Syntheses of Polypeptides by Hydrogenolysis of *N*-Benzyloxycarbonyl-Amino Acid Anhydrides

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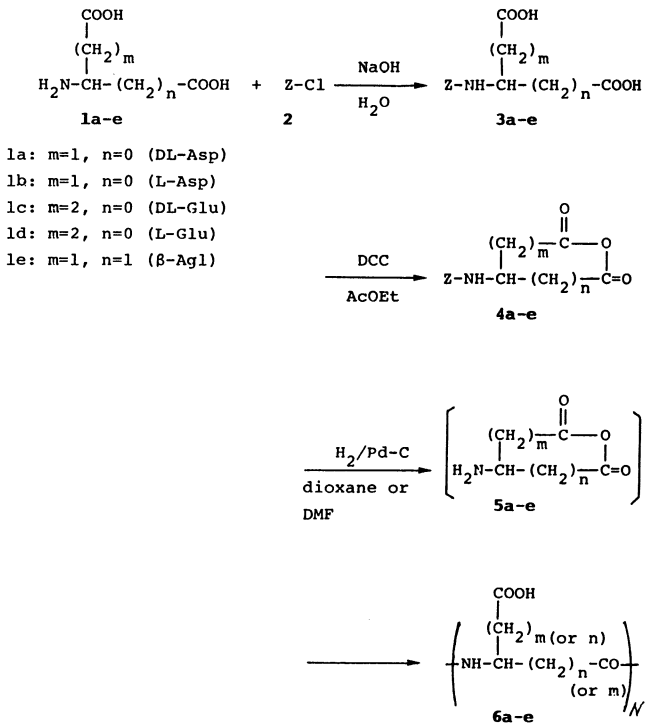
(Received March 27, 1989)

Synopsis. When anhydrides of *N*-benzyloxycarbonyl-DL-aspartic acid (Z-DL-Asp), Z-L-Asp, *N*-Z-DL-glutamic acid (Z-DL-Glu), Z-L-Glu, and *N*-Z-3-aminoglutaric acid (Z-β-Agl) were hydrogenolyzed in *N*,*N*-dimethylformamide (DMF), polypeptides were obtained in high yields. Hydrogenolyses of Z-DL-Glu and Z-L-Glu in dioxane gave pyroglutamic acid.

Although there are many investigations on the syntheses of polyamino acids, there have been few studies on the polymerization of amino acid anhydrides because of their instability and difficulty of isolation.[1] One way to overcome this difficulty would be to generate the amino acid anhydride in situ,[1-3] which would then react with each other to form polypeptides. The hydrogenation of 2-azidosuccinic anhydride in dioxane to afford polyaspartic acid has been reported.[2] However, the molecular weight and the structure of the product were not characterized in detail.[2] Aspartic acid anhydride was considered as the intermediate (actual monomer) in the polymerization.

In this paper, we wish to describe the polymerization reactions of amino acid anhydrides generated by the hydrogenolyses of anhydrides of *N*-benzyloxycarbonyl-DL-aspartic acid (Z-DL-Asp), Z-L-Asp, Z-DL-glutamic acid (Z-DL-Glu), Z-L-Glu, and Z-3-aminoglutaric acid (Z-β-Agl) in dioxane or *N*,*N*-dimethylformamide (DMF) over palladium on charcoal. Benzyloxycarbonyl (Z) group is a convenient protecting group and is easily removable by catalytic hydrogenolysis without any side reactions.[5] The Z-amino acid anhydrides (**4a—e**) were prepared and then hydrogenolyzed as shown in Scheme 1. The catalytic hydrogenolyses were carried out at room temperature in dioxane or DMF for 72 h. After hydrogenolysis, the solution obtained by filtration of the reaction mixture was evaporated and the residual product was purified by gel filtration.



Scheme 1. Preparation of Z-amino acid anhydride and polymerization reaction by hydrogenolyses. Z: benzyloxycarbonyl-, β-Agl: 3-aminoglutaric acid, DCC: dicyclohexylcarbodiimide, AcOEt: ethyl acetate, DMF: *N*,*N*-dimethylformamide.

Results and Discussion

The yields of polypeptides, reaction conditions, amino acid recovery in acid-hydrolysates are listed in Table 1. Catalytic hydrogenolyses of Z-DL-Asp anhydride (**4a**) and Z-L-Asp anhydride (**4b**) in dioxane or DMF gave white powder whose molecular weights were estimated to be in the range of 3000 to 5000 by means of G-25 gel filtration. Amino acid recoveries after acid-hydrolysis were more than 90%. The IR spectrum of the white powder showed typical absorption bands of acidic polypeptides (1700 cm^{-1}: carboxylic acid; 1640 cm^{-1}: amide I; 1540 cm^{-1}: amide II).

Hydrogenolyses of anhydrides (**4c—e**) of Z-DL-Glu(**3c**), Z-L-Glu (**3d**), and Z-3-aminoglutaric acid (**3e**) in DMF gave peptides similar to those obtained after hydrogenolysis of Z-Asp anhydrides (**4a—b**). However, Z-DL-Glu anhydride (**4c**) and Z-L-Glu anhydride were hydrogenolyzed in dioxane to afford pyroglutamic acid in high yields (79, 80%). The hydrogenolysis of Z-L-Glu anhydride (**4d**) in glacial acetic acid or ethyl acetate also gave pyroglutamic acid.[4]

Although the polymerization reactions were carried out at room temperature, it is important to estimate the optical purity of Asp composing the poly-L-Asp, due to the possibility of racemization during the polymerization. If optically pure poly-L-Asp was obtained, it would be an interesting material for various studies. The actual ratio of D-Asp to L-Asp in the poly-L-Asp obtained from Z-L-Asp anhydride (**4b**) which was optically pure was determined by the gas chromatographic resolution of the derivatives of the enantiomers in the hydrolysate of the poly-L-Asp. Figure 1 shows the amino acid recovery and the D/L ratio of Asp against the hydrolysis time in 6 M HCl (1 M=1 mol dm^{-3}) at 110 °C. Free amino acid recovery reached 90% after 8 hours' hydrolysis. The D/L ratio of Asp increased proportionally with the hydrolysis time. After 8 hours' hydrolysis, the D/L ratio was 5%.

Table 1. Formation of Polypeptides by Hydrogenolyses of Z-Amino Acid Anhydrides (**4a—e**) in Dioxane or *N,N*-Dimethylformamide (DMF)

Substrate	Solvent	Product[a]	Yield/%[b]	AAA/%[c]	Elemental analysis/%	(C	H	N)
Z-DL-Asp Anhydride (**4a**)	Dioxane	Poly-Asp	71	101	Calcd for $C_4H_5NO_3 \cdot 0.81\ H_2O$	: 37.09	5.14	10.82
					Found	: 37.32	4.82	10.69
	DMF	Poly-Asp	87	95	Calcd for $C_4H_5NO_3 \cdot 0.41\ H_2O$	: 39.28	4.78	11.45
					Found	: 39.22	4.85	11.71
Z-L-Asp Anhydride (**4b**)	Dioxane	Poly-Asp	81		Calcd for $C_4H_5NO_3 \cdot 0.15\ H_2O$	: 40.78	4.54	11.89
					Found	: 40.79	4.73	11.64
	DMF	Poly-Asp	90	100	Calcd for $C_4H_5NO_3 \cdot 0.79\ H_2O$	: 37.09	5.14	10.82
					Found	: 37.17	4.93	10.64
Z-DL-Glu Anhydride (**4c**)	Dioxane	Pyro-Glu	80					
	DMF	Poly-Glu	55	88	Calcd for $C_5H_7NO_3 \cdot 0.51\ H_2O$	: 43.48	5.84	10.14
					Found	: 43.42	5.76	9.99
Z-L-Glu Anhydride (**4d**)	Dioxane	Pyro-Glu	79		Calcd for $C_5H_7NO_3$	: 46.51	5.46	10.85
					Found	: 46.04	5.51	10.65
	DMF	Poly-Glu	56	94	Calcd for $C_5H_7NO_3 \cdot 0.69\ H_2O$	: 42.43	5.96	9.89
					Found	: 42.42	5.63	9.78
Z-β-Agl Anhydride (**4e**)	Dioxane		—	—				
	DMF	Poly-β-Agl	91	78	Calcd for $C_5H_7NO_3 \cdot 0.32\ H_2O$	: 44.52	5.71	10.38
					Found	: 44.53	6.06	10.00

a) Molecular weights of polypeptides estimated by GPC were 3000—5000. b) Yield is expressed by the molar ratio of amino acid residue in product to substrate. c) Amino acid recovery in hydrolysate of polypeptide.

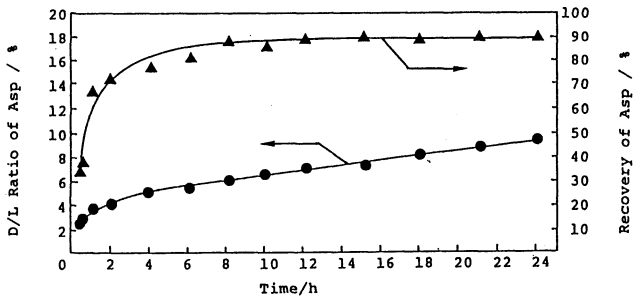


Fig. 1. Recovery and D/L ratio of Asp in acid-hydrolysate of the polypeptide which was obtained by the hydrogenolysis of Z-Asp anhydride (**4b**) in DMF.

The actual D/L ratio (3%) in poly-Asp before hydrolysis was estimated by extrapolating the value of D/L ratio to 0 hour. The existence of small amount of D-Asp(3%) would be due to the following three reasons: (1) racemization of L-Asp during the polymerization, (2) racemization during the hydrolysis of poly-Asp, and (3) existence of D-Asp in the starting material (Z-L-Asp anhydride). The third one would be negligible, because the optical purity of Z-L-Asp anhydride was almost 100% according to the gas chromatographic analysis of the derivative (*N*-trifluoroacetyl-Asp isopropyl ester) from Z-L-Asp anhydride. Similar experiments were carried out for the polypeptides derived from Z-L-Glu anhydride (**4d**) to show almost same racemization (3%).

In the present paper, we demonstrate a new synthetic method of polypeptides using catalytic hydrogenolysis. According to the best of our knowledge, this report would afford the first examples of the syntheses of poly-Glu and poly-β-Agl by hydrogenolysis of the corresponding amino acid anhydrides. Further characterizations such as determination of the ratios of β- or γ-carboxyl group to α-carboxyl group in the side chains are under investigation.

Experimental

All the melting points were not corrected. Optical rotations were measured with a JASCO DIP Polarimeter. All the gas chromatographic analyses were carried out with a Hitachi 163 gas chromatograph, and the peaks on the chromatograms were integrated with a Sic Chromatocoda 11. NMR spectra were measured on a JEOL FT-100 NMR spectrometer. IR spectra were measured with a Jasco Infrared spectrometer. Palladium on charcoal (5%) was purchased from Nippon Engelhald.

Materials. Amino Acids (1a—d). DL-Aspartic acid (**1a**), L-aspartic acid (**1b**), DL-glutamic acid (**1c**), and L-glutamic acid (**1d**) were purchased from Wako Pure Chemical Company. 3-Aminoglutaric acid (**1e**) was prepared by heating glutaconic acid monohydrate (Aldrich Chemical Company) and ammonia. Yield, 60%. Mp 274 °C (decomposed).

Preparation of Z-Amino Acids (3a—e). Z-Amino acids **3a—e** were prepared from benzyloxycarbonyl chloride (**2**) and amino acids (**1a—e**) by the manner in the literature.[5] **3a:** Yield, 63%, Mp 85—88 °C. **3b:** Yield, 89%, Mp 117—118 °C. $[\alpha]_D$ +8.8, *c* 1.0 (acetic acid). **3c:** Yield, 72%, Mp 117—119 °C. **3d:** Yield, 90%, Mp 112—113 °C. $[\alpha]_D^{25}$ −0.76 (*c* 10, acetic acid). **3e:** Yield, 73%, Mp 157—159 °C. ^{1}H NMR (CD_3OD): δ=2.59 (4H, d, *J*=6.4 Hz), 4.32 (1H, t, *J*=6.8 Hz), 5.06 (2H, s), 7.32 (5H, s). IR (KBr): 3350, 1720, 1540, 1430, 1240—1300 cm^{-1}. Calcd for $C_{13}H_{15}NO_6$: C, 55.51; H, 5.37; N, 4.99%. Found: C, 55.49; H, 5.01; N, 4.99%.

Preparation of Z-Amino Acid Anhydrides (4a—e).[5-7] **4a.**[5,6] To a cooled ethyl acetate solution (150 ml) of Z-DL-

Asp (**3a**) (13.3 g, 50 mmol), was added dicyclohexylcarbodiimide (DCC) (11.3 g, 55 mmol) dissolved in 50 ml ethyl acetate. The cyclization reaction was carried out for 1 h at 0 °C and for 24 h at room temperature. After usual work-up, Z-DL-Asp anhydride (**4a**) was obtained. Yield, 89%. Mp 120—122 °C. ^{1}H NMR ($CDCl_3$): δ=2.23 (2H, m), 4.36—4.64 (1H, m), 5.15 (2H,s), 7.36 (5H, s). IR (KBr): 3400, 1860, 1780, 1700, 1540, 1260 cm^{-1}. Calcd for $C_{12}H_{11}NO_5$: C, 57.83; H, 4.44; N, 5.62%; Found: C, 58.08; H, 4.68; N, 5.88%. **4b,** Yield, 98%. Mp 106—107 °C. $[\alpha]_D^{25}$ −29.1 (*c* 1.0, acetic acid). ^{1}H NMR ($CDCl_3$): δ=2.23 (2H, m), 4.36—4.64 (1H, m), 5.15 (2H, s), 7.36 (5H, s). IR (KBr): 3400, 1860, 1780, 1700, 1540, 1260 cm^{-1}. Calcd for $C_{12}H_{11}NO_5$: C, 57.83; H, 4.44; N, 5.62%; Found: C, 58.22; H, 4.50; N, 5.57%. **4c**, Yield, 96%. Mp 103—105 °C. ^{1}H NMR ($CDCl_3$) δ=1.8—2.2 (2H, m), 2.8—3.1 (2H, m), 4.3—4.6 (1H, m), 5.15 (2H, s), 5.6 (1H, br), 7.36 (1H, s). IR (KBr): 3400, 1820, 1770, 1730, 1540, 1260 cm^{-1}. Calcd for $C_{13}H_{13}NO_6$: C, 59.31; H, 4.97; N, 5.32%; Found: C, 59.04; H, 5.01; N, 5.27%. **4d**, Yield, 60%. Mp 76—77 °C. $[\alpha]_D^{20}$ −41.6 (*c* 9.98, acetic acid). ^{1}H NMR ($CDCl_3$): δ=1.8—2.2 (2H, m), 2.8—3.1 (2H, m), 4.3—4.6 (1H, m), 5.15 (2H, s), 5.6 (1H, br), 7.36 (1H, s). IR (KBr): 3400, 1820, 1770, 1730, 1540 cm^{-1}. Calcd for $C_{13}H_{13}NO_6$: C, 59.31; H, 4.98; N, 5.32%. Found: C, 59.19; H, 5.07; N, 5.35%. **4e,** Yield, 73%. Oil. ^{1}H NMR (DMSO-d_6): δ=2.64—3.20 (4H, m), 4.00—4.20 (1H, m), 5.04 (2H, s), 7.35 (5H, s), 7.90 (1H, br). IR (KBr): 3350, 1820, 1780, 1700, 1540, 1220—1280 cm^{-1}. Calcd for $C_{13}H_{13}NO_6$: C, 59.31; H, 5.98; N, 5.32%. Found: C, 59.71; H, 5.73; N, 5.64%.

Hydrogenolyses of Z-Amino Acid Anhydrides (4a—e) Followed by Polymerization. Z-DL-Asp anhydride (**4a**) (747 mg, 3.0 mmol) was dissolved in 5.0 ml dioxane or DMF, and was hydrogenolyzed over 5% palladium on charcoal (0.34 g) for 72 h under hydrogen atmosphere. After 3 hours' heating at 40 °C, the reaction mixture was filtered and the filtrate was evaporated in vacuo to give a residual product. The product was dissolved in water and was lyophilyzed to give white powder. The resulted white powder was dissolved in 5 ml of 0.5 M acetic acid and was loaded into a Sephadex column (G-10). Elution was carried out with 0.5 M acetic acid. The fractions which have the absorption at UV 230 nm and did not show ninhydrin color on TLC, were lyophilized to give white solid. Other Z-amino acid anhydrides (**4b—e**) were hydrogenolyzed by the similar manner to that described for **4a**.

Estimation of Molecular Weight of Polypeptides (6a—e). Molecular weights of all the purified polypeptides were estimated with Sephadex G-25 using 0.1 M sodium phosphate buffer (pH 6.8). The polypeptides were eluted earlier than oxidized Insulin (A and B chains, MW ca. 3000)[8] and later than the size exlusion limit (MW 5000) of this gel.

Determination of D/L Ratio of Asp in the Poly-L-Asp. The purified polypeptide **6b** (10 mg) was dissolved in 5 ml 6 M HCl and the solution in a sealed tube under vacuum was heated at 110 °C for 1 to 24 h. The D/L ratios of Asp were determined by the gas chromatographic resolution of enantiomeric *N*-trifluoroacetyl-Asp isopropyl esters using a chiral glass capillary column (Chirasil-Val[9] III).

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