THE CARBOHYDRATE–PROTEIN LINKAGE IN GLYCOPROTEINS

PART II. THE SYNTHESIS OF N-L-SERYL-D-GLUCOSAMINE AND N-L-THREONYL-D-GLUCOSAMINE

J. K. N. JONES, J. P. MILLINGTON, AND M. B. PERRY

Department of Organic Chemistry, Queen's University, Kingston, Ontario

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ABSTRACT

N-L-Seryl-D-glucosamine has been prepared by the condensation of *N*-carbobenzoxy-L-serine with D-glucosamine in the presence of dicyclohexyl carbodiimide followed by hydrogenolysis of the resulting *N*-(*N*-carbobenzoxy-L-seryl)-D-glucosamine to remove the *N*-carbobenzoxy protecting group.

N-L-Threonyl-D-glucosamine has been prepared by (*a*) the condensation of 1,3,4,6-tetra-*O*-acetyl-α- (and β-) D-glucosamine and *N*-carbobenzoxy-*O*-tetrahydropyranyl-L-threonine in the presence of diisopropyl carbodiimide followed by the removal of the protecting groups from the resulting *N*-(*N*-carbobenzoxy-*O*-tetrahydropyranyl-L-threonyl)-1,3,4,6-tetra-*O*-acetyl-α- (and β-) D-glucosamine, and (*b*) by the condensation of *N*-carbobenzoxy-L-threonine with D-glucosamine in the presence of dicyclohexyl carbodiimide followed by removal of the *N*-carbobenzoxy group from the resulting *N*-(*N*-carbobenzoxy-L-threonyl)-D-glucosamine by hydrogenolysis.

INTRODUCTION

As part of a program in this laboratory aimed at the determination of the nature of the carbohydrate–peptide bonds occurring in natural glycoproteins (1) the synthesis of model amino acid – glycose compounds linked by *N*-glycoside, *O*-glycoside, ester, and amide bonds is being undertaken. A study of the stability of these model compounds and of the conditions required for their successful paper chromatographic and electrophoretic separation will aid in the selection of experimental conditions required for the isolation of such derivatives from glycoproteins. The derivatives may subsequently be of use as reference compounds for the identification of the hydrolytic degradation products of glycoproteins and mucoproteins.

This paper records the synthesis of two *N*-amino acyl derivatives of D-glucosamine. The original methods used in the synthesis of this type of compound involved the reaction of *N*-carbobenzoxy amino acyl chlorides with fully *O*-acetylated 2-amino-2-deoxyaldohexoses (2) or the condensation of suitably protected 2-amino-2-deoxyhexoses with a *N*-carbobenzoxy amino acid derivative by the carbodiimide method (1, 3, 4). In either case the protecting groups must be subsequently removed in order to obtain the unsubstituted *N*-amino acyl aminohexose compounds.

A simple and convenient method for the preparation of amino acyl derivatives of glycoses has recently been reported by Kochetkov and his co-workers (5, 6) which involves the condensation of an unprotected glycose with a *N*-carbobenzoxy amino acid in the presence of carbodiimide. It was found that *N*-carbobenzoxy amino acids condensed readily with 2-amino-2-deoxyhexoses in the presence of dicyclohexyl carbodiimide in aqueous pyridine solution at room temperature to form *N*-(*N*-carbobenzoxy amino acyl)-hexosamines, the exclusive formation of the *N*-substituted derivatives under these conditions being attributed to the specific action of carbodiimide and the instability of *O*-acyl derivatives in aqueous pyridine solution. This procedure has now been applied to the synthesis of *N*-seryl-D-glucosamine and *N*-threonyl-D-glucosamine.

EXPERIMENTAL

All melting points were determined using the Fisher–Johns block and are uncorrected. Solutions were concentrated by rotary evaporation under reduced pressure below 40° C.

Paper chromatography was performed by the descending method on Whatman No. 1 paper, using butan-1-ol/ethanol/water (3:1:1 v/v). The following spray reagents were used to detect the compounds on the developed chromatograms: (A) 2% solution of *p*-anisidine hydrochloride in butan-1-ol (7), (B) 1% solution of silver nitrate in acetone, followed by 2% ethanolic sodium hydroxide solution (8), and (C) 2% ninhydrin in butan-1-ol (9). The rate of movement of the compounds on the chromatograms is given relative to that of D-glucosamine hydrochloride ($R_{GN.HCl}$) or *N*-acetyl-D-glucosamine (R_{GNAc}).

The infrared spectra of the compounds were measured as 0.8% dispersions in potassium bromide disks, or as 6% solutions in chloroform or methylene chloride, using the Perkin–Elmer Model 21 spectrophotometer.

1. N-L-Seryl-D-glucosamine

(a) N-(N-Carbobenzoxy-L-seryl)-D-glucosamine

The procedure of Kochetkov and his co-workers was followed (5, 6). D-Glucosamine hydrochloride (2.15 g, 0.01 mole) was dissolved in water (5 ml) and to the cooled, mechanically stirred solution was added 2 *N* sodium hydroxide solution (5 ml) followed by *N*-carbobenzoxy-L-serine (2.39 g, 0.01 mole) dissolved in dry redistilled pyridine, and dicyclohexyl carbodiimide (3.09 g, 0.015 mole) dissolved in pyridine (20 ml). The solution was allowed to warm to room temperature and stirring was continued for 24 hours. The precipitated dicyclohexyl urea (ca. 0.01 mole) was removed by filtration and the unreacted dicyclohexyl carbodiimide was removed by extraction with ether (2×50 ml).

The aqueous solution was concentrated to a small volume and the product which crystallized out was collected by filtration and was then recrystallized from methanol solution. The *N*-(*N*-carbobenzoxy-L-seryl)-D-glucosamine (38% yield) had m.p. 217° C (decomp.) and $[\alpha]_D^{23}$ +73.5° (*c*, 4.0 chloroform).

The compound had R_{GNAc} 2.09 on paper chromatograms and was free from D-glucosamine and L-serine. The infrared spectrum exhibited absorption bands at 3460 cm^{-1} (s, hydroxyl) and 1682 and 1495 cm^{-1} (amide). Anal. Found: C, 50.7; H, 6.5; N, 6.8%. $C_{17}H_{24}O_9N_2$ requires: C, 50.99; H, 6.04; N, 7.0%.

(b) N-(L-Seryl hydrochloride)-D-glucosamine

The *N*-carbobenzoxy group was removed from the *N*-(*N*-carbobenzoxy-L-seryl)-D-glucosamine by hydrogenolysis in dilute hydrochloric acid solution using palladized charcoal as a catalyst according to the procedure of Jones *et al.* (1). The *N*-(L-seryl hydrochloride)-D-glucosamine, obtained as fine crystals from methanol/ether mixture (85% yield), had $R_{GN.HCl}$ 0.55 on paper chromatograms and its infrared spectrum exhibited absorption bands at 3460 cm^{-1} (wide, hydroxyl), 1682 cm^{-1} (amide), and 1495 cm^{-1} (ionic nitrogen). Attempts to prepare the free amine failed since the initial product blackened before it could be isolated.

2. N-L-Threonyl-D-glucosamine

Method A

(a) N-Carbobenzoxy-L-threonine

N-Carbobenzoxy-L-threonine was prepared in 90% yield by the method of Moore *et al.* (3). The product after recrystallization from ethyl acetate had m.p. 102° C and $[\alpha]_D^{21}$ −4.3° (*c*, 4.25 acetic acid). Anal. Found: C, 57.0; H, 5.9; N, 5.6%. $C_{12}H_{15}O_5N$ requires: C, 56.91; H, 5.97; N, 5.53%.

(b) Methyl N-Carbobenzoxy-L-threonate

N-Carbobenzoxy-L-threonine (8.0 g) was dissolved in 2.5% methanolic hydrogen chloride solution (100 ml), and after 12 hours at room temperature the solution was neutralized by passage through a column of Duolite A4 (OH^-) ion-exchange resin. The solution was concentrated to a syrup which gave crystalline methyl *N*-carbobenzoxy-L-threonate (89% yield) having m.p. 90° C and $[\alpha]_D^{20}$ −16.1° (*c*, 4.25 methanol). Anal. Found: C, 57.9; H, 6.5; N, 5.3%. $C_{13}H_{17}O_5N$ requires: C, 58.42; H, 6.41; N, 5.24%.

(c) Methyl N-Carbobenzoxy-O-tetrahydropyranyl-L-threonate

The procedure of Iselin and Schwyzer (10) was followed. Hydrogen chloride (2 *N*) in anhydrous ethyl acetate (0.5 ml) was added to a solution of methyl *N*-carbobenzoxy-L-threonate (7.5 g) in purified 2,3-dihydro-4-pyran (4 g). The reaction mixture was kept at room temperature for 24 hours after which it was diluted with ether and then washed with dilute sodium bicarbonate solution followed by water. The ether solution was dried (anhyd. sodium sulphate), filtered, and then concentrated to a yellow oil (80% yield).

(d) Hydrolysis of Methyl N-Carbobenzoxy-O-tetrahydropyranyl-L-threonate

Methyl *N*-carbobenzoxy-*O*-tetrahydropyranyl-L-threonate (8.0 g) was dissolved in methanol (25 ml) and was then saponified by the addition of *N* methanolic sodium hydroxide solution (50 ml) according to the method of Iselin and Schwyzer (10) to yield *N*-carbobenzoxy-*O*-tetrahydropyranyl-L-threonine (4.21 g, 55% yield) as a yellow syrup which failed to crystallize. The syrup had equivalent weight 345 (theory 337) and its infrared spectrum showed absorption bands at 987 and 1125 cm^{-1} (tetrahydropyranyl group).

A second compound crystallized from the reaction product which had m.p. 183° C and equivalent weight 238. Anal. Found: C, 60.8; H, 5.7; N, 6.0%. $C_{12}H_{13}O_4N$ requires: C, 61.27; H, 5.57; N, 5.96%.

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

These derivatives were prepared by the method described by Jones *et al.* (1). α-Anomer: 65% yield, m.p. 118° C, $[\alpha]_D^{20}$ +145° (*c*, 2 chloroform); β-anomer: 65% yield, m.p. 143° C, $[\alpha]_D^{20}$ +27° (*c*, 2 chloroform).

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

N-Carbobenzoxy-*O*-tetrahydropyranyl-L-threonine (0.460 g), 1,3,4,6-tetra-*O*-acetyl-α-D-glucosamine (0.510 g), and diisopropyl carbodiimide (0.178 g) were dissolved in methylene chloride (6 ml) and the mixture was shaken mechanically at room temperature for 24 hours. The precipitated diisopropyl urea by-product was removed by filtration and the filtrate was washed with dilute hydrochloric acid, dilute sodium bicarbonate solution, and finally with water. The dried (anhyd. magnesium sulphate) methylene chloride solution on concentration gave a white solid (5% yield) which had m.p. 185° C and $[\alpha]_D^{20}$ +74.4° (*c*, 0.5 methanol).

The above procedure was repeated using 1,3,4,6-tetra-*O*-acetyl-β-D-glucosamine to give the β-anomer as a syrup (50% yield) which failed to crystallize. It had $[\alpha]_D^{20}$ +12.3° (*c*, 4.0 methanol).

The infrared absorption spectra of both the above products exhibited absorption bands at 1745 cm^{-1} (carbonyl, *O*-acetyl), 1670 cm^{-1} (amide I), 1555 cm^{-1} (amide II), 1130 and 987 cm^{-1} (tetrahydropyranyl group), and 695 cm^{-1} (phenyl group).

(g) N-(N-Carbobenzoxy-L-threonyl)-1,3,4,6-tetra-O-acetyl-β-D-glucosamine

N-(*N*-Carbobenzoxy-*O*-tetrahydropyranyl-L-threonyl)-1,3,4,6-tetra-*O*-acetyl-β-D-glucosamine (0.50 g) was heated in boiling 50% acetic acid solution (10 ml) for 10 minutes and the cooled solution was diluted with water and extracted with chloroform (4×20 ml). The dried (anhyd. magnesium sulphate) chloroform extract was concentrated to dryness to yield a syrup (0.25 g, 58% yield) which had $[\alpha]_D^{18}$ +30° (*c*, 3.0 chloroform) and whose infrared spectrum did not show absorption bands due to the tetrahydropyranyl group at either 1130 or 987 cm^{-1}.

(h) N-(N-Carbobenzoxy-L-threonyl)-D-glucosamine

N-(*N*-Carbobenzoxy-L-threonyl)-1,3,4,6-tetra-*O*-acetyl-β-D-glucosamine (100 mg) dissolved in dry methanol (4 ml) was treated with 0.4 *N* barium methoxide in methanol (2 ml) and the mixture was kept at 5° C for 24 hours. Slightly less than the equivalent amount of 0.01 *N* sulphuric acid was added to the reaction mixture, the precipitated barium sulphate was removed by filtration, and the filtrate was further deionized by passage through a column of mixed IR 120 (H^+) and Duolite A4 (OH^-) ion-exchange resins. On concentration of the methanol solution, *N*-(*N*-carbobenzoxy-L-threonyl)-D-glucosamine was obtained as a white solid (30 mg). It had m.p. 209° (decomp.) and $[\alpha]_D^{14}$ +20.4° (*c*, 2.8 methanol). On paper chromatography and detection with spray reagents A and B the compound was revealed as a single spot having R_{GNAc} 2.90 and was not contaminated with either D-glucosamine or L-threonine.

The infrared spectrum of the compound exhibited absorption bands at 3360 cm^{-1} (broad, hydroxyl group) and 1670 and 1550 cm^{-1} (amide) but no absorption band at 1745 cm^{-1} (carbonyl, *O*-acetyl).

Method B

(a) N-(N-Carbobenzoxy-L-threonyl)-D-glucosamine

The procedure of Kochetkov and his co-workers (5, 6) was used. D-Glucosamine hydrochloride (2.15 g, 0.01 mole) dissolved in water (5 ml) was cooled to 0° C and neutralized by the addition of 2 *N* sodium hydroxide solution (5 ml). The mechanically stirred solution was immediately treated with a solution of *N*-carbobenzoxy-L-threonine (2.53 g, 0.01 mole) in dry pyridine (7 ml) followed by a solution of dicyclohexyl carbodiimide (3.09 g, 0.015 mole) in dry pyridine (20 ml) and the stirring was continued for a further 24 hours. The precipitated dicyclohexyl urea was removed by filtration and the unreacted dicyclohexyl carbodiimide was removed by extraction with ether (2×50 ml). The *N*-(*N*-carbobenzoxy-L-threonyl)-D-glucosamine isolated from the concentrated aqueous solution was obtained as crystals which, after several recrystallizations from methanol (final yield 0.415 g), had m.p. 209° C (decomp.) and $[\alpha]_D^{20}$ +21.0° (*c*, 3.0 methanol). The compound was indistinguishable on paper chromatograms from the product obtained by method A and the infrared spectra of the two products were identical. Anal. Found: C, 51.7; H, 6.2; N, 6.7%. $C_{18}H_{26}O_9N_2$ requires: C, 52.17; H, 6.32; N, 6.76%.

(b) N-L-Threonyl-D-glucosamine

N-(*N*-Carbobenzoxy-L-threonyl)-D-glucosamine (25 mg) was dissolved in chloroform/ethanol mixture, and palladized charcoal (25 mg) was added. The mixture was shaken for 24 hours at room temperature in an atmosphere of hydrogen (1.3 atm). The catalyst was removed by filtration and the filtrate was concentrated to a dark yellow syrup (7 mg). On paper chromatography a single spot, which had $R_{GN.HCl}$ 0.44, was revealed by the ninhydrin spray whereas the silver nitrate spray revealed a series of spots indicating the presence of decomposition products.

(c) N-(L-Threonyl hydrochloride)-D-glucosamine

N-(*N*-Carbobenzoxy-L-threonyl)-D-glucosamine (28 mg) was subjected to hydrogenolysis as described above with the inclusion of *N* hydrochloric acid (0.7 ml) in the reaction mixture. The *N*-(L-threonyl

hydrochloride)-D-glucosamine (9.5 mg) was obtained as a clear syrup which failed to crystallize. The syrup had $[\alpha]_D^{20}$ −13° (*c*, 1.0 methanol) and on paper chromatograms it gave a single ninhydrin and silver nitrate positive spot having $R_{GN.HCl}$ 0.46. The infrared absorption spectrum of the compound showed a broad band at 3360 cm^{-1} due to hydroxyl and amino group absorption, but no bands due to the phenyl group corresponding to the original carbobenzoxy group.

Attempts to prepare the free amine from *N*-(L-threonyl hydrochloride)-D-glucosamine using Duolite A4 ($CO_3^=$) ion-exchange resin or Duolite A4 (OH^-) resin failed owing to the rapid decomposition of the initial product.

DISCUSSION

N-(L-Seryl hydrochloride)-D-glucosamine was prepared according to the general procedure of Kochetkov and his co-workers (5, 6). D-Glucosamine was condensed with *N*-carbobenzoxy-L-serine in aqueous pyridine solution in the presence of dicyclohexyl carbodiimide to give *N*-(*N*-carbobenzoxy-L-seryl)-D-glucosamine, from which the protecting *N*-carbobenzoxy group was removed by hydrogenolysis in the presence of dilute hydrochloric acid to yield *N*-(L-seryl hydrochloride)-D-glucosamine in 32% overall yield. The compound was stable in aqueous solution, but attempts to prepare the free *N*-L-seryl-D-glucosamine by careful neutralization with dilute alkali or by the use of mild basic ion-exchange resins failed owing to the rapid decomposition of the free amine under the conditions used.

The *N*-(L-seryl hydrochloride)-D-glucosamine was chromatographically identical with, and had an infrared spectrum indistinguishable from that of, the same derivative prepared earlier by Jones *et al.* (1) in greatly reduced overall yield by the condensation of *N*-carbobenzoxy-*O*-tetrahydropyranyl-L-serine with 1,3,4,6-tetra-*O*-acetyl-D-glucosamine in the presence of diisopropyl carbodiimide to give *N*-(*N*-carbobenzoxy-*O*-tetrahydropyranyl-L-seryl)-1,3,4,6-tetra-*O*-acetyl-D-glucosamine, from which the protecting groups were subsequently removed to yield the required product.

N-(L-Threonyl hydrochloride)-D-glucosamine was prepared in low overall yield by the condensation of *N*-carbobenzoxy-*O*-tetrahydropyranyl-L-threonine with 1,3,4,6-tetra-*O*-acetyl-D-glucosamine in the presence of diisopropyl carbodiimide to give *N*-(*N*-carbobenzoxy-*O*-tetrahydropyranyl-L-threonyl)-1,3,4,6-tetra-*O*-acetyl-D-glucosamine, from which the protecting groups were removed to yield the amino acyl-glycosamine derivative.

An unexpected crystalline product was obtained from the saponification product of methyl-*N*-carbobenzoxy-*O*-tetrahydropyranyl-L-threonate which had an equivalent weight of 238 and whose analysis agrees with the formula $C_{12}H_{13}O_4N$. The compound is tentatively identified as *N*-carbobenzoxy-2-amino-buten-3-oic acid, possibly formed by the elimination of a molecule of water from *N*-carbobenzoxy-L-threonine.

A greatly improved yield of *N*-(L-threonyl hydrochloride)-D-glucosamine was achieved when the general procedure of Kochetkov *et al.* (5, 6) was used. D-Glucosamine was condensed with *N*-carbobenzoxy-L-threonine in aqueous pyridine solution in the presence of dicyclohexyl carbodiimide to give *N*-(*N*-carbobenzoxy-L-threonyl)-D-glucosamine, which on hydrogenolysis in the presence of dilute hydrochloric acid afforded *N*-(L-threonyl hydrochloride)-D-glucosamine in 5% overall yield. The final products obtained by the two different routes were chromatographically identical and their infrared spectra were indistinguishable from each other.

Attempts to prepare *N*-L-threonyl-D-glucosamine by hydrogenolysis of the *N*-(*N*-carbobenzoxy-L-threonyl)-D-glucosamine in neutral media, or by neutralization of the *N*-(L-threonyl hydrochloride)-D-glucosamine by dilute alkali or by using mild basic ion-exchange resins failed to give a pure product owing to the rapid decomposition of the initial free amine. The hydrochloride salt, however, was stable in aqueous solution.

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