature after which it was diluted with ether and then washed with dilute sodium bicarbonate solution followed by water. The solution was dried (anhyd. sodium sulphate), filtered, and then concentrated to a yellow oil (15 g; 88% yield).

*(e) N-Carbobenzoxy-O-tetrahydropyranyl-*L*-serine*

The methyl ester (*d*) was dissolved in methanol (50 ml) and saponified by the method of Iselin and Schwyzer (15). Addition of ether to the final product in ethyl acetate resulted in the separation of one isomer in a crystalline form (I_{e1}), in 14% yield, which had m.p. 129°.

The second isomer (I_{e2}) was obtained from the concentrated mother liquor which gave crystals after 2 days (30% yield) which had m.p. 150°.

The infrared spectra of the products showed absorption bands at 987 cm^{-1} and 1130 cm^{-1} (in chloroform solution). These bands are due to the tetrahydropyranyl group, and are not present in the spectrum of *N*-carbobenzoxy-L-serine. The phenyl band at 695 cm^{-1} was also present.

*(f) 1,3,4,6-Tetra-O-acetyl-N-acetyl-β-*D*-glucosamine*

D-Glucosamine hydrochloride (30 g) was dissolved in a solution of zinc chloride (36 g) in acetic anhydride (250 ml). The solution was kept at 70–80° for 10 minutes and was then poured into ice water (400 ml). Sodium bicarbonate was slowly added to neutralize the excess acid and on allowing the reaction mixture to stand overnight in a refrigerator, a white precipitate of the pentaacetate separated. The material was collected, washed with water, dried, and recrystallized from ethanol solution to give the product (30.8 g;

52% yield) which had m.p. 187° which rose to m.p. 190–192° after recrystallization. $[\alpha]_D^{25}$ +3° (*c*, 4.0 chloroform).

The infrared spectrum of the product showed the following absorption bands: carbonyl of *O*-acetyl (1740 cm^{-1}); amide I (1682 cm^{-1}); and amide II (1512 cm^{-1}). Important bands referred to later occurred at 1238, 1078, 1042, and 900 cm^{-1}.

The corresponding α-anomer was prepared in a similar manner, but the reaction was carried out at room temperature. Yield 56%, m.p. 139°, $[\alpha]_D^{22}$ +91° (*c*, 4.0 chloroform).

(g) 1,3,4,6-Tetra-O-acetyl-α- (and β-) D-*glucosamine Hydrobromide*

The technique of Inouye *et al.* (18) was used. 1,3,4,6-Tetra-*O*-acetyl-*N*-acetyl-α-D-glucosamine (10 g) was added to a stirred ice-cold solution of dry hydrogen bromide in acetic acid (30 ml) to give the product (4.5 g; 45% yield), which had $[\alpha]_D^{22}$ +129° (*c*, 4.0 water), chars at 205–220°.

The β-anomer was prepared in a similar manner from 1,3,4,6-tetra-*O*-acetyl-*N*-acetyl-β-D-glucosamine and the product (4.5 g; 45% yield) had $[\alpha]_D^{21}$ +12.1° (*c*, 4.0 water), chars above 200°.

(h) 1,3,4,6-Tetra-O-acetyl-α- (and β-) D-*glucosamine*

The hydrobromides (1 g) were respectively dissolved in a slurry of Amberlite IR45 ($CO_3^{=}$) exchange resin (4 g hydrobromide to 50 ml resin). The mixture was stirred at 5° for 1 hour and then filtered. The filtrates were extracted with chloroform (4×80 ml) and the combined extracts were dried (anhyd. sodium sulphate) and then concentrated to a small volume. On the addition of ether to the concentrates, the products separated as needle-like crystals, which were collected and recrystallized from ethanol. The α-anomer (65% yield) had m.p. 118° and $[\alpha]_D^{23}$ +145.5° (*c*, 4.0 chloroform). The β-anomer (65% yield) had m.p. 143° and $[\alpha]_D^{22}$ +27° (*c*, 3.6 chloroform).

*(i) N-(N-Carbobenzoxy-O-tetrahydropyranyl-*L-*seryl)-1,3,4,6-tetra-O-acetyl-α- (and β-)* D-*glucosamine*

N-Carbobenzoxy-*O*-tetrahydropyranyl-L-serine (isomer I_{c1}) (2.0 g; 6.2 mmoles), 1,3,4,6-tetra-*O*-acetyl-β-D-glucosamine (2.3 g; 6.2 mmoles), and diisopropyl carbodiimide (0.8 g; 6.2 mmoles) were dissolved in methylene chloride (25 ml) and the solution was shaken mechanically for 20 hours at room temperature. One drop of dilute acetic acid was added, and the shaking was continued for a further 1 hour. The diisopropyl urea by-product was filtered off, and the filtrate was stored at −10° for 24 hours. The solution was refiltered, and the filtrate then washed with dilute hydrochloric acid, followed by 2 *N* sodium carbonate solution and finally with water. The solution was dried (anhyd. sodium sulphate) and the methylene chloride removed by distillation to leave a syrup. The syrup was taken up in the minimum amount of hot ethanol from which the α-anomer was obtained as crystals (40% yield) which had m.p. 201° and $[\alpha]_D^{23}$ +10° (*c*, 4.0 chloroform). Anal. Found: C, 55.8; H, 6.16; N, 4.3%. $C_{30}H_{40}O_{14}N_2$ requires: C, 55.2; H, 6.18; N, 4.3%.

The above synthesis was repeated using 1,3,4,6-tetra-*O*-acetyl-α-D-glucosamine to give the α-anomer as a syrup (35% yield) which could not be crystallized and which had $[\alpha]_D^{23}$ +91° (*c*, 4.0 chloroform).

The second isomer (I_{c2}) of *N*-carbobenzoxy-*O*-tetrahydropyranyl-L-serine was treated in the same way as described above with 1,3,4,6-tetra-*O*-acetyl-β-D-glucosamine to yield a crystalline product (29% yield) which had m.p. 160° and $[\alpha]_D^{23}$ +2° (*c*, 5.0 chloroform). Anal. Found: C, 55.5; H, 6.18; N, 4.6%.

The infrared absorption spectra of the products showed absorption band at the following wave numbers: carbonyl of *O*-acetyl (1745 cm^{-1}), amide I (1682 cm^{-1}), and amide II

(1495 cm^{-1}). The spectra showed the following absorption bands in common with 1,3,4,6-tetra-*O*-acetyl-β-D-glucosamine: 1235 (s), 1078 (s), 1041 (s), and 900 cm^{-1} (s). Bands characteristic of *N*-carbobenzoxy-*O*-tetrahydropyranyl-L-serine were detected at 1130, 987, and 695 cm^{-1}. The spectra of the isomers were almost identical.

*(j) N-(N-Carbobenzoxy-*L*-seryl)-1,3,4,6-tetra-O-acetyl-α- (and β-)* D*-glucosamine*

The *O*-tetrahydropyranyl group was removed from the products (*i*) by the following method. *N*-(*N*-Carbobenzoxy-*O*-tetrahydropyranyl-L-seryl)-1,3,4,6-tetra-*O*-acetyl-β-D-glucosamine (600 mg) was boiled for 10 minutes in 50% aqueous acetic acid (10 ml) and the cooled solution was then diluted with water (50 ml). The solution was extracted with chloroform (4×20 ml), the combined extracts were washed with water, dried (anhyd. sodium sulphate), and concentrated to a small volume. The residue was dissolved in the minimum quantity of hot ethanol from which the product crystallized slowly in the form of long colorless needles (75% yield) which had m.p. 168° and $[\alpha]_D^{23}$ +25° (*c*, 4.0 chloroform). Anal. Found: C, 53.3; H, 5.73; N, 5.0%. $C_{25}H_{32}O_{13}N_2$ requires: C, 52.8; H, 5.67; N, 4.93%.

The α-anomer was similarly prepared from *N*-(*N*-carbobenzoxy-*O*-tetrahydropyranyl-L-seryl)-1,3,4,6-tetra-*O*-acetyl-α-D-glucosamine as a crystalline product (65% yield) which had m.p. 145° and $[\alpha]_D^{23}$ +102° (*c*, 3.7 chloroform).

The infrared absorption spectra of these products were the same as those of the precursors except that the bands at 1130 cm^{-1} and 987 cm^{-1} due to the tetrahydropyranyl group were absent. The isomerism due to the forms (I_{e1}) and (I_{e2}) disappeared, as the same product was obtained from both isomers.

*(k) N-(N-Carbobenzoxy-*L*-seryl)-α- (and β-)* D*-glucosamine*

N-(*N*-Carbobenzoxy-L-seryl)-1,3,4,6-tetra-*O*-acetyl-α-D-glucosamine (0.4 g) dissolved in anhydrous methanol (8 ml) was treated with 0.4 *N* barium methoxide in methanol (2 ml) at 5° for 20 hours. After testing for the presence of excess barium, a little water was added and the remaining alkali was precipitated by carbonation of the solution. The barium carbonate was removed by centrifugation, and the clear supernatant was concentrated to a small volume. The residue gave a crystalline product (24% yield) from ethanol solution which had m.p. 195° and $[\alpha]_D^{20}$ +75° (*c*, 4.0 chloroform).

The β-anomer was similarly prepared from *N*-(*N*-carbobenzoxy-L-seryl)-1,3,4,6-tetra-*O*-acetyl-β-D-glucosamine as a crystalline product (30% yield) which had m.p. 198° and $[\alpha]_D^{21}$ +8° (*c*, 4.0 chloroform).

The products had R_{GNAc} 2.10 on paper chromatograms and was free from D-glucosamine and L-serine.

The infrared spectra of the two products were very similar, and differed from the spectra of the precursors in that the carbonyl of *O*-acetyl at 1750 cm^{-1} had disappeared, and the hydroxyl band at 3460 cm^{-1} appeared very strongly. The amide linkage bands at 1682 cm^{-1} and 1495 cm^{-1} were still present, though the latter was slightly displaced.

*(l) N-(*L*-Seryl hydrochloride)-*D*-glucosamine*

The carbobenzoxy group was removed from (1*k*) by hydrogenolysis using palladized charcoal as a catalyst. The catalyst was prepared by adding palladous chloride (0.1 g) in a little water to animal charcoal (2 g) and the mixture was then heated to 130° to remove water. Hydrazine sulphate was added, followed by a little *N* sodium hydroxide and the mixture was then stored for 24 hours at −5°. The charcoal was then collected by filtration and dried.

N-(*N*-Carbobenzoxy-L-seryl)-D-glucosamine (26 mg) was dissolved in a mixture of methanol and chloroform (5 ml) and the solution was added to water (5 ml) containing *N* hydrochloric acid (0.07 ml). Palladized charcoal was added and the mixture was shaken mechanically for 20 hours at room temperature under a slight pressure of hydrogen (2 lb above atmospheric pressure). The charcoal was removed from the product by filtration and the filtrate was concentrated to a syrup which was insoluble in chloroform and alcohol, but soluble in water.

The product was obtained from methanol/ether mixture as fine colorless crystals (19 mg; 86% yield) which decomposed over the temperature range 200–250°. On paper chromatograms developed in the 1-butanol/ethanol/water system the compound was revealed by the ninhydrin spray reagent as an orange spot which reddened on standing, and had $R_{GN\cdot HCl}$ 0.55. Anal. Found: N, 8.9; Cl, 11.7%. $C_9H_{19}O_7N_2Cl$ requires: N, 9.2; Cl, 11.7%.

The infrared spectrum showed a strong hydroxyl band at 3460 cm^{-1} which was very wide due to the amino group absorption. Amide I absorption was present at 1682 cm^{-1}, but was absent at 1495 cm^{-1}. Evidence for the ionic nitrogen was found at 1495 cm^{-1}.

The product prepared from the α-anomer failed to crystallize, and the yield of crude material was very poor.

(m) N-L-Seryl-D-glucosamine

Attempts to synthesize the free amine from *N*-(*N*-carbobenzoxy-L-seryl)-β-D-glucosamine by direct hydrogenolysis in aqueous solution failed, owing to the rapid decomposition of the product in the atmosphere. An indirect method was investigated. A little of the hydrochloride (*l*) (15 mg) was dissolved in an aqueous slurry of Amberlite IR 45 ($CO_3^=$) exchange resin (5 ml) and after removal of the resin the water was removed by distillation under reduced pressure at 30°, methanol being added to speed the evaporation of the water. The product which was a solid, blackened before it could be isolated.

(n) N-(N-Acetyl-O-acetyl-L-seryl)-1,3,4,6-tetra-O-acetyl-β-D-glucosamine

N-(L-Seryl hydrochloride)-β-D-glucosamine (10 mg) was acetylated with pyridine/acetic anhydride mixture (5 ml) at 0° for 2 days. The product was obtained as a syrup (10 mg) which had R_{GNpAc} 1.08 on paper chromatograms developed in the neutral solvent system. The infrared absorption spectrum of the product showed strong carbonyl of *O*-acetyl absorption at 1750 cm^{-1} but no band due to hydroxyl at 3460 cm^{-1}.

2. N-Glycyl-D-glucosamine

(a) N-Carbobenzoxyglycine

Glycine (25 g) was treated with carbobenzoxy chloride in toluene (60 g) using the method of Moore *et al.*, to give *N*-carbobenzoxyglycine (70 g).

(b) N-Carbobenzoxyglycyl p-Nitrophenyl Ester

N-Carbobenzoxyglycine (10 g) was dissolved in ethyl acetate (100 ml) containing *p*-nitrophenol (6.4 g) and dicyclohexycarbodiimide (9.2 g), and the mixture was kept at 0° for 1 hour and was then allowed to rise to room temperature. The precipitated dicyclohexyl urea by-product was removed by filtration and was washed free from the mother liquor with ethyl acetate. The combined filtrate and washings were concentrated and the ethanol solution of the residue gave the crystalline product (15 g; 95% yield) which had m.p. 127°. The infrared spectrum of the product showed the characteristic sharp absorption peaks due to the *p*-nitrophenyl group at 1775, 1635. 1600, 1540, 1500, 1355, and 695 cm^{-1}.

(c) N-(N-Carbobenzoxyglycyl)-1,3,4,6-tetra-O-acetyl-β-D-glucosamine

N-Carbobenzoxyglycyl *p*-nitrophenyl ester (1 g) was condensed with 1,3,4,6-tetra-*O*-acetyl-β-D-glucosamine hydrobromide (1.25 g) in the presence of dimethylformamide containing triethylamine (0.3 g) by Bodanszky and Du Vigneaud's (16) procedure to give the product, obtained as crystals (0.9 g) from methanol solution, which had m.p. 168°, and $[\alpha]_D^{22}$ +49° (*c*, 4.0 methanol).

The infrared spectrum of the product resembled that of the corresponding L-serine derivative (1*j*) showing absorption bands due to amide at 1675 cm^{-1} and carbonyl of *O*-acetyl at 1750 cm^{-1}. The "fingerprint" regions of the spectra were also similar, with peaks at 1375, 1250, 1058, and 695 cm^{-1}.

(d) N-(N-Carbobenzoxyglycyl)-D-glucosamine

N-(*N*-Carbobenzoxyglycyl)-1,3,4,6-tetra-*O*-acetyl-β-D-glucosamine (0.5 g) in dry methanol (10 ml) was treated with 0.4 *N* barium methylate (1 ml) at −5° for 20 hours and the deacetylated product was isolated as described for (1*k*), as crystals (0.2 g) which had m.p. 188° and $[\alpha]_D^{24}$ +39.5° (*c*, 4.0 methanol).

(e) N-(Glycyl hydrochloride)-D-glucosamine

N-(*N*-Carbobenzoxyglycyl)-D-glucosamine (0.15 g), dissolved in dioxan/water mixture (1:1 v/v) containing palladized charcoal (0.1 g), was placed under a slight pressure of hydrogen (2 lb above atmospheric pressure) and shaken for 24 hours. Hydrochloric acid (1 ml, 10%) was added to the mixture, the charcoal removed by filtration, and the filtrate was concentrated *in vacuo* to a syrup. The syrup was dissolved in hot ethanol from which the product was obtained as crystals (70 mg) which had m.p. 172° and $[\alpha]_D^{23}$ +31.5° (*c*, 1.0 water). Reported constants (8) for the product obtained by a different route are: m.p. 170° and $[\alpha]_D^{18}$ +29.7° (water).

The product was detected on paper chromatograms, developed in the neutral solvent system, by the ninhydrin and silver nitrate spray reagents as a single spot which had $R_{GN.HCl}$ 0.53.

(f) N-Glycyl-D-glucosamine

Attempts to remove the carbobenzoxy group from *N*-(*N*-carbobenzoxyglycyl)-1,3,4,6-tetra-*O*-acetyl-β-D-glucosamine and *N*-(*N*-carbobenzoxyglycyl)-D-glucosamine by hydrogenolysis in neutral solution, to form the free base, failed owing to the rapid decomposition of the product when it was exposed to the atmosphere. Attempts were made to deionize the salt (2*e*) with Amberlite IR 45($CO_3^=$) exchange resin but the product decomposed immediately on isolation.

3. N-L-Glutamyl-D-glucosamine

(a) N-Carbobenzoxy-L-glutamic Acid

L-Glutamic acid (25 g) was treated with 75% carbobenzoxychloride in toluene (44 ml) according to the procedure of Moore *et al.* (14), to give the crystalline product (51 g; 95% yield), which had m.p. 124°.

(b) N-Carbobenzoxy-L-glutamic Acid Di-p-nitrophenyl Ester

The ester was prepared by treating *N*-carbobenzoxy-L-glutamic acid (21 g) dissolved in ethyl acetate (200 ml) with *p*-nitrophenol (26 g) and dicyclohexylcarbodiimide (32 g) for 30 minutes at 0°. The reaction mixture was then kept at room temperature for 1 hour, the precipitated dicyclohexyl urea by-product was filtered off, and the filtrate was concentrated to a syrup which was dissolved in hot ethanol. On cooling, the crystalline product was obtained (21 g), which had m.p. 210° and $[\alpha]_D^{23}$ +17.1° (*c*, 4.0 benzene).

The infrared spectrum of the product showed no absorption due to hydroxy group, but showed bands due to the *p*-nitrophenyl group at 1775, 1540, 1500, 1355, and 1135 cm^{-1}.

(c) N-Carbobenzoxy-γ-L-glutamide α-p-Nitrophenyl Ester

The diester (3*b*) (10 g) dissolved in benzene/methanol mixture (100 ml; 1:1 v/v) was treated with the calculated amount of *N* methanolic ammonia, sufficient to react with one ester group, and the solution was allowed to stand overnight at room temperature. A small amount of precipitated material, identified as the diamide derivative, was removed and on the addition of benzene the crystalline monoamide was obtained (4.6 g; 60% yield) which after recrystallization from methanol had m.p. 158° and $[\alpha]_D^{21}$ −25.1° (*c*, 4.0 DMF). Anal. Found: N, 10.6%. $C_{19}H_{19}O_7N_3$ requires: N, 10.5%.

(d) 1-(1′,3′,4′,6′-Tetra-O-acetyl-β-D-glucosaminyl)-2-N-carbobenzoxy-L-glutamine

1,3,4,6-Tetra-*O*-acetyl-β-D-glucosamine hydrobromide (0.4 g) and the monoamide ester (3*c*) (0.4 g) dissolved in dimethylformamide (2 ml) were treated with excess triethylamine (0.2 g) at 95° for 20 minutes and the reaction mixture was then cooled to room temperature. Ether (4 ml) was added to precipitate the triethylamine hydrobromide which was removed by filtration, and the filtrate was then washed with *N* hydrochloric acid (3×20 ml) followed by water until the yellow color due to *p*-nitrophenol was removed. An ethanol solution of the product, diluted with chloroform, was dried (anhyd. sodium sulphate) and concentrated to a syrup which was dissolved in ethyl acetate and ether was added. The product was obtained as crystals (0.23 g) from the cooled solution which had, after recrystallization from ethanol, m.p. 178° and $[\alpha]_D^{20}$ −6.0° (*c*, 4.0 pyridine). The infrared spectrum of the product showed absorption bands in common with the D-glucosamine reactant at 1750, 1480, 1250, and 1050 cm^{-1}, and in common with the substituted amino acid at 3360, 1650, 1530, 1460, 1090, 1050, 900, and 695 cm^{-1}. The characteristic *p*-nitrophenyl bands were absent.

(e) 1-(1′,3′,4′,6′-Tetra-O-acetyl-β-D-glucosaminyl)-2-N-carbobenzoxy-L-glutamic Acid

The condensation product (3*d*) (50 mg) was treated with an excess of nitrous acid and when the reaction had subsided the solution was diluted with water and extracted with chloroform (3×10 ml). The dried (anhyd. sodium sulphate) chloroform extract was concentrated to a syrup (41 mg) which was purified by cellulose column partition chromatography using 1-butanol half saturated with water as the mobile phase. The final product (30 mg), obtained crystalline from ethanol solution, had m.p. 218° (decomp.) and $[\alpha]_D^{18}$ −7.4° (*c*, 2.0 pyridine).

(f) 1-(N-D-Glucosaminyl)-2-N-carbobenzoxy-L-glutamic Acid

The compound (3*e*) (30 mg) was treated with barium methoxide in the usual way to yield the deacetylated product (9 mg), which had m.p. 184°, and $[\alpha]_D^{18}$ +70° (*c*, 1.0 pyridine).

(g) 1-(N-D-Glucosaminyl)-L-glutamic Acid Hydrochloride

The deacetylated product (3*f*) (63 mg) dissolved in aqueous methanol (4 ml) containing 2 *N* hydrochloric acid (2 ml) was reduced with hydrogen in the presence of palladized charcoal as described for (1*l*). The product, crystallized from ethanol – light petroleum (b.p. 60–80°) mixture (30 mg), had $[\alpha]_D^{24}$ +14.1° (*c*, 1.5 water) and decomposed above 200°. Anal. Found: C, 37.9; H, 6.2; N, 8.0; Cl, 10.0%. $C_{11}H_{21}N_2O_8Cl$ requires: C, 38.3; H, 6.1; N, 8.1; Cl, 10.3%.

*4. 1-O-β-*L*-Seryl-N-acetyl-*D*-glucosaminide*

*(a) 1-Chloro-1-deoxy-N-acetyl-3,4,6-tri-O-acetyl-α-*D*-glucosamine*

The compound was prepared in 25% yield by the method of Inouye (18) and his co-workers. It had m.p. 124° and $[\alpha]_D^{25}$ +116° (*c*, 4.0 chloroform).

*(b) N-Carbobenzoxy-*L*-seryl Methyl Ester*

The methyl ester was synthesized by the action of diazomethane on the carboxylic acid (20). *N*-Carbobenzoxy-L-serine (8 g) dissolved in an alcohol/ether mixture (50 ml; 1:1 v/v) was treated with a solution of diazomethane (3 g) in ether (100 ml) and after the esterification was completed, the excess diazomethane was removed with hydrogen chloride gas. The solution was deacidified with Duolite A4(OH^-) exchange resin and then concentrated under reduced pressure to give the product as a pale yellow syrup (7 g).

*(c) 1-O-β-(N-Carbobenzoxy-*L*-seryl methyl ester)-N-acetyl-*D*-glucosaminide*

The substituted *O*-glucosaminide was synthesized by the Koenigs and Knorr (17) procedure, followed by deacetylation and hydrogenolysis of the product. Dried samples of 1-chloro-1-deoxy-*N*-acetyl-3,4,6-tri-*O*-acetyl-α-D-glucosamine (3.23 g) and *N*-carbobenzoxy-L-seryl methyl ester (2.5 g) were dissolved in anhydrous alcohol-free chloroform (50 ml) containing Drierite (10 g). Silver oxide (3 g) and iodine (5 mg catalyst) were added and the reaction mixture was shaken in the dark at room temperature for 140 hours. The insoluble material was removed by filtration through a Celite pad, the residue washed with chloroform, and the combined filtrate and washings were concentrated to a syrup. Paper chromatographic examination of the syrup in the neutral solvent system revealed the product as the major component having R_{GNAc} 3.2. The syrup was fractionated by cellulose column partition chromatography using 1-butanol half saturated with water as the mobile phase to give the chromatographically pure product (1.78 g) as a syrup which could not be crystallized. The product was deacetylated in absolute methanol (30 ml) containing 0.4 *N* barium methylate (2 ml) for 22 hours at −5°. The syrupy product (0.94 g) was found by paper chromatography to contain the required compound, which had R_{GNAc} 1.88, and *N*-acetylglucosamine. The faster-moving component was isolated by cellulose column partition chromatography using 1-butanol half saturated with water as the mobile phase. The product was crystallized from ethanol/light petroleum (b.p. 60–80°) mixture (219 mg). It had R_{GNAc} 1.89 and R_{GNDAc} 0.50, m.p. 133° and $[\alpha]_D^{23}$ +6.4° (*c*, 10 ethanol).

*(d) 1-O-β-(*L*-Seryl methyl ester)-N-acetyl-*D*-glucosaminide*

1-*O*-β-(*N*-Carbobenzoxy-L-seryl methyl ester)-*N*-acetyl-D-glucosaminide (140 mg) dissolved in aqueous ethanol (4 ml) containing palladized charcoal (200 mg), was placed under a slight pressure of hydrogen and shaken for 24 hours. The charcoal was removed by filtration and the filtrate was concentrated to a syrup (100 mg) which was shown by paper chromatography to contain the product contaminated with *N*-acetylglucosamine. The syrup was fractionated by chromatography on large sheets of Whatman 3MM paper in the neutral solvent system to give the pure derivative (60 mg) which had $R_{GN.HCl}$ 0.28, m.p. 230° (decomp.) and $[\alpha]_D^{24}$ +1.7° (*c*, 1.0 methanol).

The substituted D-glucosaminide was much more stable in air than the corresponding *N*-peptide.

*(e) 1-O-β-(*L*-Seryl methyl ester hydrochloride)-N-acetyl-*D*-glucosaminide*

1-*O*-β-(*N*-Carbobenzoxy-L-seryl methyl ester)-*N*-acetyl-D-glucosaminide (25 mg) was treated in the cold with 0.1 *N* hydrochloric acid (5 ml) for 6 hours, the excess acid was removed on Duolite A4(OH^-) exchange resin and the solution concentrated to dryness.

The product was twice recrystallized from ethanol/light petroleum (b.p. 60–80°) mixture. Anal. Found: C, 40.5; H, 6.7; N, 8.1; Cl, 9.8%. $C_{12}H_{23}N_2O_8Cl$ requires: C, 40.2; H, 6.4; N, 7.8; Cl, 9.90%.

The infrared spectrum of the product supported the assigned structure and showed absorption peaks at 1750 cm^{-1} due to ester carbonyl, 1610 cm^{-1} due to *N*-acetyl, and an ether-type absorption peak at 1100 cm^{-1}.

*(f) 1-O-β-*L*-Seryl-N-acetyl-*D*-glucosaminide*

The glucosaminide methyl ester (4*d*) (48 mg) dissolved in methanol (0.5 ml) was diluted with water (2 ml) and 0.5 *N* sodium hydroxide solution (3 ml) was slowly added and the saponification was allowed to proceed for 3 hours at room temperature. The solution was made slightly acidic by the addition of 0.1 *N* sulphuric acid (0.24 ml) and the solution was concentrated under reduced pressure. The residue was extracted with methanol from ionic material and the concentrated extract was dissolved in ethanol/light petroleum (b.p. 60–80°) mixture which on cooling gave the crystalline product (27 mg) which had m.p. 236°, and $[\alpha]_D^{24}$ −31.0° (*c*, 1.0 water). Anal. Found: C, 42.85; H, 6.4; N, 9.0%. $C_{11}H_{20}N_2O_8$ requires: C, 42.9; H, 6.5; N, 9.1%.

The infrared absorption spectrum of the product showed no absorption peak due to ester group at 1750 cm^{-1} but the strong absorption band at 1610 cm^{-1} due to *N*-acetyl was present.

DISCUSSION

The object of this investigation was to determine the properties of D-glucosamine derivatives of some amino acids, particularly their stability in solution.

The prepared derivatives were dissolved in acidic media in order to investigate their behavior under conditions of acid hydrolysis. The *N*-peptide hydrochlorides were found to be unaffected by *N* hydrochloric acid at 60° for 24 hours, but the 1-*O*-β-L-seryl-D-glucosaminide gave L-serine, *N*-acetyl-D-glucosamine, and D-glucosamine after 1 hour under the same conditions.

The *N*-peptides were stable in neutral or basic media but degraded rapidly in the presence of air, and immediately upon isolation. It is apparent that the *N*-peptides may only be obtained as their salts, from acid media, whereas the D-glucosaminides examined are relatively unstable in acid solution and should be separated only from neutral or basic media.

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