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Studies on *N*-Glycopeptides. II. Separation of α- and β-Amide of *N*-(L-Aspartyl)-β-D-glucopyranosylamine Obtained *via* *N*-(Benzyloxycarbonyl)-L-aspartic Anhydride

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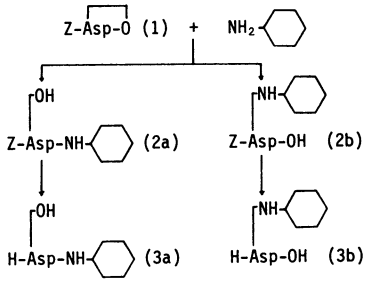
A mixture of *N*-(L-α-aspartyl)- and *N*-(L-β-aspartyl)-β-D-glucopyranosylamine was obtained by the condensation of *N*-(benzyloxycarbonyl)-L-aspartic anhydride with β-D-glucopyranosylamine. Attempts to separate the mixture by using cation-exchange chromatography, a copper(II) complex, and fractional recrystallization were carried out. These separations gave good yields of the desired β-amine.

In a previous paper, we reported the preparation of nine *N*-glycopeptides, obtained by the condensation of *N*-hydroxysuccinimide esters of aspartyltripeptides with three glycosylamines.[1] This active ester method realized a carbohydrate–peptide linkage formation reaction, which was scarce in the *N*-glycopeptide synthesis. However, another synthetic route is necessary in order to prepare more complex *N*-glycopeptides for which demand has been increasing. Preparations of aspartylpeptides *via* *N*-(benzyloxycarbonyl)-L-aspartic anhydride have also been reported. Hirata *et al.* condensed *N*-(benzyloxycarbonyl)-L-aspartic anhydride with a L-histidine benzyl ester, and separated the α- and β-isomer from the reaction mixture.[2] If an efficient separation of aspartyl α- and β-amide is established, this anhydride method can be applied to *N*-glycopeptide synthesis.

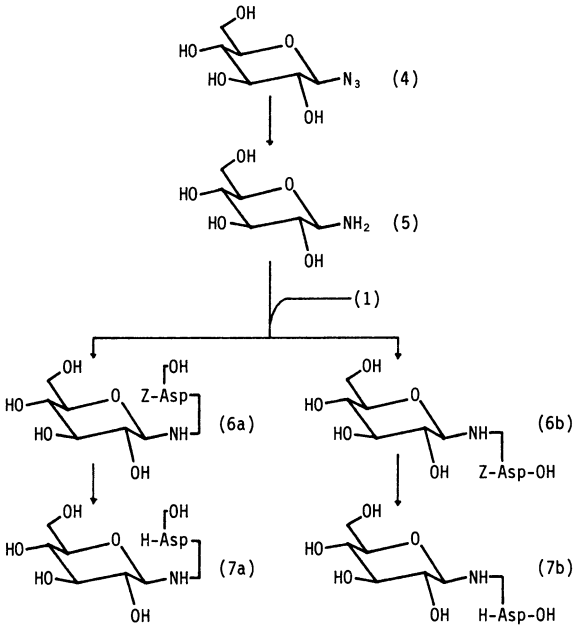
Jeanloz *et al.*, actually, prepared 2-acetamido-3,4,6-tri-*O*-acetyl-1-*N*-[*N*-(benzyloxycarbonyl)-L-β-aspartyl]-2-deoxy-β-D-glucopyranosylamine and some of their analogs by the condensation of *N*-(benzyloxycarbonyl)-L-aspartic anhydride with glycosylamines and the following fractional recrystallization or silica-gel column chromatography.[3] They however, carried out the condensation using fully *O*-protected glycosylamine, and so, the separation technique was limited. Moreover, de-*O*-acetylation at the final step requires an alkaline treatment which usually harms the aspartyl linkage. We have attempted to resolve this problem by condensing glycosylamine possessing unprotected hydroxyl groups. Futhermore, applying this idea to the synthesis of *N*-glycopeptide *via* *N*-(benzyloxycarbonyl)-L-aspartic anhydride should reap a greater variety of separations of the α- and β-aspartyl isomer than Jeanloz *et al.* has attempted. We prepared *N*-(L-α-aspartyl)cyclohexylamine, *N*-(L-β-aspartyl)cyclohexylamine, *N*-(L-α-aspartyl)-β-D-glucopyranosylamine, and *N*-(L-β-aspartyl)-β-D-glucopyranosylamine as models of carbohydrate-asparagine compounds. We then examined several separations by using cation-exchange resine chromatography, a copper (II) complex, and fractional recrystallization to find the most efficient ones.

Mixtures were prepared as shown in Schemes 1 and 2. *N*-(Benzyloxycarbonyl)-L-aspartic anhydride (**1**) was condensed with cyclohexylamine to give a mixture of **2a** and **2b**. This was hydrogenated in the presence of palladium black, and the desired mixture of **3a** and **3b** was obtained. *N*-(Benzyloxycarbonyl)-L-aspartic anhydride (**1**) was also condensed with β-D-glucopyranosylamine (**5**), which was prepared by the hydrogenation of β-D-glucopyranosyl azide[1] (**4**) in the presence of Adams' catalyst (PtO_2), to give a mixture of **6a** and **6b**. Hydrogenation of the mixture in the presence of palladium black gave the desired mixture of **7a** and **7b**.

It should be noted here that Kornguth *et al.* reported



Scheme 1.



Scheme 2.

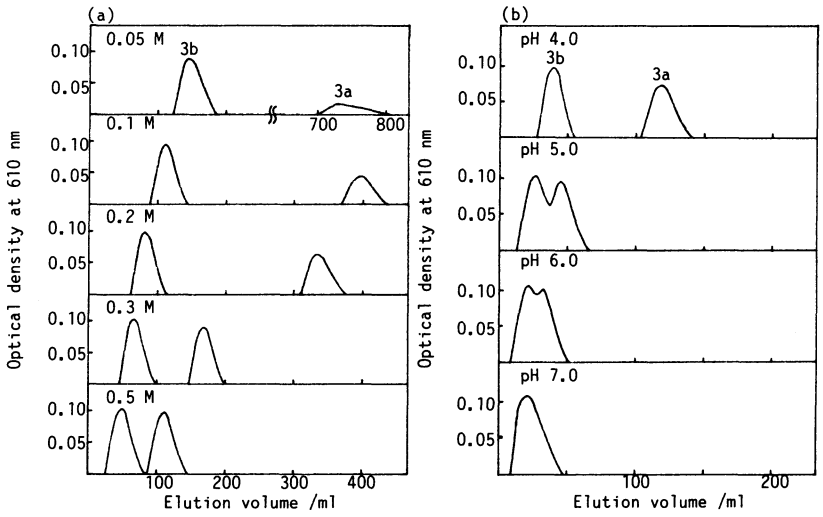


Fig. 1. Effect of salt concentration (a) and pH (b) in pyridinium acetate solvent system on separation of *N*-(L-α-aspartyl)cyclohexylamine (**3a**) and *N*-(L-β-aspartyl)cyclohexylamine (**3b**).

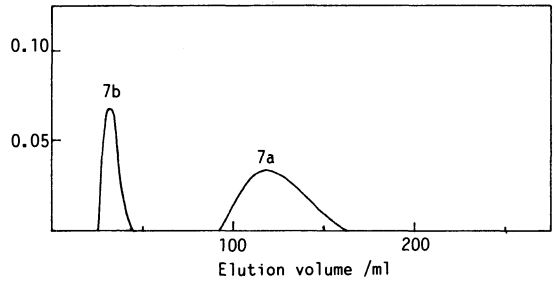


Fig. 2. Separation of *N*-(L-α-aspartyl)-β-D-glucopyranosylamine (**7a**) and *N*-(L-β-aspartyl)-β-D-glucopyranosylamine (**7b**) on a small column.
Amberlite CG-120 type II (pyridinium form, 1.2× 10 cm) Buffer solution ; 0.1 M pyridinium acetate (pH 4.0).

the separation of a mixture of ε-(L-α-glutamyl)-L-lysine and ε-(L-γ-glutamyl)-L-lysine using an amino-acid analyzer,[4] and Hirata *et al.* reported the separation of a mixture of L-α-aspartyl-L-histidine and L-β-aspartyl-L-histidine, or L-α-glutamyl-L-histidine and L-γ-glutamyl-L-histidine using a cation-exchange resin.[2] With this in mind, we first examined the separation of mixtures **3a** and **3b**, and **7a** and **7b** by cation-exchange resin chromatography. Preliminary experiments on a mixture of **3a** and **3b** were performed with a small column of Amberlite CG-120 using pyridinium acetate buffers at different pH and salt concentrations in order to find an optimal condition for the separation. The elution patterns of a mixture composed of **3a** and **3b** are indicated in Figs. 1-a and 1-b. 0.1 M† Pyridinium acetate (pH 4.0) separated the mixture most effectively. We then attempted to separate a mixture of **7a** and **7b** using the same solvent. As shown in Fig. 2, **7a** and **7b** were separated completely.

As a large-scale experiment we attempted to separate material composed of **7a** and **7b** into α- and β-amide with a large column (2.8×54 cm) of Amberlite CG-120, using 0.1 M pyridinium acetate (pH 4.0) as an eluent.

† 1M=1 mol dm^{-3}.

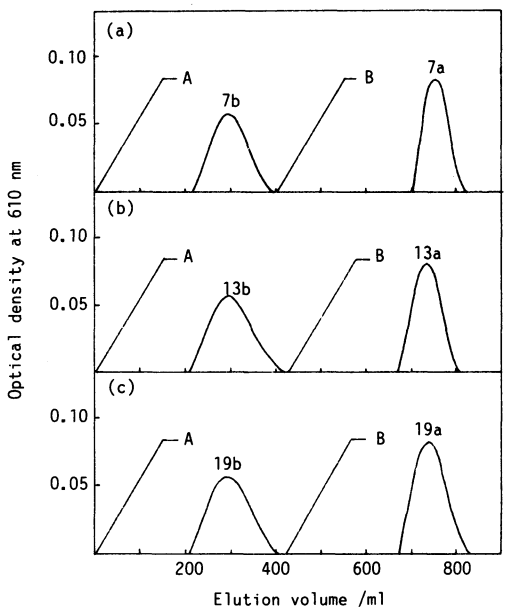
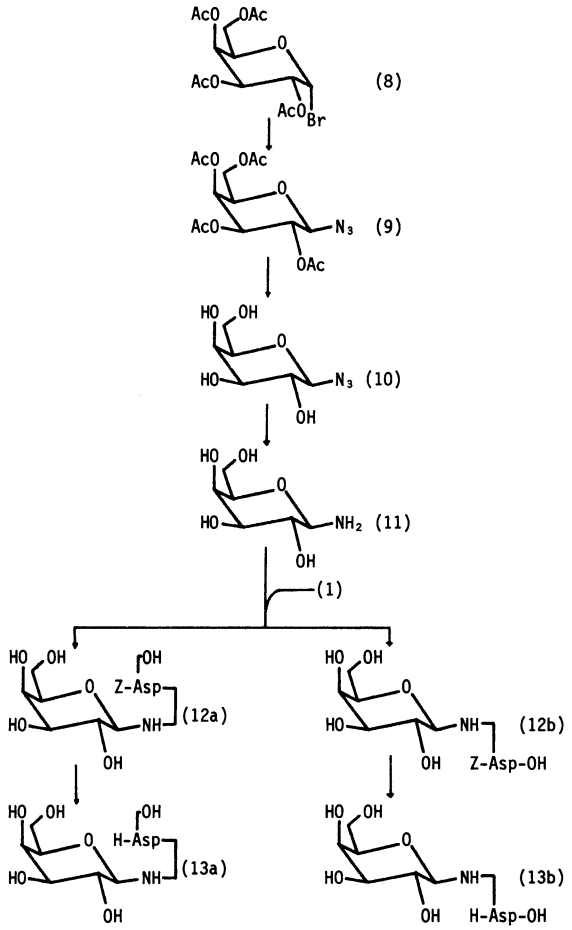


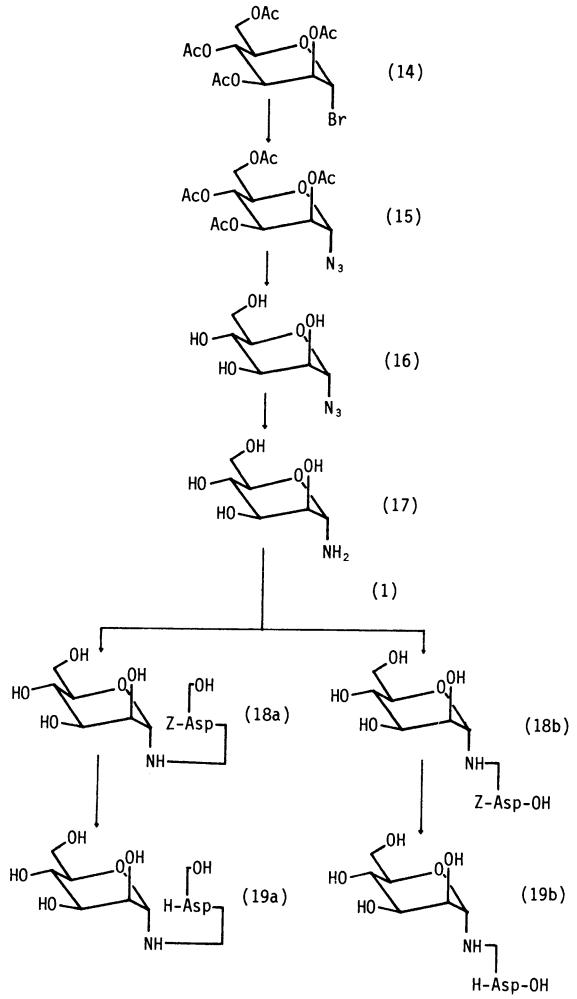
Fig. 3. Preparative separation of **7a** and **7b** (a), **13a** and **13b** (b), and **19a** and **19b** (c).
Amberlite CG-120 type I (pyridinium form, 2.8× 50 cm) Buffer solution ; A : 0.1 M pyridinium acetate (pH 4.0) B : 0.2 M pyridinium acetate (pH 4.0).

After the first peak was eluted, the eluent was changed to 0.2 M pyridinium acetate (pH 4.0). Compounds **7a** and **7b** were separated completely (see Fig. 3-a) and provided high yields (250 mg and 120 mg, respectively).

We further prepared mixtures of α- and β-amide of β-D-galactopyranosylamine and α-D-mannopyranosylamine as shown in Schemes 3 and 4, and treated them using the same method as described for the separation of **7a** and **7b** in order to investigate the limit of separation using a cation-exchange resin. Both galactosyl (**13a** and **13b**) and mannosyl (**19a** and **19b**) derivatives were separated completely, as shown in Figs. 3-b and 3-c. From these results, the usefulness of the separation of



Scheme 3.



Scheme 4.

α- and β-amide of aspartyl residue from the reaction mixture (obtained by the condensation of *N*-(benzyloxycarbonyl)-L-aspartic anhydride with various glycosylamines) by the use of cation-exchange resin chromatography was proved.

We next applied a mixture of **7a** and **7b** to an amino acid analyzer. Compounds **7a** and **7b** were separated completely (see Fig. 4). The percentage of color yield of **7a** and **7b**, based on L-aspartic acid as 100%, were observed to be 89% and 162%, respectively. We used an amino acid analyzer to check the purity of the mixture after the following separations.

Secondly, we examined a separation using a copper (II) complex. By the addition of dicopper carbonate dihydroxide to **7a** and **7b**, 6-membered and 5-membered rings were formed, respectively. It is well-known that a 5-membered copper (II) complex is more stable than a 6-membered salt (see Fig. 5). We tried to separated **7a** and **7b** using the difference in their stabilities. The experiment was performed as shown in Scheme 5. The crystals (II) obtained were de-salted and apllied to an amino acid analyzer. We found that purification was as follows: **7a**:**7b**=9:91. The purification by the differential stability of copper (II) salts of **7a** and **7b** may be suitable in the preparation of *N*-glycopeptide.

Thirdly, we attempted to isolate **6b** from the mixture by fractional recrystallization. We prepared diastereomeric salts of **6a** and **6b** with cyclohexylamine and dicyclohexylamine, and these salts were recrystallized from methanol. The crystals obtained were hydrogenated, applied to an amino acid analyzer to confirm the purity of the mixture, and the results are listed in Table 1. We discovered an interesting and useful fact; the dicyclohexylammonium salt of **6b** is more soluble than

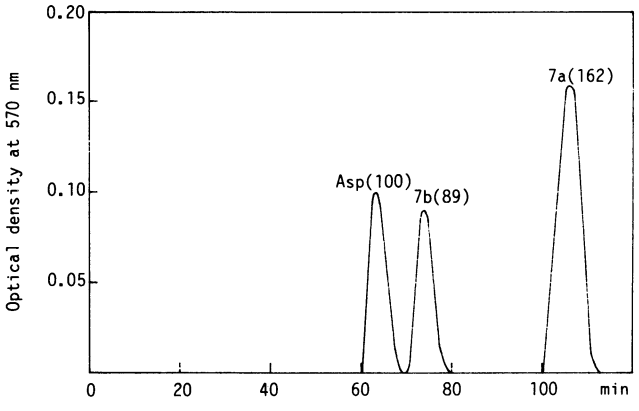


Fig. 4. Elution pattern of aspartic acid, *N*-(L-β-aspartyl) β-D-glucopyranosylamine (**7b**), and *N*-(L-α-aspartyl)-β-D-glucopyranosylamine (**7a**) by amino acid analyzer. Sample; 0.1 μmol/ml solution.

that of **6a**, whereas the cyclohexylammonium salt of **6b** is less soluble than that of **6a**. These facts were applied to separate a mixture of **6a** and **6b**. At the outset, we attempted to obtain **6b** by the repetitious recrystallization of a cyclohexylammonium salt as shown in Scheme 6, however, pure **6b** was obtained in a very low yield. Therefore, we used both a cyclohexylammonium salt and a dicylohexylammonium salt (shown in Scheme 7) to obtain a pure cyclohexylammonium salt of **6b** with a high yield.

We considered that fractional recrystallization has several merits, to wit; (a) fractional recrystallization is performed in organic solution, in which aspartyl residue is more stable than an acidic or basic solution of water, and (b) the resulting β-amide is suitable to elongate the peptide chain toward the C-terminal since fractional recrystallization is carried out using an *N*-protected aspartyl derivative.

We established separation techniques to obtain β-amide formed by the reaction of *N*-(benzyloxycarbonyl)-L-aspartic anhydride with glycosylamine. Each separation gave a good yield and compared favorably with that of Jeanloz *et al.* Additionally,and equally important, is the fact that this method goes to lesser extremes, yet is much more refined than the method of Nerberger *et al.* in which they condensed *N*-(benzyloxycarbonyl)-L-aspartic acid α-benzyl ester with 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosylamine in the presence of dicyclohexylcarbodimide.[5]

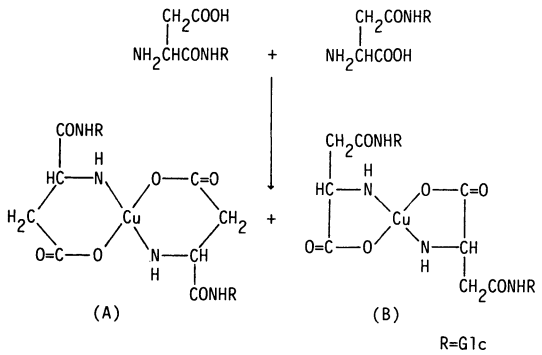
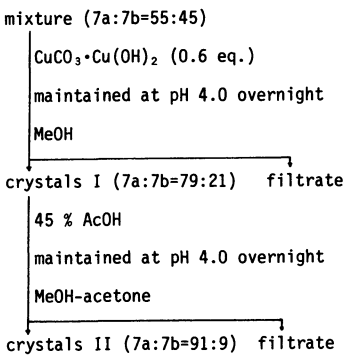


Fig. 5. Copper (II) complex of **7a** and **7b** (B).

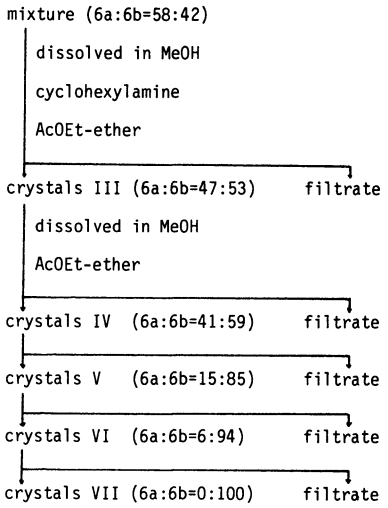


Scheme 5.

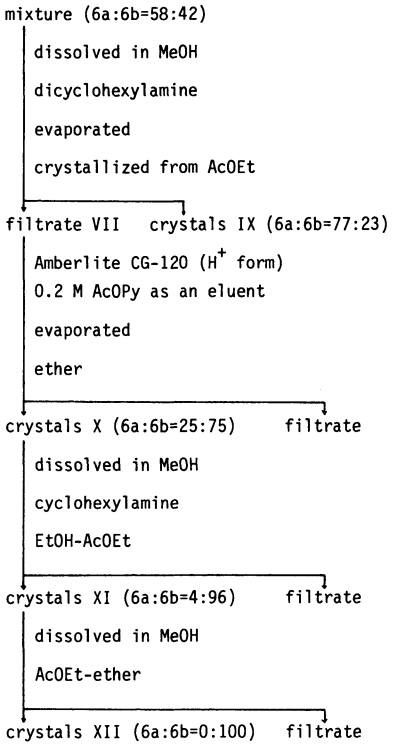
TABLE 1. RESULTS OF PRELIMINARY EXPERIMENTS OF FRACTIONAL RECRYSTALLIZATIONS BY THE USE OF ORGANIC BASES

Base	Yield of 1st crop*	Yield of 2nd crop**
Cyclohexylamine	79% (**6a** : **6b**=39 : 61)	12% (**6a** : **6b**=52 : 48)
Dicyclohexylamine	22% (**6a** : **6b**=100 : 0)	34% (**6a** : **6b**=0 : 100)

* 1st crop was crystallized by the aid of AcOEt-ether.
** 2nd crop was crystallized by the aid of petroleum ether.



Scheme 6.



Scheme 7.

Experimental

All the melting points were uncorrected. The optical rotations were measured on a Union PM-101 Polarimeter. IR spectra were taken on a Jasco spectrophotometer Model IRA-1. Thin-layer chromatography was carried out on a Kieselgel G nach Stahl (Type 60) with 1-butanol–acetic acid–pyridine–water (4:1:1:2 v/v). Spots of material possessing a free amino group in a thin-layer plate were detected by ninhydrin, and those of the amino group-blocked material by spraying 25% HBr/AcOH and then ninhydrin. A quantitative analysis of the aspartyl derivatives was performed with a Hitachi Model KLA-5 amino acid analyzer. The amounts of color developed were determined using an Atago AG-4 Densitometer (slit 1×18 mm, 610 nm). Samples for elemental analysis were dried at 60 °C over phosphorus pentaoxide *in vacuo*.

Preparation of Mixtures. N-*(L-α-Aspartyl)- and* N-*(L-β-Aspartyl)cyclohexylamine (3a and 3b)*: A solution of *N*-(benzyloxycarbonyl)-L-aspartic anhydride[6] (**1**) (1.25 g) in tetrahydrofuran (5 ml) was added to a solution of cyclohexylamine (0.7 ml) and triethylamine (0.56 ml) in tetrahydrofuran (5 ml) under vigorous stirring at room temperature overnight. The reaction mixture was evaporated to dryness and the residue was dissolved in ethyl acetate. The solution was washed with 4% sodium hydrogencarbonate and water, successively, dried over anhydrous sodium sulfate, and evaporated. A solid of **2a** and **2b** (1.40 g, 80%) was obtained by the aid of ether–petroleum ether.

The mixture of **2a** and **2b** (0.70 g) was dissolved in acetic acid (30 ml) and treated with hydrogen in the presence of palladium black. The filtrate from the catalyst was evaporated to dryness and a mixture of **3a** and **3b** was obtained after crystallization from ether; yield, 0.43 g.

Found: C, 56.00; H, 8.49; N, 13.01%. Calcd for $C_{10}H_{18}N_2O_3$: C, 56.05; H, 8.47; N, 13.08%.

N*[N-(Benzyloxycarbonyl)-L-α-aspartyl]- and* N-*[N-(Benzyloxycarbpnyl)-L-β-aspartyl]-β-D-glucopyranosylamine (6a and 6b)*: To a solution of β-D-glucopyranosylamine[1] (**5**) (4.30 g), obtained by the hydrogenation of β-D-glucopyranosyl azide[1] (**4**), and triethylamine (3.36 ml) in water (20 ml), a solution of **1** (4.98 g) in tetrahydrofuran (20 ml) was added under vigorous stirring at room temperature. The stirring continued overnight. The reaction mixture was evaporated to dryness and the residue was dissolved in water (40 ml). The solution was adjusted to pH 4.0 by the addition of 2% hydrochloric acid and extracted with ethyl acetate (total 500 ml). The organic layer was dried over anhydrous sodium sulfate and evaporated. The residual oil was crystallized using ether; yield, 4.79 g (56%); R_f 0.79 (**6a**) and 0.72 (**6b**).

Found: C, 50.49; H, 5.66 N, 6.52%. Calcd for $C_{18}H_{24}N_2O_{10}$ C, 50.49; H, 5.65; N, 6.54%.

N-*(L-α-Aspartyl)- and* N-*(L-β-Aspartyl)-β-D-glucopyranosylamine (7a and 7b)*: A mixture of **6a** and **6b** (1.28 g) was dissolved in methanol and hydrogenated in the presence of palladium black. After 4 h, the catalyst was filtered off and the filtrate was evaporated to dryness. A mixture of **7a** and **7b** was crystallized using ethanol; yield 0.58 g (66%); R_f 0.34.

Found: C, 40.14; H, 6.17; N, 9.52%. Calcd for $C_{10}H_{18}N_2O_8$: C, 40.18; H, 6.17; N, 9.52%.

Tetra-O-acetyl-β-D-galactopyranosyl Azide (9): This compound was prepared by the method of W. Pfleiderer and B. Bühler.[7] The yield was 84% and the product had a mp of 92—93 °C; lit,[7] mp 96 °C. ν_{max}^{KBr} 1760 (OAc) and 2100 cm^{-1} (N_3).

Found: C, 45.00; H, 5.14; N, 11.22%

N-*(L-α-Aspartyl)- and* N-*(L-β-Aspartyl)-β-D-galactopyranosylamine (13a and 13b)*: Compound (**9**) (7.46 g) was dissolved in methanol (20 ml) saturated with dry ammonia at 0 °C and was stored in a glass-stoppered bottle at room temperature overnight. The reaction mixture was then evaporated *in vacuo*. The residual oil was dissolved in methanol and hydrogenated in the presence of PtO_2. The filtrate from the catalyst was evaporated to dryness and the residue was solidified using ethanol; yield, 2.45 g.

This β-D-galactopyranosylamine (**11**) (4.30 g) and **1** (4.98 g) were treated as described for the preparation of a mixture of **6a** and **6b**, to give **12a** and **12b**; yield, 4.54 g (53%); R_f 0.82 (**12a**) and 0.74 (**12b**).

A mixture of **12a** and **12b** (1.28 g) was hydrogenated as described for the preparation of **7a** and **7b**; yield, 0.52 g (59%); R_f 0.38.

Found: C, 40.13; H, 6.19; N, 9.56%. Calcd for $C_{10}H_{18}N_2O_8$: C, 40.18; H, 6.27; N, 9.52%.

Tetra-O-acetyl-α-D-mannopyranosyl Azide (15): This was prepared by the method of Paulsen *et al.*[8] The yield was 71% and the product had $[\alpha]_D^{20}$ +87° (*c* 1, chloroform): lit,[8] $[\alpha]_D^{20}$ +91° (*c* 1, dioxane), ν_{max}^{nujol} 1750 (OAx) and 2110 cm^{-1} (N_2).

Found: C, 45.12; H, 5.11; N, 11.21%.

N-*(L-α-Aspartyl)- and* N-*(L-β-Aspartyl)-α-D-mannopyranosylamine (19a and 19b)*: **15** (7.46 g) was de-*O*-acetylated and hydrogenated as described for preparation of **11** to give **17**. **17** (4.30 g) and **1** (4.98 g) were treated as described for preparation of the mixture of **6a** and **6b**; yield, 4.88 g (57%); R_f 0.68 (**18a**) and 0.64 (**18b**).

A mixture of **18a** and **18b** (1.28 g) was hydrogenated as described for the preparation of **7a** and **7b**; yield, 0.51 g (58%); R_f 0.31.

Found: C, 40.22; H, 6.20; N, 9.50%. Calcd for $C_{10}H_{18}N_2O_8$: C, 40.18; H, 6.17; N, 9.52%.

Separation of α- and β-Amide by Cation-exchange Resin Chromatography. Separation of 3a and 3b on a Small Column: A column (1.2×10 cm) with Amberlite CG-120 (pyridinium form) was equilibrated by washing with pyridinium acetate (pH 4.0) of a definite salt concentration (0.05 M, 0.1 M, 0.2 M, 0.3 M, or 0.5 M) or 0.2 M pyridinium acetate of definite pH (4.0, 5.0, 6.0, or 7.0), and the mixture was applied to a column. The column was eluted with the same buffer at room temperature, and two ml fractions were collected at a flow rate of about 10 ml/h. Approximately 0.01 ml from each tube was spotted on a strip of filter paper. The strip was dried at 100 °C for 5 min, immersed in a 0.2% ninhydrin–acetone solution, and again heated. The degrees of coloration developed were determined by a densitometer. The resulting patterns are shown in Fig. 1.

Separation of 7a and 7b on a Small Column: A mixture of **7a** and **7b** (50 mg) was treated on a column of Amberlite CG-120 (pyridinium form) in same manner as the saparation of **3a** and **3b** using 0.1 M pyridinium acetate (pH 4.0). The resulting patterns are shown in Fig. 2.

Preparative Separation of 7a and 7b: A mixture of **7a** and **7b** (500 mg) was applied to a large column (2.8×54 cm) of Amberlite CG-120 (pyridinium form) using 0.1 M pyridinium acetate (pH 4.0) as an eluent. For the elution of the slower peak, 0.2 M pyridinium acetate (pH 4.0) was used as an eluent. The elution patterns are shown in Fig. 3-a.

7a: Yield 256 mg (51%); mp 183—185 °C (decomp); $[\alpha]_D^{20}$ +13.5° (*c* 2, water). ν_{max}^{KBr} 3300 (broad OH and NH) and 1630 cm^{-1} (CONH).

Found: C, 40.90; H, 6.14; N, 9.51%. Calcd for $C_{10}H_{18}N_2O_8$: C, 40.81; H, 6.17; N, 9.52%.

7b: Yield, 120 mg (24%); mp 204 °C (decomp) $[\alpha]_D^{20}$ −5.5° (*c* 2, water). ν_{max}^{KBr} 3300 (broad OH and NH) and 1650 cm^{-1} (CONH).

Found: C, 40.87; H, 6.15; N, 9.49%. Calcd for $C_{10}H_{18}N_2O_8$: C, 40.81; H, 6.17; N, 9.52%.

Preparative Separation of 13a and 13b: A mixture of **13a** and **13b** (500 mg) was treated in same manner as has been described for the separation of **7a** and **7b**. The elution patterns are shown in Fig. 3-b.

13a: Yield, 211 mg (42%); mp 186—189 °C (decomp); $[\alpha]_D^{20}$ +16.5° (*c* 2, water). ν_{max}^{KBr} 3300 (broad OH and NH) and 1640 cm^{-1} (CONH).

Found: C, 40.84; H, 6.14; N, 9.55%. Calcd for $C_{10}H_{18}N_2O_8$: C, 40.81; H, 6.17; N, 9.52%.

13b: Yield, 163 mg (33%); mp 202—216 °C (decomp); $[\alpha]_D^{20}$ −6.0° (*c* 2, water). ν_{max}^{KBr} 3300 (broad OH and NH) and 1650 cm^{-1} (CONH).

Found: C, 40.81; H, 6.14; N, 9.51%. Calcd for $C_{10}H_{18}N_2O_8$: C, 40.81; H, 6.17; N, 9.52%.

Preparative Separation of 19a and 19b: A mixture of **19a** and **19b** (500 mg) was treated in the same manner as for the separation of **7a** and **7b**. The elution patterns are shown in Fig. 3-c.

19a: Yield, 158 mg (52%); mp 187—188 °C (decomp); $[\alpha]_D^{20}$ +16.5° (*c* 2, water). ν_{max}^{KBr} 3300 (broad OH and NH) and 1640 cm^{-1} (CONH).

Found: C, 40.79; H, 6.16; N, 9.54%. Calcd for $C_{10}H_{18}N_2O_8$: C, 40.81; H, 6.17; N, 9.52%.

19b: Yield, 119 mg (39%); mp 204—221 °C (decomp); $[\alpha]_D^{20}$ −4.0° (*c* 2, water). ν_{max}^{KBr} 3300 (broad OH and NH) and 1660 cm^{-1} (CONH).

Found: C, 40.85; H, 6.16; N, 9.50%. Calcd for $C_{10}H_{18}N_2O_8$: C, 40.81; H, 6.17; N, 9.52%.

Separation of α- and β-Amide by Copper (II) Complex. To a solution of **7a** and **7b** (**7a**:**7b**=55:45, 294 mg) in water (6 ml), dicopper carbonate dihydroxide (66 mg) was added. The solution was heated for 5 min and then 45% acetic acid was added. It was maintained at pH 4.0 onernight. Type-I crystals (139 mg, 43%) were obtained using methanol. A small amount of these were treated on a column of Amberlite CG-120 (H^+ form) (1.3×8.0 cm) with water and 0.2 M pyridinium acetate (pH 5.0) as an eluent. The eluting fraction was collected and evaporated to dryness. The residue was applied to an amino acid analyzer (**7a**:**7b**=36:64).

Type-I crystals were dissolved in hot water and 45% acetic acid was added to the solution. The mixture was maintained at pH 4.0 overnight. Type-II crystals (46 mg, 33%) were obtained using methanol-acetone. A small amount of these were treated on a column of Amberlite CG-120 (H^+ form) as described above (**7a**:**7b**=9:91).

Separation of α- and β-Amide by Fractional Recrystallization. Preliminary Experiments: To a solution of **6a** and **6b** (428 mg) in methanol (5 ml), cyclohexylamine or dicyclohexylamine (2 mmol each) was added. Crystals were obtained using ether (5 ml), and the filtrates were evaporated. Crystals were obtained using ether-petroleum ether as second crop. These crystals were dissolved in methanol, and hydrogenated in the presence of palladium black. Filtrates from the catalyst were evaporated and applied to an amino acid analyzer. The results are listed in Table 1.

Separation of 6a and 6b by the Use of Cyclohexylammonium Salt: A mixture of **6a** and **6b** (**6a**:**6b**=58:42, 428 mg) was dissolved in methanol (5 ml) and cyclohexylamine (0.23 ml) was added to the solution. Type-III crystals (295 mg, 56%) were obtained using ethyl acetate-ether. A small amount of these crystals were dissolved in methanol and hydrogenated in the presence of palladium black. The catalyst was filtered off the filtrate was evaporated. The residual oil was applied to an amino acid analyzer (**6a**:**6b**=47:53).

Type-III crystals were dissolved in methanol (5 ml) in methanol (5 ml) and type-IV crystals (210 mg, 71%) were obtained using ethyl acetate-ether. A small amount of these crystals were hydrogenated as described above, and applied to an amino acid analyzer (**6a**:**6b**=41:59).

Type-IV crystals were dissolved in methanol (5 ml) and type-V crystals (152 mg, 72%) were obtained using ethyl acetate-ether. A small amount of these crystals were hydrogenated and applied to an amino acid analyzer (**6a**:**6b**=15:85).

Type-V crystals were treated as described for the prepartion of type-IV crystals, and type-VI crystals (107 mg, 70%, **6a**:**6b**=6:94) was obtained.

Type-VI crystals were treated as described for the preparation of type-VI crystals, and type-VII crystals (75 mg, 70%, **6a**:**6b**=0:100) were obtained.

Separation of 6a and 6b by the Use of Both Cyclohexylammonium and Dicyclohexylammonium Salt: To a solution of **6a** and **6b** (428 mg, **6a**:**6b**=58:42) in methanol (5 ml), dicyclohexylamine (0.197 ml) was added. The mixture was evaporated to dryness and type-IX crystals (396 mg, 65%) were obtained by the recrystallization from hot ethyl acetate. A small amount of type-IX crystals were hydrogenated, applied to an amino acid analyzer (**6a**:**6b**=77:23).

A filtrate (VIII) was evaporated to dryness, and residual oil was dissolved in water (10 ml). The solution was treated on a column Amberlite CG-120 (H^+ form) using 0.2 M pyridinium acetate (pH 5.0) as an eluent. The eluting fractions was collected and evaporated to dryness. Type-X cryltals (123 mg, 29%) were obtained using ether. A small amount of type-X crystals were hydrogenated and applied to an amino acid analyzer (**6a**:**6b**=25:75).

Type-X crystals were dissolved in methanol (3 ml) and cyclohexylamine (0.05 ml) was added to the solution. The solution was evaporated to dryness and the residual oil was crystallized from ethanol-ethyl acetate to give type-XI crystals (120 mg, 79%). A small amount of type-XI crystals were hydrogenated and applied to an amino acid analyzer (**6a**:**6b**=4:96).

Type-XI crystals were dissolved in methanol (3 ml) and the solution was evaporated to dryness. Type-XII crystals (103 mg, 86%) were obtained using ethyl acetate-ether. A small amount of type-XII crystals were hydrogenated and applied to an amino acid analyzer (**6a**:**6b**=0:100).

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