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# Polypeptide Formation by Heating N-*t*-Butyloxycarbonyl Acidic Amino Acid Derivatives

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An acid labile N-protecting group for amino acids, *t*-butyloxycarbonyl (Boc) group has deprotected at elevated temperatures. The study describes an application of the lability on heating to synthesis of polypeptides from acidic amino acids. *t*-Butyloxycarbonyl-acidic amino acids (aspartic acid, glutamic acid and β-aminoglutaric acid) and their anhydrides were heated at the higher temperatures than their melting points. Anhydrides of *t*-butyloxycarbonyl-aspartic acid and *t*-butyloxycarbonyl-β-aminoglutaric acid gave polypeptides. Thermal analyses of the substrates clarified the pathway of the polypeptide formation.

**Keywords: Acidic amino acid, Heating, *t*-Butyloxycarbonyl group, Thermal analysis.**

## INTRODUCTION

Aspartic acid and glutamic acid can be found as components in proteins and the research on these amino acids has been performed in the viewpoints of peptide synthesis using prebiotic[1-8], conventional organic chemistry[9] and molecular biological methods[10], as well as their chemical[11] and physiological properties[12].

Free aspartic acid[1-3] and malic acid mono-ammonium salt[4,5] gave polyaspartic acid by heating without solvents at elevated temperatures or by microwave induction[6,8]. The mechanism of dehydration from aspartic acid to polypeptides has been reported in the literatures[1-3,13]. The reaction mixture during heating aspartic acid was monitored[13] by infrared spectrophotometry, thermal gravitmetry and differential thermal analysis. The residues after the heating reactions showed IR absorption corresponding to peptide formation (amide I and amide II). The DTA record of the heating reaction showed that the decrease in weight was consistent with the point of endothermal peak due to the formation of peptide bonds on the DTA line. However, these heating reactions have been carried out at the higher temperature than 180 °C, at which anhydrous polyaspartic acid formation and racemization will proceed. The lower temperature seems to be suitable to depress such side reactions.

In the previous report[14], we described heating reactions of N-*t*-butyloxyl-carbonyl-aspartic acid (*t*-butyloxycarbonyl-L-Asp) anhydride at 130 °C to afford polyaspartic acid with a little amount of racemized residues (D/L ratio: 7.5 % during 4 h' heating at 130 °C). The purpose of the protection with N-*t*-butyloxylcarbonyl group is to achieve melting at the lower temperatures. Under the reaction conditions, *t*-butyloxycarbonyl-L-Asp anhydride seemed to release carbon dioxide and 2-butene gradually to afford L-Asp anhydride, which was suggested to react itself to give polyaspartic acid. However, detailed mechanism and experimental procedure have not been reported.

This research describes the mechanism and experimental procedure of the polypeptide formation at the lower temperatures using acidic N-*t*-butyloxylcarbonyl-amino acids (*t*-butyloxycarbonyl-amino acids) and their anhydrides as shown in Fig. 1.

## EXPERIMENTAL

A Hitachi 835 amino acid (Hitachi, Tokyo, Japan) analyser was used for amino acid analysis. A Hitachi Model 260-50 infrared spectrophotometer (Hitachi, Tokyo, Japan) was used for the record of infrared spectra. A Shimadzu DT-40 (Shimadzu, Kyoto, Japan) was used for thermal analysis. A JOEL EX-270 NMR system (Joel, Tokyo, Japan) was used for measurement of $^1$H NMR spectra. Molecular weight stimulation was performed using a high performance liquid chromatography system composed of an ultraviolet detector, Jasco Uvdec-100-V UV Spectrophotometer and a flow pump, Jasco TRI Rotor-V equipped with a Sephadex G-25F column (450 × 10 mm I.D.). The flow rate was 0.15 mL/min (0.1 M sodium phosphate, pH 6.8) and the absorbance of the samples was detected at 230 nm. The peaks on the chromatograms were integrated with a SIC Chromatocoda. Enantiomer separation of amino acids

was performed with a Hitachi 163 gas chromatograph equipped with a chiral glass capillary column (Chirasil-Val[15-17]), 25m × 0.3 mm I.D.). Nitrogen was used as a carrier gas at a flow rate 30 mL/min. The temperature was programmed from 80 to 170 °C at a rate of 4 °C/min. The detection was carried out with a flame ionization detector. Optical rotation of the synthetic compounds from L-aspartic acid was determined with a Jasco DIP-181 Digital Polarimeter.

**Amino acids (1a-c):** DL-aspartic acids (**1a**), L-aspartic acid (**1b**) and DL-glutamic acid (**1c**) were purchased from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan). Di-*t*-butyloxycarbonyl-carbonate was purchased from Watanabe Chemical Industries, LTD. (Hiroshima, Japan). Glutaconic acid (*trans*-2-pentendioic acid) was purchased from Sigma-Aldrich.

**β-Amino glutaric acid (1d):** To a solution of glutaconic acid (5.2 g, 20 mmol) in 15 mL water was added concentrated ammonia (5.6 mL, 40 mmol) to give a crystal at 0 °C. The crystal was recrystallized with water to give 5 g glutaconic acid mono-ammonium (85 %). m.p. 179-181 °C. Elemental analysis calcd. for $C_5H_9NO_4$: C, 40.81; H, 6.16; N, 9.52 %. Found: C, 40.62; H, 6.18; N, 9.52 %. Glutaconic acid mono-ammonium (0.294 g, 2 mmol) was heated at 170 °C for 2 h under nitrogen stream. To the resulted mixture was added methanol to a suspension, which was filtered to give β-amino glutaric acid (0.093 g, 32 %). m.p. 285 °C (dec.). Elemental analysis: Calcd. for $C_5H_9NO_4$: C, 40.81; H, 6.16; N, 9.52 %. Found: C, 40.73; H, 6.24; N, 9.52 %. $^1$H NMR (1M-DCl/$D_2O$): δ = 2.95 (4H, d), 4.07 ppm (1H, t). IR: 3200, 1720, 1600, 1490, 1410-1320 $cm^{-1}$.

**N-*t*-Butyloxycarbonyl-amino acids (2a-d):** A typical preparation procedure for *t*-butyloxycarbonyl-DL-aspartic acid (**2a**) is shown as follows: To a mixture of DL-aspartic acid (**1a**) (13.3 g, 0.10 mmol), 4 M sodium hydroxide (80 mL, 0.20 mmol), pure water (20 mL) and 1, 4-dioxane (100 mL), was added dropwise a solution containing di-*t*-butyloxy-dicarbonate (24 g, 0.11 mol) over stirring at 10-20 °C. And then 1,4-dioxane (40 mL) with 4M-sodium hydroxide (40 mL) was added. After overnight stirring at room temperature, the reaction mixture was evaporated *in vacuo* to about 100 mL solution, which was cooled and acidified with 5 % potassium hydrogen sulfate to pH 2. The resulted solution was extracted with ethyl acetate (40 mL × 3) and the combined ethyl acetate layer was washed with a little water. The obtained ethyl acetate solution was dried with anhydrous magnesium sulphate and evaporated *in vacuo* to give 21.4 g (92 %). m.p. 145-146 °C. IR: 3450, 3000-2700, 1740-1680, 1500, 1440 $cm^{-1}$.

**N-*t*-Butyloxycarbonyl-amino acids:** Compunds **2b**, **2c**, **2d** were prepared from the corresponding amino acids **(1a-d)** in the similar manner of the preparation of **2a**. **2b:** Yield, 78 %. m.p. 119-121 °C. $[\alpha]_D^{25}$-4.5 (c = 1, methanol). **2c:** Yield, 89 %. m.p. 125-128 °C. **2d:** Yield, 86 %. m.p. 139-142 °C.

**N-*t*-Butyloxycarbonyl-amino acid anhydrides (3a-d):** A typical preparation procedure for N-*t*-butyloxycarbonyl-DL-aspartic acid anhydride (**3a**) is shown as follows: N-*t*-butyloxy-carbonyl-DL-aspartic acid (11.8 g, 50 mmol) was dissolved in 150 mL ethyl acetate. To this cooled solution at 0 °C was added a dicyclohexylcarbodiimide (11.4 g, 55 mmol) dissolved in 50 mL ethyl acetate. The reaction was carried out over stirring for 1 h at 0 °C and for 24 h at room temperature. The reaction solution was filtered and the resulted filtrate was evaporated *in vacuo* to give a crude crystal (10.1g, 94 %), which was recrystallized with acetone-petroleum ether to give 9.8 g (91 %). m.p. 122-124 °C.

**N-*t*-Butyloxycarbonyl-amino acid anhydrides:** Compounds **3b**, **3c**, **3d** were prepared from the corresponding N-*t*-butyloxycarbonyl-amino acids (**2a-d**) in the similar manner of the preparation of **3a**. **3b:** Yield, 94 %. m.p. 137-139 °C. $[\alpha]_D^{25}$38.7 (c = 1, acetic acid). **3c:** Yield, 88 %. m.p. 104-106 °C. **3d:** Yield, 83 %. m.p. 160-162 °C.

**Heating reaction of N-*t*-butyloxycarbonyl-amino acids (2a-d) and N-*t*-butyloxycarbonyl-amino acid anhydrides (3a-d):** The compounds **2a-d** (2 mmol) and **3a-d** (2 mmol) were put into each test tube (180 mm × 16 mm I.D.), which was heated under a nitrogen stream in a silicone oil bath controlled at a constant temperature. After heating, the reaction mixture was weighed and 180 mg of each sample was dissolved in 0.5 M acetic acid (5 mL). The solution was loaded onto a Sephadex-10 (120 mm × 1.8 mm I.D.). The elution was carried out with 0.5 M acetic acid and the eluted fractions were collected by a fraction collector, monitoring UV absorption of each fraction at 230 nm. Remaining samples of the resulted residue was injected to Sephadex G-25F for molecular weight analysis.

**General procedure of thermal analysis:** Samples of *t*-butyloxycarbonyl-amino acids (**2a-d**) and their anhydrides (**3a-d**) (8-13 mg, weighed to within 0.1 mg) were placed in the aluminum sample pans of the α-aluminum reference disk was placed in the center of the second aluminum pan. The temperature of the heating chamber was programmed from 27 to 400 °C using a heating rate of 10 °C/min, under a nitrogen flow (50 mL/min).

## RESULTS AND DISCUSSION

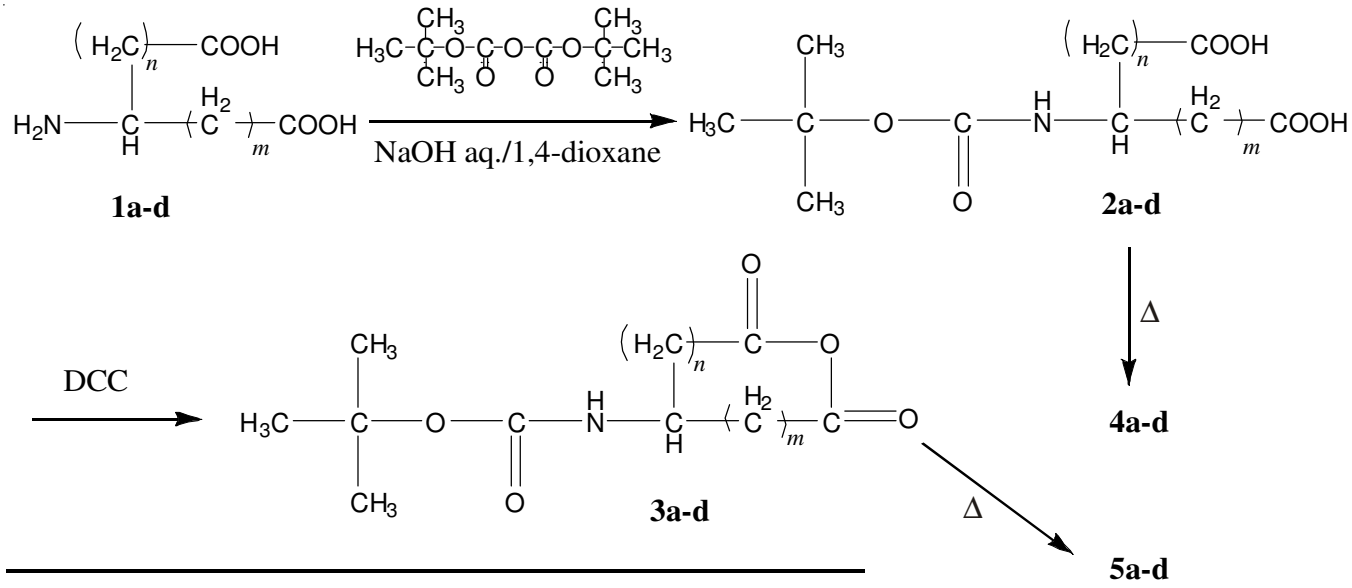
**Heating reaction of N-*t*-butyloxycarbonyl-amino acids and amino acid recovery:** N-*t*-Butyloxycarbonyl-L-alanine (Boc-L-Ala) (**2e**), N-*t*-butyloxycarbonyl-L-valine (Boc-L-Val) (**2f**) and N-*t*-butyloxy-carbonyl-L-aspartic acid (Boc-L-Asp) (**2b**) were heated at each temperature and for 1 or 2 h to free corresponding amino acids as shown in Table-1.

TABLE-1
FREE AMINO ACID RECOVERY AFTER HEATING N-*t*-BUTYLOXYCARBONYL-AMINO ACIDS

| Boc-amino acid | m.p. (°C) | Reaction conditions | | Free amino acid recovery (%) |
|---|---|---|---|---|
| | | Temp. (°C) | Time (h) | |
| Boc-L-Asp (**2b**) | 119-120 | 130 | 1 | 81 |
| Boc-L-Ala (**2e**) | 83-84 | 140 | 2 | 80 |
| Boc-L-Val (**2f**) | 80 | 140 | 2 | 70 |

The results show a kind of elimination from *t*-butyloxy-carbonyl-amino acid proceeded to give 2-butene (**6**), carbon dioxide (**7**) and corresponding amino acids (**8b**, **8e**, **8f**) as shown in Fig. 2.

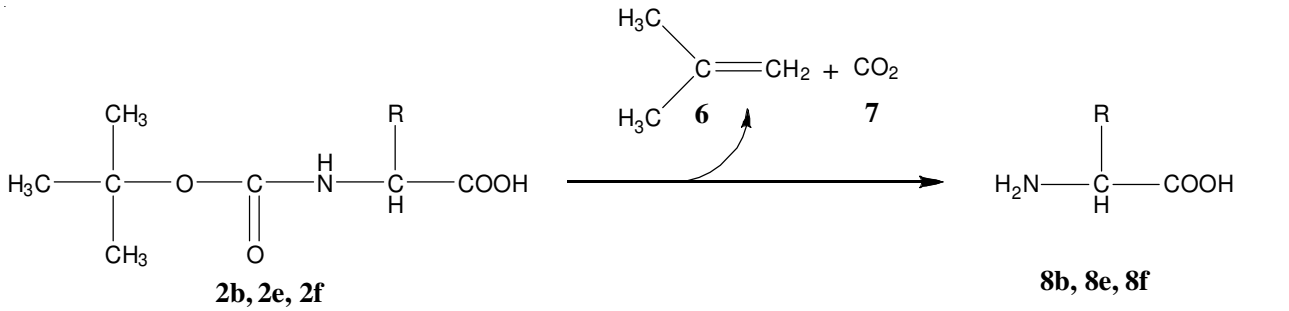
The results are obtained by just short time heating, but further longer reactions are not monitored. This study describes further longer reactions of *t*-butyloxycarbonyl-acidic amino acids (**2a-d**). Heating reaction of **2e**, **2f** and other neutral




| Compound | *m* | *n* | Amino acid | Configuration |
|---|---|---|---|---|
| **2a, 3a** | 0 | 1 | Aspartic acid (Asp) | DL |
| **2b, 3b** | 0 | 1 | Aspartic acid (Asp) | L |
| **2c, 3c** | 0 | 2 | Glutamic acid (Glu) | DL |
| **2d, 3d** | 1 | 1 | β-Amino-glutaric acid (β-Glu) | Non |

DCC:Dicyclohexylcarbodiimide

Fig. 1. Preparation of *t*-butyloxycarbony-amino acids (**2a-d**) and their anhydrides (**3a-d**)




**2b: R=-$CH_2COOH$ (L-Asp)**
**2e: R=-$CH_3$ (L-Ala)**
**2f: R=-$CH(CH_3)_2$ (L-Val)**

**8b: R=-$CH_2COOH$ (L-Asp)**
**8e: R=-$CH_3$ (L-Ala)**
**8f: R=-$CH(CH_3)_2$ (L-Val)**

Fig. 2. Plausible degradation pathway of *t*-butyloxycarbony-amino acids (**2b**, **2e**, **2f**)

*t*-butyloxycarbonyl-amino acids will be discussed in other opportunity.

**Heating reactions of *t*-butyloxycarbonyl-acidic amino acids (2a, 2c, 2d)**

**(1) IR spectrum of the products of heating *t*-butyloxycarbonyl-amino acids (2a, 2c, 2d):** Fig. 3 shows a heating product of *t*-butyloxycarbonyl-amino acid **2a** due to imide structure, which suggests the formation of anhydro-polyaspartic acid through polyaspartic acid during the heating reaction. This is supported by the observation in the products of heating reactions of aspartic acids[10]. Fig. 4 shows an IR spectrum of heating product of *t*-butyloxycarbonyl-β-aminoglutaric acid (**2c**). The spectrum was identical to that of β-aminoglutaric acid (**1c**). Fig. 5 shows IR spectrum of a heating product of *t*-butyloxycarbonyl-DL-glutamic acid (**2d**). The spectrum was identical to that of pyroglutamic acid.

**(2) Thermal analyses of *t*-butyloxycarbonyl-amino acids (2a, 2d):** Fig. 6 shows differential thermal analysis and thermal gravitmetry of *t*-butyloxycarbonyl-amino acid (**2a**). The peak (A) on DTA line corresponds to the endothermal peak due to melting of the sample, because the decrease in weight was not observed until the peak (A). However, with the peak (A), the weight of the sample rapidly decreased as

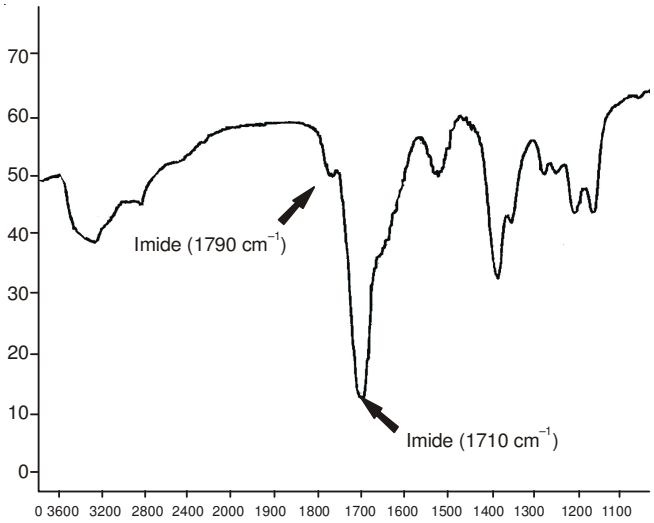



Fig. 3. IR spectrum of the product obtained by heating Boc-DL-Asp at 150 °C for 4 h

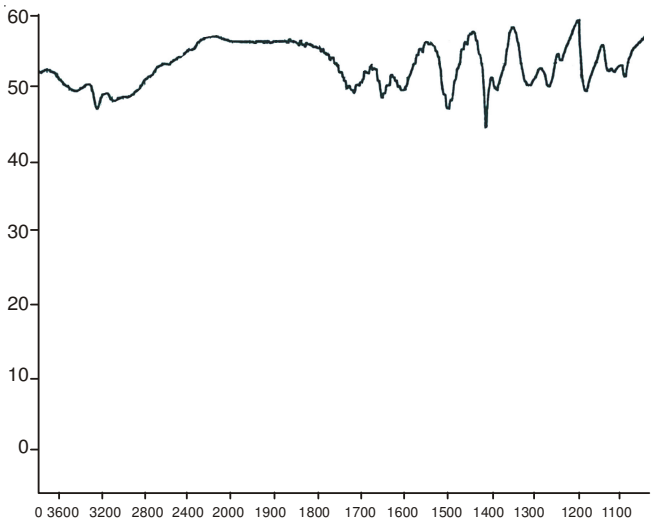



Fig. 4. IR spectrum of the product obtained by heating *t*-butyloxycarbony-β-aminoglutaric acid at 150 °C for 4 h

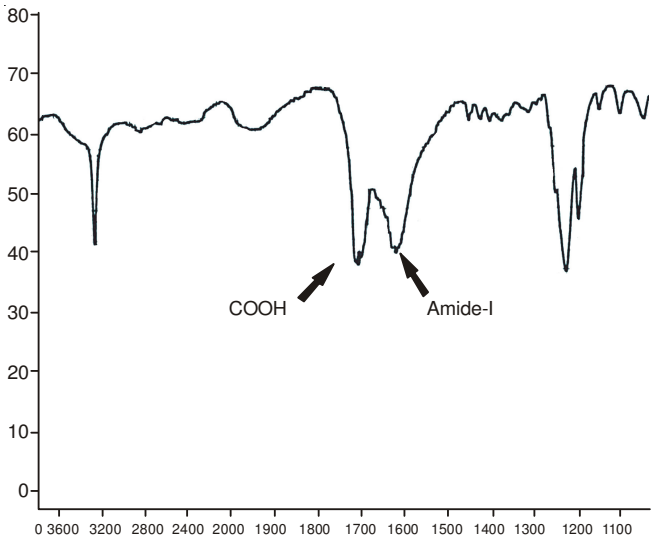



Fig. 5. IR spectrum of the product obtained by heating Boc-DL-Glu at 150 °C for 4 h

shown in TG line. The weight loss is due to 44.6 % of the total weight of the sample (12.3 mg) after peaks B and C, which seem to be the decomposition of *t*-butyloxycarbonyl-group. The weight loss of *t*-butyloxycarbonyl-group is 43.3 %, which almost equal to 44.6 %. During the peak D reveals on the DTA line, 14 % weight loss due to the weight of two water molecules (15.4 %) was observed. The last two broad peaks mean gradual degradation. The degradation is composed of three steps after melting.

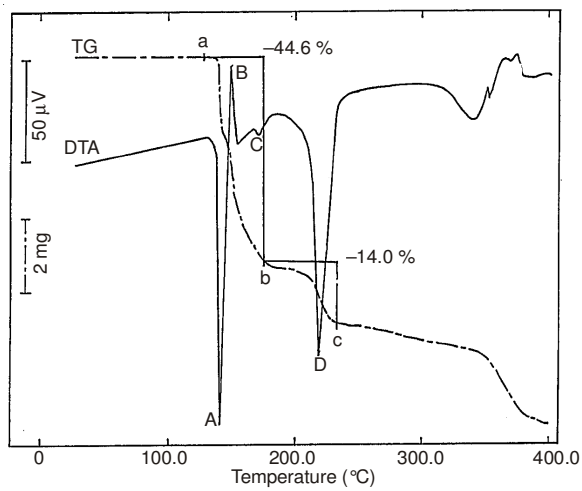



Fig. 6. Thermal analysis of Boc-DL-Asp (**2a**)

Fig. 7 shows differential analysis and thermal gravitmetry of *t*-butyloxycarbonyl-amino acid (**2d**). The peak (A) on DTA line corresponds to the endothermal peak due to melting of the sample, because the decrease in weight was not observed until the peak (A). However, with the peak (A), the weight of the sample rapidly decreased as shown in TG line. The weight loss is due to 43.9 % of the total weight of the sample (11.8 mg) after peaks B and C, which seem to be the decomposition of *t*-butyloxycarbonyl-group. The weight loss of *t*-butyloxycarbonyl-group is 43.9 %, which almost equal to 40.9 %. However, the three step weight losses on the TG line as shown in thermal analysis of **2a** (Fig. 6) was not observed in that of **2d**. After melting, TG line is composed of two step weight losses and the later step includes whole degradation without clear dehydration step.

Table-2 shows the summarized results of heating reactions of Boc-amino acids (**2a, 2c, 2d**).

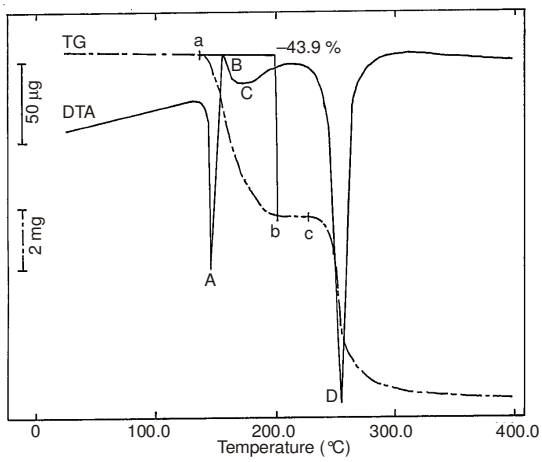



Fig. 7. Thermal analysis of *t*-butyloxycarbony-β-aminoglutaric acid

TABLE-2
REACTION CONDITIONS OF HEATING REACTION OF BOC-AMINO ACIDS

| Boc-amino acid | m.p. (°C) | Temp. (°C) | Time (h) |
|---|---|---|---|
| **2a** | 145-146 | 150 | 4 |
| | | 150 | 12 |
| | | 170 | 4 |
| | | 170 | 12 |
| **2c** | 125-128 | 130 | 4 |
| **2d** | 139-142 | 150 | 4 |

**Heating reactions of *t*-butyloxycarbonyl-acidic amino acids anhydrides (3a-d)**

**(1) Observation of reaction mixture upon heating:** The prepared anhydrides of *t*-butyloxycarbonyl-acidic amino acids (**3a-d**) were heated at different temperatures for each as shown in Table-3. The common process of morphologic change during the heating reaction is as follows: At first the crystal of the sample melted to a liquid, which released bubbles and then solidified again. Substrates **3a-b** gave polyaspartic acid and **3d** gave poly-β-amino-glutaric acid. The proof of the peptide structure of the product from substrate **3a** and **3d** is shown in the IR of Fig. 8. However, the product from substrate **3b** gave identical IR spectrum to that of pyroglutamic acid (Fig. 9). The formation mechanism can be explained by self-cyclization shown in Fig. 10.

The free amino group by the destruction of *t*-butyloxy-carbonyl-group attacked α-carboxyl carbon to afford a five-membered ring compound, which is pyroglutamic acid.

**(2) Thermal analyses of Boc-amino acids anhydrides (3a, 3c, 3d):** Although thermal analysis of Boc-amino acid anhydrides (**3a**, **3b**, **3d**) was carried out, the data did not give us meaningful information because of very noisy lines of DTA and TA. The lines look a vibration probably caused by a rapid bubbling of removing gasses.

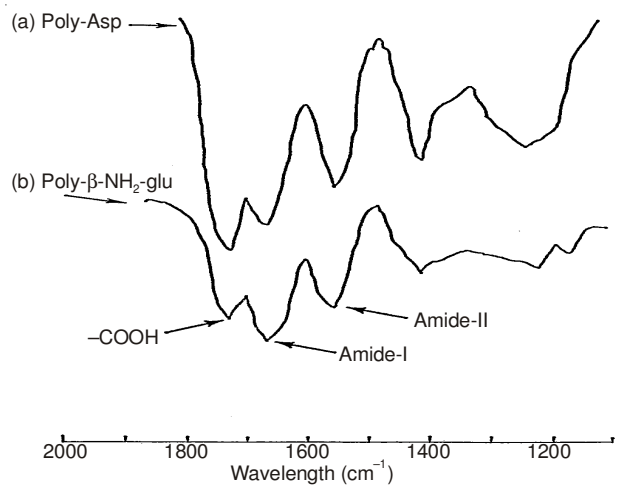



Fig. 8. IR spectrum of polyaspartic acid and poly-β-aminoglutaric acid

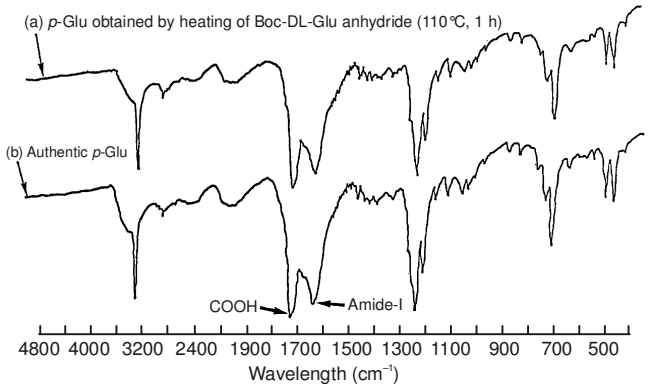



Fig. 9. IR spectrum of the product obtained by heating Boc-DL-Glu and standard pyro-glutamic acid

TABLE-3
HEATING REACTION OF Boc-AMINO ACID ANHYDRIDES (**3a-c**)

| Boc-amino acid anhydride | m.p. (°C ) | Reaction conditions | | Product | Yield (%) | Amino acid recovery (%) | D/L ratio (%) |
|---|---|---|---|---|---|---|---|
| | | Temp. (°C ) | Time (h) | | | | |
| **3a** | 122-124 | 130 | 1 | Polyasp[a] | 91 | 87 | - |
| | | 130 | 4 | | 89 | 99 | - |
| | | 130 | 12 | | 93 | 97 | - |
| | | 140 | 1 | | 89 | 97 | - |
| | | 150 | 1 | | 90 | 95 | - |
| **3b** | 135-137 | 130 | 1 | Polyasp[a] | 90 | 93 | 10 |
| | | 140 | 1 | | 89 | 87 | 14 |
| | | 140 | 4 | | 89 | 91 | 14 |
| | | 140 | 12 | | 93 | 89 | 17 |
| | | 150 | 1 | | 89 | 95 | 14 |
| | | 160 | 1 | | 93 | 94 | 18 |
| **3c** | 104-106 | 110 | 1 | Polyasp[a] | 93 | - | - |
| | | 110 | 4 | | 85 | - | - |
| | | 110 | 12 | | 92 | - | - |
| | | 140 | 1 | | 83 | - | - |
| | | 170 | 1 | | 75 | - | - |
| **3d** | 160-162 | 165 | 1 | Polyasp[a] | 90 | - | - |
| | | 165 | 4 | | 81 | - | - |
| | | 165 | 12 | | 90 | - | - |
| | | 175 | 1 | | 85 | - | - |
| | | 185 | 1 | | 88 | - | - |

[a]Polyaspartic acid; [b]Pyroglutamic acid; [c]Poly-β-amino glutaric acid

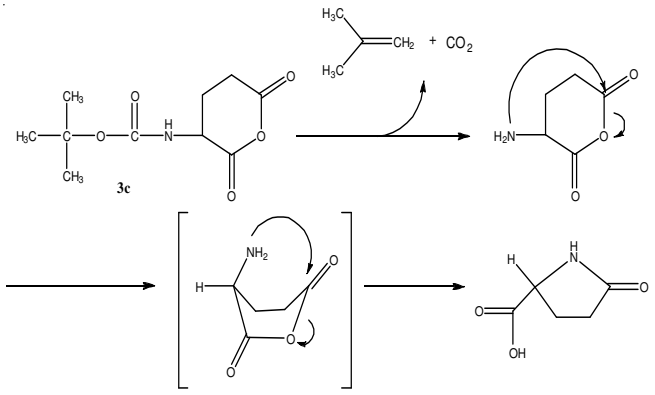



Fig. 10. Plausible mechanism of the formation of pyroglutamic acid from Boc-DL-Glu anhydride

Elementary analysis for some products obtained by heating **3a**, **3b** and **3d** was carried out. Polyaspartic acid (**5a**) by heting **3a** at 130 °C for 1 h; calcd. for $C_4H_5NO_3 \cdot 0.4H_2O$: C, 39.28; H, 4.78; N, 11.45 %. Found: C, 39.22; H, 5.04; N, 11.00 %. Polyaspartic acid (**5b**) by heating **3b** at 130 °C for 1 h: calcd. for $C_4H_5NO_3 \cdot 0.3H_2O$: C, 39.87; H, 4.68; N, 11.62 %. Found: C, 40.08; H, 5.03; N, 11.08 %. Poly-β-aminoglutaric acid (**5d**) by heating 165 °C for 1 h: calcd. for $C_4H_5NO_3 \cdot 0.3H_2O$: C, 44.64; H, 5.69; N, 10.41 %. Found: C, 44.64; H, 5.85; N, 10.48 %. Data of the elementary analysis almost agree with those of the calculated values based on the corresponding amino acid residue.

**(3) GPC of mixture of heating reaction:** Fig. 11 shows a gel permeation chromatography of the product obtained by heating *t*-butyloxycarbonyl-DL-Asp anhydride (**3a**). The peak of the polypeptide shows that the averaged molecular weight is in the range between 3,000 and 12,400 Da. The product from **3b** and **3d** gave similar molecular weight.

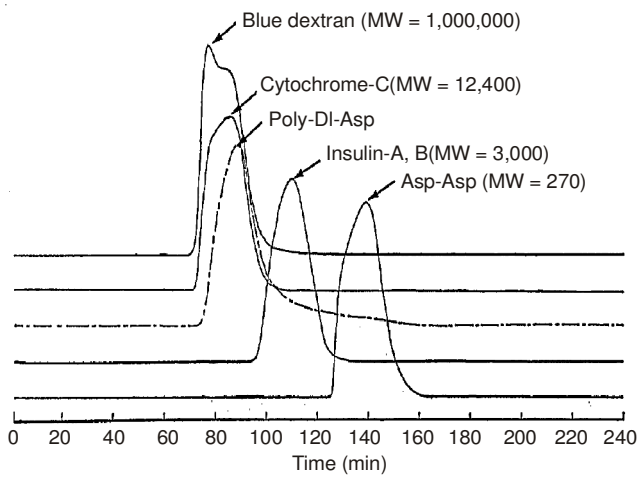



Fig. 11. Gel permeation chromatography (Sephadex G-25F) of the product obtained by heating Boc-DL-Asp anhydride

**(4) D/L ratio of the product from heating reaction of 3b:** Hydrolyzates of the heating products were evaporated *in vacuo* to give residues containing amino acids. The products were derivatized[15-17] to N-trifluoroacetyl-amino acid 2-propyl esters, which were injected to a gas chromatography equipped with a chiral capillary column. A typical chromatograph is shown in Fig. 12. About 10 % D/L ratio was observed from the product by heating reaction at 130 °C for 1 h as shown in Table-3. The longer reaction time and the higher reaction temperature gave a little higher racemization (D/L ratio: 14 to 18 %).

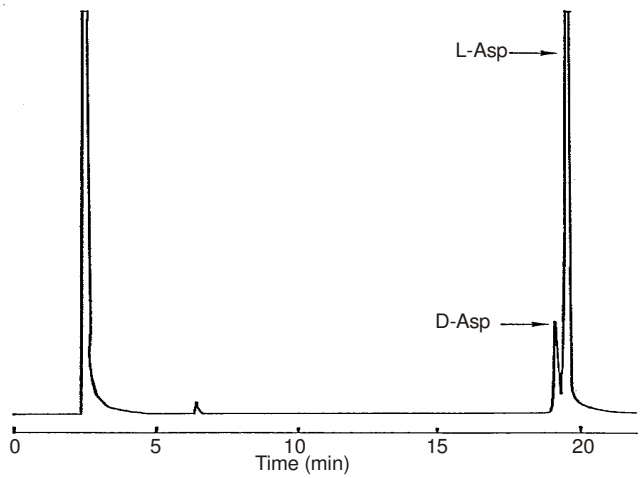



Fig. 12. Gas chromatogram of DL-aspartic acid. Aspartic acids were derivertized to N-trifluoroacetyl-aspartic acid 2-propyl esters, which were injected to gas chromatograph equipped with a chiral glass capillary column, Chirasil-Val

## Conclusions

• *t*-Butyloxycarbonyl-acidic amino acids (DL-, L-aspartic acid, DL-glutamic acid and β-amino glutaric acid) **2a-d** and their anhydrides **3a-d** were heated at the higher temperatures than their melting points to give corresponding main products as follows: Polyaspartic acid from compounds **2a-b** and **3a-b**; pyroglutamic acid from compounds **2c**, **3c**; β-amino glutaric acid from **2d**; poly-β-aminoglutaric acid from **3d**.

• Averaged molecular weight of polyaspartic acid was estimated as 3000 to 12,400 Da with a gel permeation chromatography.

• Simultaneous measurements of thermal gravitmetry (TG) and differential thermal analysis showed the gradual weight decrease of compounds **2b-d** corresponding to 2-butene, carbon dioxide and water molecules.

• Polyaspartic acid synthesized upon heating *t*-butyloxycarbonyl-L-Asp anhydride (**3b**) showed a slight degree of racemization (D/L: 10 %, for 1 h at 130 °C).

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