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Heating Reactions of *N*-*t*-Butyloxycarbonyl-Asparagine and Related Compounds

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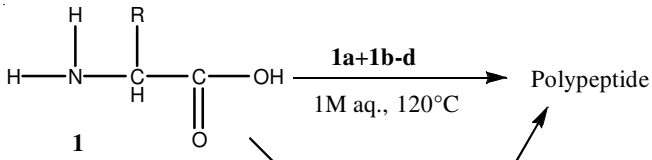
N-*t*-Butyloxycarbonyl-amino acids (Boc-) are labile on heating to afford free amino acids, but Boc-aspartic acid gives a kind of polypeptide. This chemical feature of Boc-aspartic acid may be caused by dehydration between two carboxyl groups as well as the formation of a free amino group. Boc-Asparagine may have a similar reactivity to Boc-aspartic acid. This research describes polypeptide formation by heating Boc-asparagine and its isomer Boc-aspartic acid amide.

Keywords: Boc-asparagine, Polypeptide, Heating reaction.

INTRODUCTION

Asparagine (**1a**), which is a neutral proteinous amino acid, has a carboxamide group at the side chain. Asparagine (**1a**) reacts with itself[1,2] and with other amino acids[2] (**1b-d**) in aqueous solutions upon heating to afford polypeptides (**Scheme-I**).



1a: R= -CH_2CONH_2
1b: R= -H
1c: R= -CH_3
1d: R= -$CH(CH_3)_2$
1e: R= -CH_2COOH

Scheme-I

A similar amino acid, aspartic acid (**1e**), has a carboxyl group at the side chain instead of the carboxamide group.Solid aspartic acid (**1e**) polymerizes[3-6] with itself and other amino acids[7]) upon heating to afford polypeptides (**Scheme-I**). These have been considered basic reactions in chemical evolutionary processes in abiotic environments[1-8].

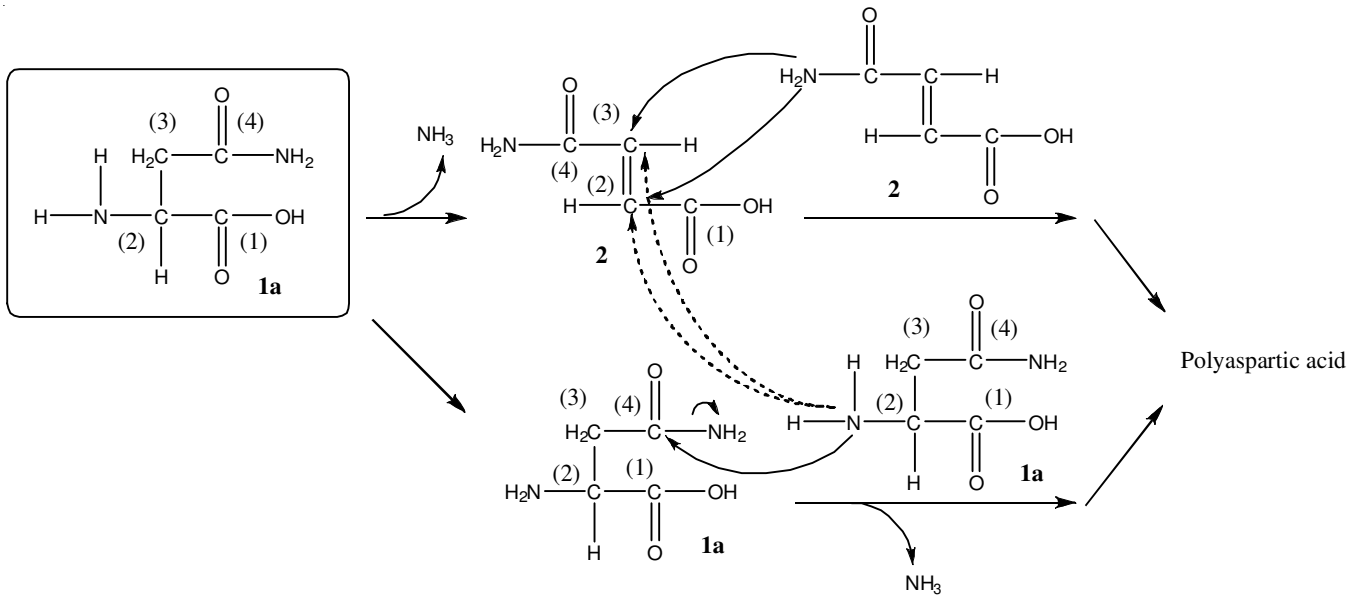
The mechanism of the heating reaction of asparagine has been explained by the deamination[9] from the C2-position and the deamidation[1,2] from the C4-position (**Scheme-II**). The elimination of amino groups at these positions yields fumaramide (**2**) and polyaspartic acid, respectively. Fumaramide (**2**) molecules can react with each other through the nucleophilic addition of the remaining amino group at the C2-position to the C=C double bond to produce polyaspartic acid. Asparagine (**1a**), aspartic acid (**1e**), fumaramide (**2**) and some other dicarboxylic acids can exist in equilibrium[2] among those compounds. Similar equilibrium containing deamination from aspartic acid has been observed[10-13].

On the other hand, the heating reactions of solid aspartic acid (**1e**) pass through water elimination to give polyaspartic acid. The intermediate aspartic acid anhydride (**3**) is suggested, but it has not been isolated because of its lability (**Scheme-III**).

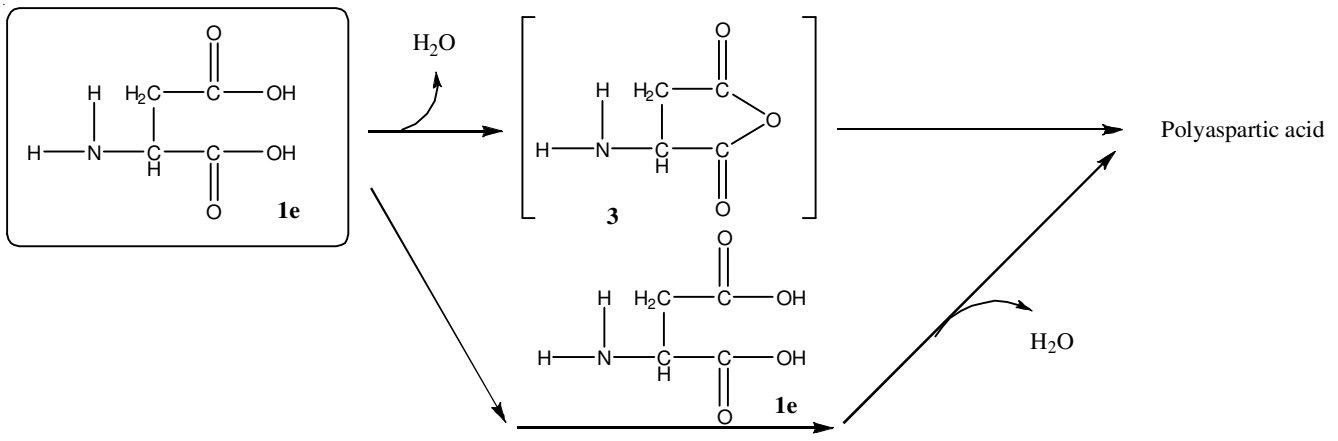
We have previously reported[14,15] the heating reactions of *N*-*t*-butyloxycarbonyl-L-aspartic acid (**4e**), which has a lower melting point (119-120 °C) than that of free aspartic acid (**1b**) (m.p. 270-271 °C in a sealed tube). The heating reaction was carried out at 140-150 °C to afford anhydrous polyaspartic acids. Because asparagine possesses a similar chemical structure to aspartic acid, it may react to give polyaspartic acid. This paper addresses the heating reactions of *N*-*t*-butyloxycarbonyl-L-asparagine (Boc-L-Asn (**4a**), **Scheme-IV**) and its isomer Boc-L-aspartic acid amide (Boc-Asp-NH_2 (**8**), **Scheme-V**), which was derived from **1e**.

EXPERIMENTAL

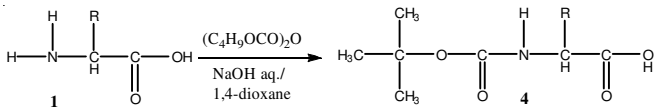
L-Asparagine (**1a**) and L-aspartic acid (**1e**) were purchased from Nacalai Tesque (Kyoto, Japan). Glycine (**1b**), L-alanine (**1c**) and L-valine (**1d**) were supplied by Nippon Rika Co.,



Scheme-II



Scheme-III

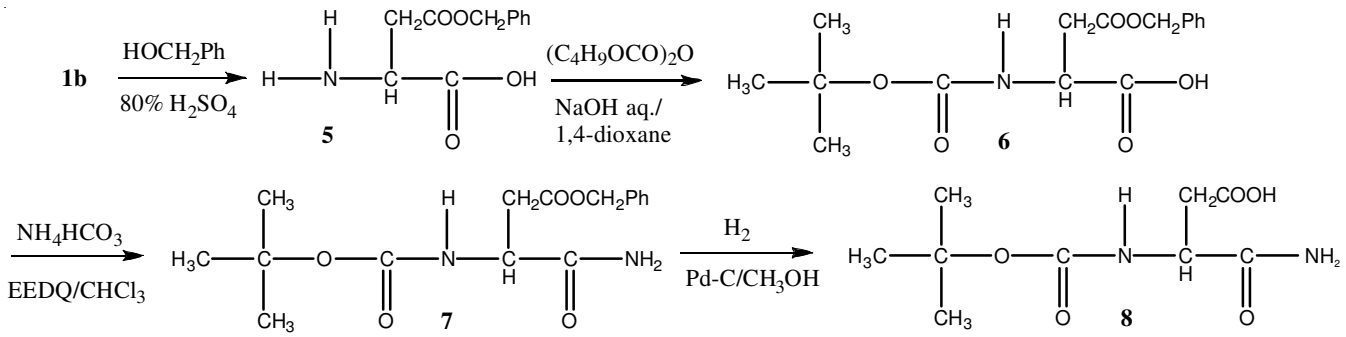


1a: R= -CH_2CONH_2
1b: R= -H
1c: R= -CH_3
1d: R= -$CH(CH_3)_2$
1e: R= -CH_2COOH

4a: R= -CH_2CONH_2
4b: R= -H
4c: R= -CH_3
4d: R= -$CH(CH_3)_2$
4e: R= -CH_2COOH

Scheme-IV

Ltd (Tokyo, Japan). Di-*t*-butyloxycarbonyl carbonate was purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). 6 M Hydrochloric acid for hydrolysis of peptides was purchased from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan). Trifluoroacetic anhydride was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). The standard proteins (Bovine serum albumin (BSA): MW = 66340; ovalbumin (OVA): MW = 44,287; carbonic anhydrase: MW = 29,000; cytochrome C: MW=12,384; myoglobin:



EEDQ: N-ethoxycarbonyl-2-ethoxy-1, 2-dihydroquinoline

Scheme-V

MW=17,800; clupeine: MW = 4110; insulin A-chain: MW = 2340) and glycine hexamer (Gly_6: MW = 360) for the calibration of the molecular weight of the peptides were purchased from SIGMA-Aldrich (St. Louis, USA). The pentamer of prolyl-prolyl-glycine ($(Pro\text{-}Pro\text{-}Gly)_5$: MW = 1274) was purchased from the Peptide Institute, Inc. (Osaka, Japan).

A Hitachi model 260-50 infrared spectrophotometer (Hitachi, Tokyo, Japan) was used to record the infrared spectra. A Hitachi Model 200-10 Spectrophotometer was used to record the ultraviolet-visible spectra. Optical rotation of the synthetic compounds from L-aspartic acid was determined with a Jasco DIP-181 digital polarimeter. A Hitachi 835 amino acid analyzer (Hitachi, Tokyo, Japan) was used for amino acid analysis. Molecular weight determination was performed using a high-performance liquid chromatography (HPLC) system composed of an ultraviolet detector, Jasco Uvdec-100-V UV spectrophotometer and a flow pump, Jasco TRI Rotor-V, equipped with a TSK GEL G3000PW (300 mm × 7.5 mm I.D.). The flow rate was 0.7 mL/min (0.1 M sodium phosphate, pH 6.9) and the absorbance of the samples was recorded at 230 nm. The peaks on the chromatograms were integrated with an SIC Chromatocorder II. Enantiomer separation of amino acids was performed with a Hitachi 163 gas chromatograph equipped with a chiral glass capillary column (Chirasil-Val[16-19]), 25 m × 0.3 mm I.D.). Nitrogen was used as the carrier gas at a flow rate of 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 thermionic detector. A Shimadzu DT-40 (Shimadzu, Kyoto, Japan) was used for thermal analysis. A Shimadzu GCMS-QP 1000A mass spectrometer was used for measuring mass spectra. A JEOL EX-270 NMR system (JEOL, Tokyo, Japan) was used for the measurement of ^{1}H NMR spectra.

Preparation of substrates

***N-t*-Butyloxycarbonyl-L-asparagine (4a):** To a solution containing L-asparagine monohydrate (**1a**) (15 g, 0.10 mol) in a mixture of 1 M sodium hydroxide (100 mL) and water (180 mL) was added a solution containing di-*t*-butyloxy-dicarbonate (24 g, 0.11 mol) in 1,4-dioxane (170 mL) dropwise with stirring at 10 °C. After overnight stirring at room temperature, the reaction mixture was evaporated *in vacuo* to an oily product, which was cooled and acidified to pH 2 with 10 % potassium hydrogen sulfate to give a precipitate. The precipitate was filtered, washed with water and recrystallized with 500 mL ethanol to give 14.8 g of crystals (64 %). m.p. 177-179 °C (lit.[20]) 181-182). ^{1}H NMR (DMSO-d_6): δ = 1.38 ppm (9H, s, CH_3), 2.09 (1H, s, NH), 2.48 (2H, d, CH_2), 4.22 (1H, q, CH), 6.88 (2H, d, NH_2). IR (KBr, ν_{max}, cm^{-1}): 1720 (COOH), 1690 (amide I), 1663 (amide II (side chain)), 1591 (amide II (side chain)), 1535 (amide II). Elemental analysis: Calcd. for $C_9H_{16}N_2O_5$: C, 46.54; H, 6.94; N, 12.07 %. Found: C, 46.70; H, 7.05; N, 11.99 %. $[\alpha]_D^{15}$-7.8° (c = 1.22, DMF) (lit.[21]) $[\alpha_D]^{20}$ -7.8 (c = 1, DMF)).

***N-t*-Butyloxycarbonyl-glycine (4b):** Yield: 12.6 g (72 %), m.p. 89-91 °C (lit.[21]) 88.5-89 °C). ^{1}H NMR (DMSO-d_6): δ = 1.38 ppm (9H, s, CH_3), 3.58 (2H, d, CH_2), 7.04 (1H, t, NH). IR (KBr, ν_{max}, cm^{-1}): 1750 (COOH), 1690 (amide I), 1671 (amide I), 1538 (amide II). Elementary analysis: Calcd. for $C_7H_{13}NO_4$: C, 47.99; H, 7.48; N, 8 %. Found: C, 48.01; H, 7.40; N, 7.99 %.

***N-t*-Butyloxycarbonyl-L-alanine (4c):** Yield: 28.8 g (76 %). m.p. 87-88 °C (lit.[22]) 83-84 °C). ^{1}H NMR (DMSO-d_6): δ = 1.38 ppm (9H, s, CH_3), 2.09 (1H, s, NH), 1.18-1.26 (3H, d, CH_3), 3.77-4.07 (1H, q, CH), 7.03-7.11 (1H, d, NH). IR (KBr, ν_{max}, cm^{-1}): 3386 (NH), 1742 (COOH), 1690 (amide I), 1520 (amide II). Elemental analysis: Calcd. for $C_8H_{15}NO_4$: C, 50.78; H, 7.99; N, 7.40 %. Found: C, 50.78; H, 8.10; N, 7.27 %. $[\alpha]_D^{15}$-24.2° (c = 1.30, acetic acid) (lit.[21]) $[\alpha]_D^{20}$-22.4 (c = 2.1, acetic acid).

***N-t*-Butyloxycarbonyl-L-valine (4d):** Yield: 23.4 g (93 %). m.p. 78-79 °C (lit.[22]) 77-79 °C). ^{1}H NMR (DMSO-d_6): δ = 0.83-0.91 ppm (6H, q, CH_3), 1.39 (9H, s, CH_3), 1.84-2.11 (1H, m, CH), 3.72-3.87 (1H, q, CH), 6.85-6.94 (1H, d, NH). IR (KBr, ν_{max}, cm^{-1}): 3312 (NH), 1740 (COOH), 1649 (amide I), 1500 (amide II). Elemental analysis: Calcd. for $C_{10}H_{19}NO_4$: C, 55.28; H, 8.81; N, 6.45 %. Found: C, 55.42; H, 8.86; N, 6.45 %. $[\alpha]_D^{15}$-5.1° (c = 0.97, acetic acid) (lit.[21]) $[\alpha]_D^{20}$-5.8 (c = 1.2, acetic acid).

L-Aspartic acid β-benzyl ester (5): L-Aspartic acid (**1b**) (50 g, 0.38 mol), benzyl alcohol (125 g, 1.16 mol), a mixture of concentrated sulfuric acid (40.5 g) and water (10 g) were mixed in a water bath at 70 °C for 0.5 h to give a clear solution, which was stirred for s further 2 h. The resulting solution was evaporated *in vacuo* to an oily product, which was added to a cold solution containing sodium hydrogen carbonate (70 g) and water (250 mL) at 0 °C. The obtained cooled solution was mixed with ether (150 mL) to give a precipitate, which was filtered and washed with cold water to give another precipitate (88 g). This was recrystallized twice with water to give 34 g (41 %). m.p. 218-219 °C (lit.[23]) 222 °C).

***N-t*-Butyloxycarbonyl-L-aspartic acid β-benzyl ester (6):** L-Aspartic acid β-benzyl ester (**5**) (3 g, 13.4 mmol) was dissolved in 1 M sodium hydroxide (13 mL) and tetrahydrofuran-acetonitrile (1:1 (v/v), 26 mL). To the cooled solution was added dropwise an acetonitrile solution containing di-*t*-butyloxy-dicarbonate (3.22 g, 14.7 mmol) over 0.5 h. The solution was adjusted to pH 8 by adding 1 M sodium hydroxide (5 mL). The reaction solution was stirred for 24 h and evaporated *in vacuo* to give an oily product. The cooled oil was acidified with 10 % potassium hydrogen sulfate (30 mL) to pH 2 and the resulting solution was extracted with ethyl acetate (30 mL × 3). The extracts were combined and dried with anhydrous magnesium sulfate and the evaporation of the filtrate gave a precipitate. Twice recrystallization with ethyl acetate-petroleum ether gave 2.5 g (56 %). m.p. 98 °C (lit.[24]) 99 °C).

***N-t*-Butyloxycarbonyl-L-aspartic acid β-benzyl ester α-amide[23] (7):** *N-t*-butyloxycarbonyl-L-aspartic acid β-benzyl ester (11.3 g, 35 mmol), ammonium hydrogen carbonate (7.99 g,105 mmol), N-ethoxycarbonyl-2-ethoxy-1 and 2-dihydroquinolone (9.52 g, 38.5 mmol) were suspended in chloroform (65 mL). The reaction mixture was stirred overnight at room temperature. The reaction mixture was extracted with water and the resulting chloroform layer was evaporated *in vacuo* to give a precipitate, which was recrystallized with ethyl acetate-hexane to give 6.48 g (58 %). m.p. 153-155 °C. (lit. 157-160). $[\alpha]_D^{25}$-2.1 (c = 1, ethanol). (lit.[25])$[\alpha]_D^{25}$ -2.6 (c = 1, ethanol)).

***N-t*-Butyloxycarbonyl-L-aspartic acid α-amide (8):** *N-t*-Butyloxycarbonyl-L-aspartic acid (4.01 g, 12 mmol) was dissolved in a solution of methanol (50 mL), ethanol (20 mL) and N,N-dimethyl formamide (30 mL). The solution was stirred in a hydrogen atmosphere over 5 % palladium on charcoal for 24 h. After filtration of the reaction mixture, the filtrate was evaporated to give 2.3 g (80 %). m.p: 138-139 °C. Elemental analysis: Calcd. for $C_9H_{16}N_2O_5$: C, 46.55; H, 6.94; N, 12.06 %. Found: C, 46.21; H, 6.96; N, 11.78 %. $[\alpha]_D^{25}$ + 52 (c = 0.44, ethanol-DMF (1:1)).

Thermal analysisof Boc-L-Asn and mass spectrometry of evolved gas from chamber: A sample of Boc-L-asparagine (7.5 mg, weighed to within 0.1 mg) was placed on a platinum sample pan and the α-aluminum reference was placed in the center of the second platinum pan. The temperature of the heating chamber was programmed from 27 to 250 °C using a heating rate of 5 °C/min under a helium flow (50 mL/min). The evolved gases during the heating were directly introduced to the mass spectrometer and analyzed as time proceeded. The pipe heater from the thermal analyzer chamber was maintained at 280 °C. Electron impact with 70 eV was carried out at an ion source temperature of 250 °C. Mass spectra were monitored at intervals of 1.5 sec.

Procedure forheating reactions of amino acids and Boc-amino acids: The heating reactions of L-Asn (**1a**) (1.0 mmol) and L-Asp (**1e**) (1 mmol) were carried out in the same manner as the reactions of Boc-L-Asn (**4a**). Boc-L-Asn (**4a**) (0.5 mmol), Boc-L-Asp-NH_2 (**8**) (0.5 mmol) and mixtures (0.5 mmol) of Boc-L-Asn (**4a**) plus other Boc-amino acids (**4b-d**) were put into different Pyrex glass tubes (165 mm × 16 mm I.D.) and stood for 5 min under a nitrogen stream. They were heated for a constant time at a constant temperature in an oil bath. After heating, the resulting samples were weighed and their infrared spectra were measured.

Determination of amino acids and purification of reaction mixture: The reaction mixture after the heating reaction was dissolved in 0.5 M acetic acid to afford 10 mL of solution. Part of the solution (0.5 mL) was evaporated in a desiccator at room temperature to dryness. The residue was redissolved in 0.01 M HCl to give a 10 mL solution, which was analyzed with an automated amino acid analyzer.

The remaining sample solution (9.5 mL) in 0.5 M acetic acid was loaded into a gel filtration column (910 mm × 15 mm I.D.) of Sephadex G-25F. The elution was carried out using 0.5 M acetic acid. Every 10 min, 3 mL of eluate was collected. The collected fractions were monitored by UV absorption at 230 nm. The higher molecular weight fractions, which were UV-positive and eluted faster than 450 min due to 135 mL at the elution point of $(Pro\text{-}Pro\text{-}Gly)_5$ (MW: 1274 Da) on the chromatograph, were combined and lyophilized to give a powdery substance. The substance was analyzed by means of HPLC equipped with the gel filtration column TSK-GEL G3000PW described above. The average molecular weight of the fraction was estimated with a calibration curve prepared with the elution data of standard proteins and peptides of known molecular weight (Fig. 1).

Hydrolysis of samples after heating reactions and amino acid analysis: A part of the powdery substances (2 mg) obtained by lyophilization of the higher molecular weight fractions was dissolved in 2 mL of 6 M hydrogen chloride in a glass tube. The tube containing solution was surrounded by a dry ice-ethanol bath and was sealed under vacuum and heated at 110 °C for 8 h. Amino acid analysis of the sample was carried out by means of the amino acid analyzer described above.

Determination of racemization: A part of the hydrolyzate was evaporated *in vacuo* to give a solid residue, which was derivatized in two steps using 1.5 M hydrogen chloride in 2-propanol first and trifluoroacetic anhydride in dichloromethane second to give N-trifluoroacetyl amino acid 2-propyl ester. The derivatives of amino acid enantiomers were analyzed with the gas chromatograph equipped with a chiral capillary column described above.

RESULTS AND DISCUSSION

L-Asparagine (**1a**) and L-aspartic acid (**1e**), Boc-L-Asn (**4a**) and Boc-L-Asp-NH_2 (**8**) were independently heated at a

TABLE -1
RESULTS OF THERMAL REACTIONS OF Asn (**1a**), Asp (**1e**), Boc-Asn (**4a**), and Boc-Asp-NH_2 (**8**)

Substrate	Temp (°C)	Time (h)	Yield[a] (mg)	Mw[b]	D/L ratio[c]	AA recovery (%)[d]	Asn residue (%)[e]
1a	200	4	19	7600	0.50	80	–
1e	200	4	30	31000	0.51	76	–
	200	16	67	45000	0.57	90	–
4a	147	4	17	3900	0.39	98	24
		8	13	4500	0.43	108	22
		16	18	4200	0.48	104	–
	150	2	15	4200	0.48	98	28
		4	15	4800	0.48	85	26
		8	13	4800	0.55	95	30
		16	10	4900	0.53	104	29
8[f]	130	4	0.21	4500	–	–	–
		8	0.81	–	–	88	–
		16	0.27	4700	–	–	–
		24	1.2	–	–	79	–
	140	8	0.66	4900	–	–	–
		16	0.73	–	–	77	–

[a]: Yield of higher molecular weight fraction. [b]: Molecular weight of higher molecular weight fraction. [c]: D/L ratio of Asp [d]: Amino acid recovery in acid hydrolysate of higher molecular weight fraction [e]: Estimated asparagine composition from the increase of ammonia in hydrolysate. [f]: The decreases in weight of heating reaction mixture were 32.5 (2 h), 44.7 (4 h), 47.1 (16 h), 47.3 % (24 h) at 130 °C; 51.2 (8 h), 51.6 (16 h) at 140 °C

constant temperature for a constant period of time as shown in Table-1. Free amino acids asparagine (**1a**) and aspartic acid (**1e**) gave higher yields (19-67 mg) and higher molecular weights compared with the products (10-18 mg) from Boc-L-Asn (**4a**). The yield obtained from Boc-L-Asp-NH_2 (**8**) was much lower than those from Boc-L-Asn (**4a**). The theoretical yield calculated from the residue weight (115.1 Da) of aspartic acid is 115.1 mg. The products from Boc-L-Asn (**2a**) and Boc-L-Asp-NH_2 (**6**) gave similar molecular weights (3900 to 4900 Da). IR spectra of these products show typical absorption caused by peptides (Imide: 1785; -COOH: 1717; amide I: 1670; amide II: 1540 cm^{-1}), as shown in Fig. 2.

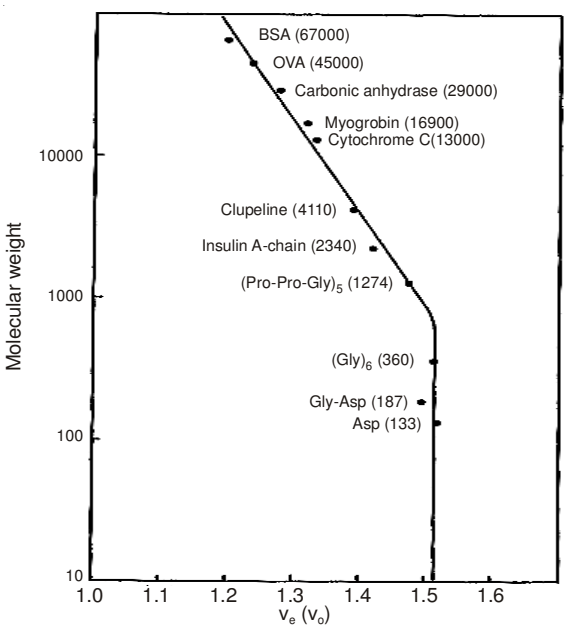


Fig. 1. Calibration curve of molecular weight against V_e divided by V_0. V_e: elution volume of proteins or peptides; V_0: void volume (6.4 mL). TSK gel G3000PW was used for the analysis at a flow rate of 0.7 mL/min of 0.1 M sodium phosphate buffer (pH 6.9). Detection was carried out at 230 nm UV absorption

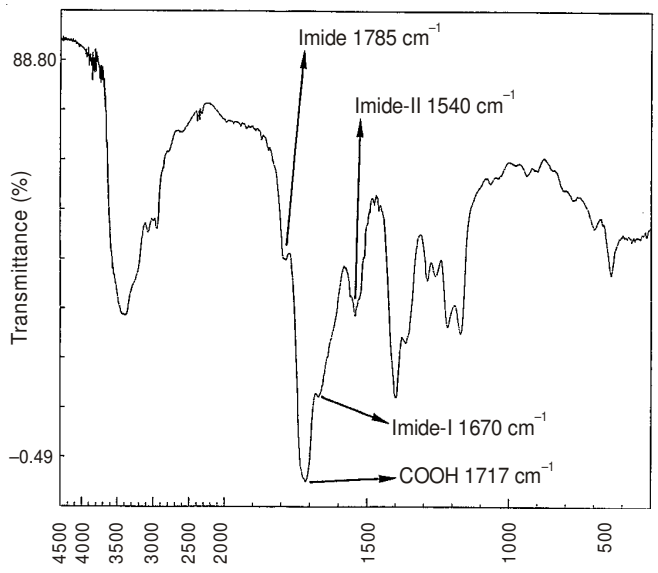


Fig. 2. IR of polypeptide obtained by thermal reaction of Boc-L-Asn at 150 °C for 2h

However, it is difficult to explain the much lower yield from Boc-L-Asp-NH_2 (**8**). The compound may have produced a cyclic dipeptide. Once such a cyclic dipeptide forms, it does not react further to yield a polypeptide. IR spectra of the lower molecular weight fractions do not show a clear absorption caused by such cyclic dipeptides, because the lower molecular weight fraction is a mixture composed of linear and cyclic peptides. Purification of the cyclic peptide must be investigated in the next step. Heating Boc-L-Asp-NH_2 (**8**) at 130 °C for 24 h and 140 °C for 16 h showed a 47.3 and 51.6 % loss of the total weight, respectively. The values are similar to the calculated value (51.1 %) for the decrease including both Boc and ammonia.

The molar ratio of D-isomer to L-isomer of aspartic acid residue was almost similar (0.39:0.57) in all cases using substrates **1a**, **1e** and **2a** to give similar racemization, although the ratio depends slightly on the reaction time and temperature. Amino acid recovery was also similar. Estimated asparagine composition in the higher molecular weight peptides was almost similar (22 to 30 %) in all corresponding cases.

Thermal reaction of Boc-L-Asn (4a) and other Boc-amino acids (4b-d): The results of the thermal reactions of Boc-L-Asn (**2a**) and other amino acids (**2b-d**) are shown in Table-2. The results show higher yield (37 to 65 mg) but lower molecular weight (900 to 2200 Da) peptide formation. The D/L ratio in the aspartic residue was lower (0.28:0.45) than when using **2a** only (0.39:0.57), because the reaction temperature is lower (130 °C) for the case in Table-2. The included Ala and Val residues in the peptides show a much lower value of 0.04. AA composition to Asp means the molar ratio of the other amino acid composition included in the peptides. Comparing the value in the same ratio (1:1) for different amino acids, the order of inclusion is Gly (0.84), Ala (0.72) and Val (0.63). The result seems to reflect the reaction rate difference caused by the steric hindrance from side chains. Although the total amino acid recovery was more than 80 % for **1a** plus **1b**, the combinations of **1a** plus **1c** and **1a** plus **1d** gave much lower recovery (57 and 50 %). The estimated composition of asparagine residue was almost the same as that for heating **1a** only, as shown in Table-1.

Thermal analysis and mass spectrum of Boc-L-Asn (2a): Figs. 3 and 4 show a typical thermal analysis profile and a mass chromatogram of the gases from the thermal analyzer, respectively. An endothermal peak caused by melting is revealed after 160 °C on the differential thermal analysis (DTA) line in Fig. 3. Just after the peak that appears at this temperature, a rapid decrease in weight startedon the thermogravimetry line to give 52.7 % loss of the initial weight. The value is larger than the weight loss (43.5 %) from the decomposition (2-butene and carbon dioxide) of the Boc group. This difference in weight decrease suggests that further reactions proceeded. The mass chromatogram in Fig. 4 shows peaks of ammonia and water as well as 2-butene and carbon dioxide to support the further reactions after the decomposition of the Boc group. The theoretical weight decreases including ammonia and water are 51.1 and 51.3 %, respectively.

Postulated reaction mechanism for heating reaction of Boc-L-Asn (4a): Scheme-VI shows a postulated reaction

TABLE-2
RESULTS OF THERMAL REACTIONS OF Boc-Asn WITH OTHER Boc-AMINO ACIDS

Substrate	Molar ratio[a]	Temp (°C)	Time (h)	Yield[b] (mg)	Mw[c]	D/L Ratio in Asp	D/L Ratio in Ala or Val	AA[d] composition to Asp	AA Recovery (%)[e]	Asn residue (%)[f]
4a+4b	(1+1)	130	2	60	1000	0.31	–	0.86	80	40
	(1+1)	130	4	42	1400	0.43	–	0.84	83	33
	(2+1)	130	4	42	1500	0.32	–	0.49	80	45
	(1+2)	130	4	37	2200	0.45	–	1.57	101	29
4a+4e	(1+1)	130	4	65	1000	0.36	0.04	0.72	57	34
4a+4d	(1+1)	130	4	59	900	0.28	0.04	0.63	50	27

[a]: Molar ratio of **4a** and **4b**: (2+1) means **4a** 1.0 mmol plus **4b** 0.5 mmol. [b]: Yield of higher molecular weight fraction. [c]: Molecular weight of higher molecular weight fraction. [d]: Other amino acid composition to aspartic acid in acid hydrolyzate of higher molecular weight fraction. [e]: Total amino acid recovery. [f]: Asparagine composition calculated from the increase of ammonia in hydrolyzate

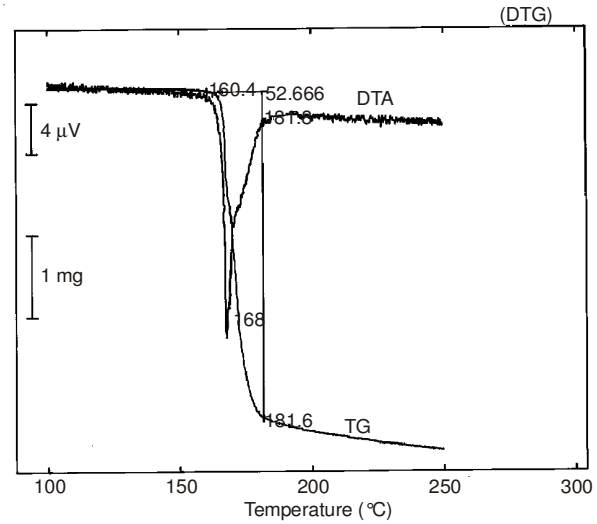


Fig. 3. Thermal analysis of Boc-L-Asn (**4a**)

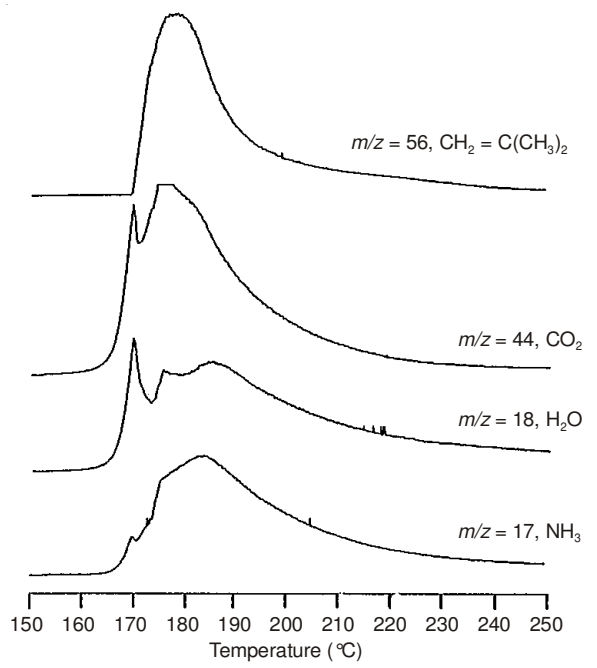


Fig. 4. Mass chromatogram of gases generated during thermal analysis of Boc-L-Asn (**4a**)

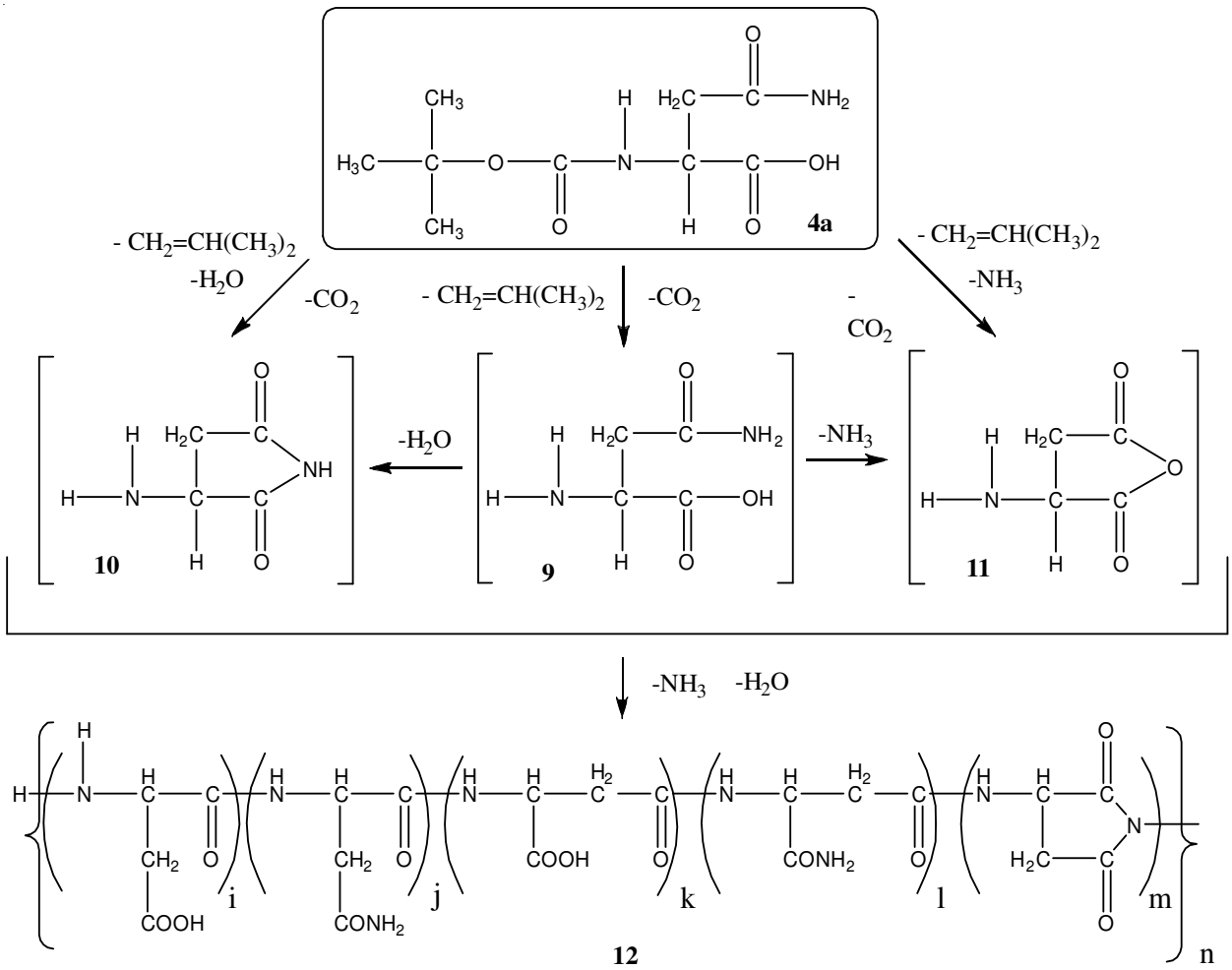
mechanism, in which three intermediates (**10**, **11** and **12**) are produced. Boc-L-Asn (**4a**) releases 2-butene and carbon dioxide to afford intermediate **9**, which may give intermediates **10** and **11** by releasing water and ammonia, respectively. However, Boc-L-Asn (**4a**) may also yield intermediates **10** and **11** without intermediate **9**, because generation of 2-butene, carbon dioxide, ammonia and water was observed at the same time according to the results of thermal analysis and the mass chromatogram as shown in Figs. 3 and 4. The order of generation of these gases is unknown. Therefore, the intermediates **10**, **11** and **12** are considered to react together as a mixture to yield polypeptide **12**.

Polypeptide 12 is a mixture of many kinds of chemical structure. It is composed of five fundamental residues bearing different side chains: 2-Methylcarboxyl, 2-methylcarboxamide, 2-carboxyl, 2-carboxamide and imide groups. Although the sequential order and number of these side chains are unknown, their existence is supported by IR spectra and ammonia generation from the acid hydrolysate of the heating products.

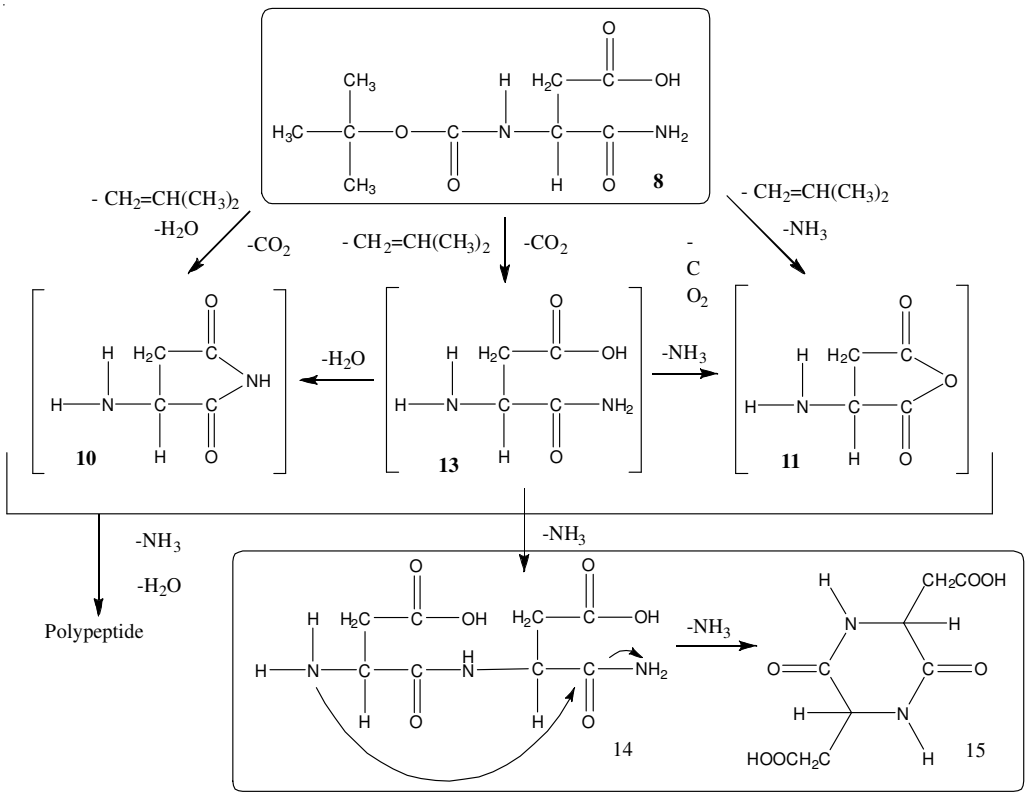
Postulated reaction mechanism of heating reaction of Boc-L-Asp-NH_2 (8): Scheme-VII shows a postulated mechanism for the heating reaction of Boc-L-Asp-NH_2 (**8**), which is suggested to give an intermediate **13** similar to intermediate **9** in **Scheme-VI** as well as intermediates **10** and **11**. However, the heating reaction did not give higher molecular weight polypeptides, as shown in Table-1. These results suggest that Boc-L-Asp-NH_2 (**8**) was deprotected to give intermediate **13**, which itself reacted, passing through a linear dipeptide to afford a cyclic dipeptide **15**. The intermediate **13** is stable and does not polymerize with itself. On the other hand, the low yield of higher molecular weight peptide suggests that intermediates **10** and **11** were maintained in very low concentration. This consideration can support the formation of cyclic dipeptide **15**.

Conclusions

- Boc-L-Asn and Boc-L-Asp-NH_2 gave higher molecular weight polypeptides up to 4900 Da upon heating at 130-150 °C.
- Racemization proceeded during heating reaction in the D/L ratio of 39 to 53 %.
- Thermal analysis with mass spectrometry clarified the formation of 2-butene, carbon dioxide, ammonia and water during heating reactions.
- Mechanisms of heating reactions of Boc-L-Asn and Boc-L-Ssp-NH_2 were proposed.



Scheme-VI



Scheme-VII

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