EtOH gave 0.2 g. of colorless plates, m.p. 187~192° (decomp.), $[\alpha]_D^{16}$ +30 (c=0.5, H_2O). *Anal.* Calcd. for $C_{10}H_{19}O_6N_3 \cdot 2.5H_2O$: C, 37.29; H, 7.45; N, 13.05. Found : C, 37.03; H, 7.03; N, 13.03.

The authors are very grateful to Chugai Seiyaku Co., Ltd. for kindly furnishing glucosamine HCl and to Mr. Matsui and Miss Soeda for spectral analyses, and to Mr. Shindo and Mr. Ishimura for microanalyses.

Summary

2-Acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucosylamine (Ⅳ) was prepared by the hydrogenation of 2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucosyl azide (Ⅲ) which was obtained by the treatment of acetochloroglucosamine with NaN_3 in formamide at 85°. Ⅳ was characterized by the acetylation as N-acetyl-2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucosylamine (Ⅵ). Ⅳ in tetrahydrofuran or pyridine was condensed in the presence of dicyclohexylcarbodiimide with *p*-nitrobenzoic acid, isonicotinic acid, aceturic acid, hippuric acid and N-benzyloxycarbonyl glycine to yield the corresponding amides, followed by the removal of the protecting groups. N-(*p*-Aminobenzoyl)2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucosylamine was obtained by the hydrogenation of the corresponding *p*-nitrobenzamido derivative over Adams' catalyst.

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[Chem. Pharm. Bull. 13(9)1041~1046(1965)]

UDC 547.457.1.07

136. Akira Yamamoto, Chieko Miyashita, and Hisao Tsukamoto : Studies on Amino Sugars. II.*1 Preparation of N-[L-α (and β)-Aspartyl]-2-acetamido-2-deoxy-β-D-glucosylamine and N-(L-γ-Glutamyl)-2-acetamido-2-deoxy-β-D-glucosylamine.*2

(*Faculty of Pharmaceutical Sciences, Kyushu University**3)

It has recently been proposed[1] for the structure of the carbohydrate-peptide bond in natural glycoproteins that the polysaccharide moiety would be attached to the amide group of asparagine residue of peptide chain through the reducing end of N-acetylglucosamine. In the previous paper of this series,*1 the preparation of six acyl derivatives of 2-acetamido-2-deoxy-β-D-glucosylamine, which was of interest in connection with the above-proposed structure, has been described. They were 2-acetamido-2-deoxy-β-D-glucosyl-amides of acetic, *p*-nitrobenzoic, isonicotinic, aceturic, hippuric acid and glycine. This paper, furthermore, reports the synthesis of three N-acyl, *i.e.*, β-aspartyl (Ⅷ), α-aspartyl (Ⅸ) and γ-glutamyl (Ⅹ) derivatives of 2-acetamido-2-deoxy-β-D-glucosylamine that were considered to be the most likely structure in the natural glycoproteins.

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*1 Part Ⅰ : This Bulletin, **13**, 1036 (1965).

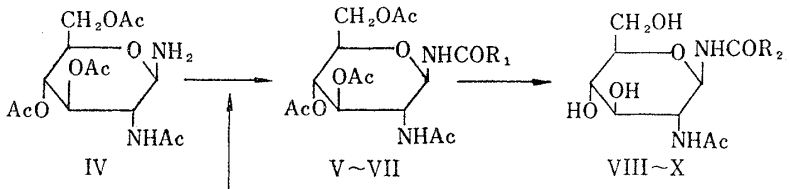
*2 Presented at the 33rd Meeting of Kyushu Branch of Pharmaceutical Society of Japan, July, 1963, Fukuoka.

*3 Katakasu, Fukuoka (山本 陽, 宮下智恵子, 塚元久雄).

1) See the Part Ⅰ of this series.

During the cource of this study, a method for the preparation of β-aspartyl derivative (Ⅷ) was developed by Marks, *et al.*[2]

The first intermediates were amino acids with protecting groups. Pure N-benzyloxycarbonyl-L-aspartic acid α-benzyl ester (I), plate, m.p. 85°, was synthesized *via* N-benzyloxycarbonyl-L-aspartic anhydride by the method of Marks, *et al.*[3] and after careful purification from its β-isomer it was characterized by the authentic specimen.[4] N-Benzyloxycarbonyl-L-aspartic acid β-benzyl ester (Ⅱ), m.p. 108°, was synthesized by the benzyloxycarbonylation of pure L-aspartic acid β-benzyl ester according to the method of Izumiya, *et al.*[4] and characterized by the authentic specimen.*4 N-Benzyloxycarbonyl-L-glutamic acid α-benzyl ester (Ⅲ) was synthesized from its dicyclohexylamine salts *via* N-benzyloxycarbonyl-L-glutamic anhydride by the method of Klieger, *et al.*[5]



I : R_3COOH
II : R_4COOH
III : R_5COOH

V : $R_1=R_3$
VI : $R_1=R_4$
VII : $R_1=R_5$

$R_3=-CH_2CHCOOCH_2Ph$ (NHCbz)

$R_4=-CHCH_2COOCH_2Ph$ (NHCbz)

$R_5=-CH_2CH_2CHCOOCH_2Ph$ (NHCbz)

VIII : $R_2=-CH_2CHCOOH$ (NH_2)

IX : $R_2=-CHCH_2COOH$ (NH_2)

X : $R_2=-CH_2CH_2CHCOOH$ (NH_2)

Ac = $-COCH_3$ Ph = $-C_6H_5$ Cbz = $-COOCH_2C_6H_5$

Chart 1.

The next step involved the condensation of the amino acid derivatives with 2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucosylamine (Ⅳ) in the presence of dicyclohexylcarbodiimide (DCC) to give the glycopeptide acetates. The analogous method as described in the previous paper[1] was undertaken, except that the reaction mixture was evaporated, extracted with chloroform and thoroughly washed with acid, alkali and water, successively. Then three glycopeptide acetates were easily obtained, *i.e.*, 1-(α-benzyl N-benzyloxycarbonyl-L-β-aspartamido)-2-acetamido-1,2-dideoxy-3,4,6-tri-O-acetyl-β-D-glucose (Ⅴ), m.p. 216~217°; 1-(β-benzyl N-benzyloxycarbonyl-L-α-aspartamido)-2-acetamido-1,2-dideoxy-3,4,6-tri-O-acetyl-β-D-glucose (Ⅵ), m.p. 187~188°; 1-(α-benzyl N-benzyloxycarbonyl-L-γ-glutamido)-2-acetamido-1,2-dideoxy-3,4,6-tri-O-acetyl-β-D-glucose (Ⅶ), 210~212°.

The removal of the protecting groups was considered in two different ways: the simultaneous removal of benzyl ester and O-acetyl group followed by the debenzyloxycarbonylation, or the simultaneous removal of benzyl ester and benzyloxycarbonyl group followed by the de-O-acetylation.

The former was attempted by Marks, *et al.*[2] who prepared Ⅷ by the treatment of Ⅴ with lithium hydroxide in aqueous acetone to cause simultaneous hydrolysis of

*4 These were kindly offered by Prof. Izumiya, Faculty of Sciences, Kyushu University.

2) G. S. Marks, R. D. Marshall, A. Neuberger: Biochem. J., **87**, 274 (1963).
3) G. S. Marks, A. Neuberger: J. Chem. Soc., **1961**, 4872.
4) N. Izumiya, S. Uchio, T. Yamashita: Nippon Kagaku Zasshi, **79**, 420 (1959).
5) E. Klieger, H. Gibian: Ann., **655**, 195 (1962).

the O-acetyl and the benzyl ester group and by the subsequent catalytic hydrogenation to remove the benzyloxycarbonyl group. The latter way was undertaken in this experiment to avoid transesterification with the formation of an α-methyl ester of aspartic moiety in the presence of magnesium methoxide in methanol.*[1,5]

Thus, the catalytic hydrogenation of V, VI, and VII in methanol over 8% palladium on charcoal by the usual method was followed by de-O-acetylation with 1% magnesium methoxide in methanol at 0°. Deacetylation was continued until only trace of ferric-hydroxamate-positive material[6] was present and the reaction was complete in 1.5 hr. for all three materials. The resulting mixture was purified through Dowex 50 (H^+) and Amberlite IRC-50 (H^+) column as described in the experimental part. Final ninhydrin-positive fractions were evaporated to dryness *in vacuo* to give crystalline materials.

In the case of VIII, after three recrystallization from cold aqueous ethanol, it gave colorless, triangular plates, VIIIa, m.p. 215~222° (decomp.), $[\alpha]_D$ +23.2, while from hot aqueous ethanol, it gave colorless needles, VIIIb, m.p. 264~266° (decomp.), $[\alpha]_D$ +26.4. The latter could be readily reconverted to original compound by recrystallization from cold aqueous ethanol. In this case, the solution of VIIIb was cooled at 0° and centrifuged and added to turbidity with ethanol and nucleated with VIIIa, otherwise it crystallized again as the mixture of VIIIa and VIIIb.

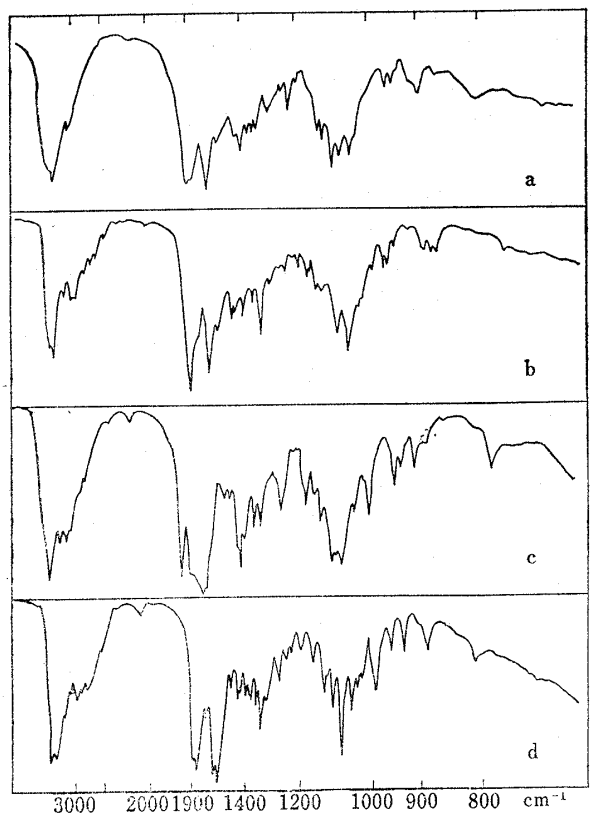


Fig. 1. Infrared Absorption Spectra (KBr)

a: N-(L-β-Aspartyl)-2-acetamido-2-deoxy-β-D-glucosylamine (Trihydrate)
b: *Ibid.* (Anhydrous form)
c: N-(L-α-Aspartyl)-2-acetamido-2-deoxy-β-D-glucosylamine (Monohydrate)
d: N-(L-γ-Glutamyl)-2-acetamido-2-deoxy-β-D-glucosylamine (Trihydrate)

From the results of elemental analyses, it was suggested that VIIIa was hydrates, which contained the crystal water of 2.5, 3, and 3.5 molecules according to the condition of drying and VIIIb was anhydrous form, however, no dehydration of VIIIa to VIIIb occurred on heating *in vacuo* at 130° for 12 hr. over phosphorus pentoxide. Furthermore, both compounds are similar in its behavior on paper electrophoresis in 5*N* AcOH and paper chromatography in butanol-acetic acid-water=12:3:5, but 3 μ and 6 μ band assigned in the infrared spectra of VIIIa may be attributed to crystal water as in Fig. 1. X-Ray powder diffraction patterns of both compound indicated complete differences of crystal structure. Finally it was observ-

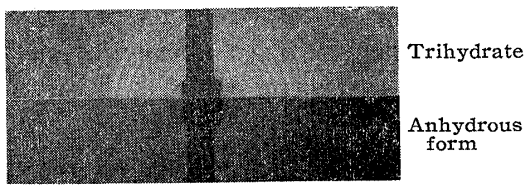


Fig. 2. X-Ray Diffraction Powder Patterns of N-(L-β-Aspartyl)-2-acetamido-2-deoxy-β-D-glucosylamine

Powder method: Cu Kα radiation, Ni filter, Camera diameter 5.027×2 cm.

6) D. R. Whitaker, M. E. Tate, C. T. Bishop: Can. J. Chem., **40**, 1885 (1962).

ed that the 2,4-dinitrophenyl derivatives of both compounds behaved chromatographically similar and were hydrolysed to dinitrophenyl-aspartic acid and free glucosamine.

Therefore the conclusion that can be drawn from above findings is that above conversion can be attributed to the presence of transition point at some temperature below which Ⅷa crystallized as the hydrate and above which it crystallized as the anhydrous form.

In the case of α-aspartyl and γ-glutamyl derivatives, the presence of the transition point in crystallization were not observed.

Marks, *et al.*,[2] prepared Ⅷa in the different procedure from us, found a small amount of contaminant together with the major product by paper chromatography and they discussed that a rearrangement from a β-aspartyl (Ⅷ) to an α-aspartyl derivative (Ⅸ) may have occurred and the contaminant may be α-aspartylglycopeptide (Ⅸ). These rearrangement has been also experienced in the conversion by alkaline hydrolysis of the ester of β-aspartyl peptides[7] into α and β mixtures of acidic aspartyl peptides.

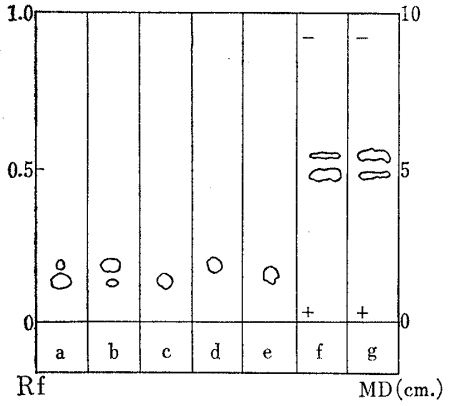


Fig. 3. Paper Chromatography and Paper Electrophoresis

Solvent: BuOH-AcOH-H_2O(12:3:5) for a~e, 5*N* AcOH f and g
a: Crude L-β-aspartamido derivative(Ⅷ)
b: Crude L-α-aspartamido derivative(Ⅸ)
c: Purified Ⅷ
d: Purified Ⅸ
e: L-γ-glutamido derivative (Ⅹ)
f: a
g: b

In this experiment, it was found that the crude β-aspartyl derivative (Ⅷ) contains two ninhydrin-positive substances on paper chromatogram and paper electrophoresis as in Fig. 3. Major product stained a specific blue color with ninhydrin reagent under LeQuesne's[8] condition. This was quite distinguishable from most amino acids and glucosamine and turned to faint green in the dark after a few days, although the color of most amino acids disappered in a few days. The other, minor product stained a purple color as same as most amino acids. The chromatographical behaviors of this material were completely similar with pure α-aspartyl peptide (Ⅸ). Furthermore, the crude α-aspartyl peptide (Ⅸ) contained a small amount of contaminant, which was found to be similar with β-aspartyl peptide (Ⅷ).

From these observations, it is considered that the rearrangement from a β-aspartyl to an α-aspartyl peptide or from α- to β-derivative occurred in alkaline de-O-acetylation. But, these crude products were purified to a chromatographically pure material by one recrystallization.

The configuration at C-1 of carbohydrate moiety of these compounds was suggested for the β-configuration by the low dextrorotatory rotation and by its mode of formation as discussed in the previous paper.*1 The above-consideration also may be supported by the presence of an absorption assignable to type 2b at 890~900 cm^{-1} in the infrared spectra of these compounds.

Experimental

1-(α-Benzyl N-Benzyloxycarbonyl-L-β-aspartamido)-2-acetamido-1,2-dideoxy-3,4,6-tri-O-acetyl-1, 2-dideoxy-β-D-glcose (Ⅴ)——To a solution of 0.45 g. of N-benzyloxycarbonyl-L-aspartic acid α-benzyl ester and 0.435 g. of 2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucosylamine in 37 ml. of dry tetrahydrofuran

7) A. R. Battery, J. C. Robinson: J. Chem. Soc., **1955**, 259.
8) W. J. LeQuesne, G. T. Young: *Ibid.*, **1952**, 24.

was added 0.3 g. of dicyclohexylcarbodiimide. The solution was stirred for 2 hr. at room temperature and allowed to stand overnight. A few drops of AcOH was added to the reaction mixture and stirred for a short time. Dicyclohexylurea separated was filtered off and the filtrate was evaporated to dryness under reduced pressure. The residue was extracted by $CHCl_3$ and filtered off to remove insoluble materials. The extracts were washed with cold dilute HCl and water, then aqueous $NaHCO_3$ and water. The solution was dried over Na_2SO_4 and was evaporated to dryness. The residue was recrystallized from MeOH or EtOH to give 0.59 g. of colorless needles, m.p. 214~215°. Several recrystallization from EtOH raised m.p. to 216~217°, $[\alpha]_D^{21}$ +10.5 (c=0.353, $CHCl_3$). *Anal.* Calcd. for $C_{33}H_{39}O_{13}N_3$: C, 57.81; H, 5.73; N,6.13. Found : C, 58.01; H, 5.80; N, 6.29.

N-(L-β-Aspartyl)-2-acetamido-2-deoxy-β-D-glucosylamine (VIII)——A solution of 0.60 g. of V in 95 ml. of MeOH was catalytically hydrogenated with 1.1 g. of 8% Pd-C at atmospheric pressure. Evolution of CO_2 ceased after 3.5 hr. and it was continued for a further 1.5 hr. The solution was filtered from the catalyst, which was washed with MeOH until the filtrate had a negative-ninhydrin test. The filtrate and washings were combined and evaporated under reduced pressure. The residue was dried over P_2O_5 overnight and subjected to the next procedure without further purification.

To a 4% solution of the residue, 0.33 g., in abs. MeOH was added an equivalent amount of 2% solution of $Mg(OCH_3)_2$ in abs. MeOH at 0°. The reaction was continued until ferric-hydroxamate test[6] became negative. After being kept at 0° for 1.5 hr., 10 ml. of water and 5 ml. of Dowex 50 (H^+) resin were added to the reaction mixture and vigorously stirred for 10 min. The mixture was poured into the column (0.9×6.2 cm.) of the same resin. The column was thoroughly washed with water and then, followed by the displacement elution with 0.15*N* aq. NH_3. The ninhydrin-positive fractions collected were passed through Amberlite IRC-50 column (0.9×6.2 cm.) to remove NH_3. The effluents were evaporated to dryness under reduced pressure to give crystalline materials. After three recrystallization from cold aqueous EtOH, it gave 0.16 g. of colorless plates, m.p. 215~222°(decomp.), but turned to brown at 205°, $[\alpha]_D^{24}$ +23.2 (c=1.5, H_2O), while from hot aqueous EtOH, it gave colorless needles, m.p. 264~266° (decomp.), $[\alpha]_D^{22}$ +23.6 (c=1.0, H_2O). *Anal.* Calcd. for $C_{12}H_{21}O_8N_3 \cdot 3H_2O$: C, 37.01; H, 6.94; N, 10.79. Found : C, 37.12; H, 7.30; N, 10.69. *Anal.* Calcd. for $C_{12}H_{21}O_8N_3$: C, 42.98; H, 6.31; N, 12.53. Found : C, 42.66; H, 6.45; N, 12.16.

1-(β-Benzyl N-Benzyloxycarbonyl-L-α-aspartamido)-2-acetamido-1,2-dideoxy-3,4,6-tri-O-acetyl-β-D-glucose (VI)——To a solution of 0.65 g. of N-benzyloxycarbonyl-L-aspartic acid β-benzyl ester and 0.63 g. of IV in 65 ml. of dry tetrahydrofuran was added 0.44 g. of DCC. After the same procedure as for V, recrystallization from EtOH gave 0.60 g. of VI, m.p. 187~188°, $[\alpha]_D^{36}$ +13.3 (c=0.750, $CHCl_3$). *Anal.* Calcd. for $C_{33}H_{39}O_{13}N_3$: C, 57.81; H, 5.73; N, 6.13. Found : C, 57.68; H, 5.71; N, 6.23.

N-(L-α-Aspartyl)-2-acetamido-2-deoxy-β-D-glucosylamine (IX)——A solution of 0.45 g. of VI in 72 ml. of MeOH was catalytically hydrogenated with 0.85 g. of 8% Pd-C at atmospheric pressure. After the same procedure as for VIII, 0.28 g. of the residue, N-(L-α-aspartyl)-2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucosylamine (VI′) was obtained.

To a 1% solution of this material in abs. MeOH was added an equal amount of 2% solution of $Mg(OCH_3)_2$ in abs. MeOH at 0°. The same procedure as for VIII was carried out except that the reaction mixture was neutralized with 20 ml. of Dowex 50 (H^+). The final residue was recrystallized from aqueous EtOH to give 0.18 g. of colorless needles, which did not melt below 300°, but turned brown at 230°, $[\alpha]_D^{36}$ +22.9 (c=0.915, H_2O). *Anal.* Calcd. for $C_{12}H_{21}O_8N_3 \cdot H_2O$: C, 40.8; H, 6.5; N, 11.9. Found : C, 40.96; H, 6.65; N, 11.84.

1-(α-Benzyl N-Benzyloxycarbonyl-L-γ-glutamido)-2-acetamido-1,2-dideoxy-3,4,6-tri-O-acetyl-β-D-glucose (VII)——To a solution of 0.50 g. of N-benzyloxycarbonyl-L-glutamic acid α-benzyl ester and 0.46 g. of IV in 41 ml. of dry tetrahydrofuran was added 0.33 g. of DCC. After the same procedure as for V, recrystallization from EtOH gave 0.39 g. of colorless needles, m.p. 210~212°, $[\alpha]_D^{20}$ +12.90 (c=0.43, $CHCl_3$). *Anal.* Calcd. for $C_{34}H_{41}O_{13}N_3$: C, 58.36; H, 5.91; N, 6.01. Found : C, 58.61; H, 5.95; N, 6.05.

N-(L-γ-Glutamyl)-2-acetamido-2-deoxy-β-D-glucosylamine (X)——A solution of 0.27 g. of VII in 30 ml. of MeOH was catalytically hydrogenated with 0.5 g. of 8% Pd-C at atmospheric pressure. After the same procedure as for VIII, 0.18 g. of the residue, N-(L-γ-glutamyl)-2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucosylamine (VII′) was obtained. The de-O-acetylation of this material was carried out by the same procedure as for VIII. After three recrystallization, it gave 0.10 g. of colorless needles, m.p. 185~190° (decomp.), $[\alpha]_D^{16}$ +18.0 (c=1.0, H_2O). *Anal.* Calcd. for $C_{13}H_{23}O_8N_3 \cdot 3H_2O$: C, 38.76; H, 7.20; N, 10.42. Found : C, 38.49; H, 7.37; N, 10.13.

Paper Chromatography and Paper electrophoresis——Irrigation was carried out by the ascending technique on Toyo-Roshi No. 51 paper. Solvent system was BuOH-AcOH-H_2O=12:3:5.[2] For paper electrophoresis, 5*N* AcOH was used with Toyo-Roshi No. 51 paper (12×24 cm.) at 800 v., 30 min. Three spray reagents were used : ninhydrin, chlorine-iodide-starch[9] and periodate-benzidine reagent. When

9) H. N. Rydon, P. W. G. Smith : Nature, **169**, 922 (1952).

paper sprayed with ninhydrin reagent (0.2% in 95 vol. of BuOH and 5 vol. of 2*N* AcOH) was dried rapidly in oven at 110°, β-aspartylglycopeptide always stained a specific blue color, otherwise, if at 80°, it was brown as reported by Marks, *et al.*[2]

The authors are very grateful to Prof. Izumiya, Faculty of Sciences, Kyushu University and his colleagues who gave us kindly pure sample of N-benzyloxycarbonyl-L-asparatic acid α-benzyl ester and N-benzyloxycarbonyl-L-aspartic acid β-benzyl ester and to Chugai Seiyaku Co., Ltd. for the supplying of glucosamine HCl. Thanks are due to the members of the Analysis Room of this Faculty for elemental and spectral analysis.

Summary

N-(L-β-Aspartyl)-2-acetamido-2-deoxy-β-D-glucosylamine has been prepared by the condensation of N-benzyloxycarbonyl-L-aspartic acid α-benzyl ester with 2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-β-D-glucosylamine in the presence of dicyclohexylcarbodiimide followed by hydrogenolysis and de-O-acetylation on the resulting 1-(α-benzyl N-benzyloxycarbonyl-L-β-aspartamido)-2-acetamido-1,2-dideoxy-3,4,6-tri-O-acetyl-β-D-glucose to remove the protecting groups.

N-(L-α-Asparty1)-2-acetamido-2-deoxy-β-D-glucosylamine and N-(L-γ-glutamyl)-2-acetamido-2-deoxy-β-D-glucosylamine have been prepared by the same method as for β-aspartylglycopeptide.

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137. Akira Yamamoto and Hisao Tsukamoto: Studies on Amino Sugars. III.*1 Isolation and Identification of Crystalline N-Acetylglucosamine-asparagine Compound from Ovalbumin.*2

(*Faculty of Pharmaceutical Sciences, Kyushu University**3)

In recent years some evidence has accumulated that the polysaccharide moiety of ovalbumin glycopeptide is attached to an aspartyl residue of peptide chain through N-acetylglucosamine.[1~6]

Concerning the chemical nature of the aspartic acid-glucosamine linkage, there are following three possibilities: (1) iminoether type,[3] the amide group of asparagine linked to C-1 of the glucosamine not through C-N-C but C-O-C bonds; (2) glucosaminyl-amine[1,2,5] type, an N-glucosaminide, involving the amide group of asparagine, analo-

*1 Part II: This Bulletin, **13**, 1041 (1965).
*2 Communicated in Biochem. Biophys. Res. Com., **15**, 151 (1964).
*3 Katakasu, Fukuoka (山本 陽, 塚元久雄).

1) F. R. Jevons: Nature, **181**, 1345 (1958).
2) R. H. Nuenke, L. W. Cunningham: J. Biol. Chem., **236**, 2452 (1961).
3) I. Yamashina, M. Makino: J. Biochem. (Tokyo), **51**, 359 (1962).
4) J. R. Clamp, L. Hough: Chem. & Ind. (London), 82 (1963).
5) G. S. Marks, R. D. Marshall, A. Neuberger: Biochem. J., **87**, 274 (1963).
6) F. Micheel, E.-A. Ostmann, G. Pielmeier: Tetrahedron Letters, No. 2, 115 (1963).