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Studies on Polypeptides. (V) Thermal Polycondensation of Alanine in the Presence of Carboxylic Acids[1)]

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Thermal polycondensation of various other amino acids than glycine was studied, especially in the presence of acetic acid. As a result, some amino acids were found to produce the oligomer as well as the diketopiperazine by the thermal condensation, and particularly marked was the reactivity of alanine, which being treated with an appropriate amount of acetic acid at a temperature of 200–220°C for 1–1.5 hrs, afforded 2,5-diketo-3,6-dimethylpiperazine and an alanine oligomer with acetylated *N*-terminal (degrees of polymerization 5–6) in 45–48% and 40–43% yields, respectively.

I. INTRODUCTION

In previous papers[2~4)] the authors reported the thermal polycondensation of glycine in the presence of a suitable amount of water or carboxylic acid, the *N*-terminal of the glycine oligomer thus obtained acylated with carboxylic acid as catalyst.

As a series of this work, the thermal polycondensation was also applied to various other amino acids than glycine, particularly in the presence of acetic acid. As shown in Table 1, some of them, such as alanine, valine, phenylalanine, aspartic acid and lysine, seemed to give oligomer judging from their positive biuret reaction. Amino acids which produced diketopiperazine were alanine, valine, leucine, isoleucine and phenylalanine, among which the most striking was alanine which produced 2,5-diketo-2,6-dimethylpiperazine as well as its oligomer, both in fairly good yields, the latter showing strong biuret reaction.

II. EXPERIMENTAL

II.1. Thermal Polycondensation

The procedures of the thermal polycondensation were the same as described previously,[2)] but the treatments after the reaction were somewhat modified. The whole contents of the sealed tube were boiled with methanol under stirring and the insoluble unreacted amino acid was filtered off. The diketopiperazine, precipitated from the filtrate by letting it stand in an ice-bath for several hours was isolated by filtration, washed with a small amount of methanol and recrystallized repeatedly from methanol, and then dried

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in a vacuum desiccator. The oligomer was obtained from the combined mother liquor and washings by concentrating it under reduced pressure and precipitating with acetone–petroleum ether (1 : 1 *v/v*). The precipitate was thoroughly washed with acetone and dried in a vacuum desiccator.

II.2. The Determination of the Molecular Weight and the Nitrogen Contents of Oligomer.

The molecular weight of the oligomer was determined by titrating its terminal carboxyl group (*C*-terminal determination) and amino group (*N*-terminal determination), while its nitrogen contents, by the method of Kjeldahl. In Table 3–6, various values for the oligomer were calculated from these data on the assumption that the *N*-terminal of the oligomer would be acylated in the same way as the polycondensation of glycine in the presence of carboxylic acid.

III. RESULTS AND DISCUSSION

III.1. The Thermal Polycondensation of Various Amino Acids in the Presence of Water or Acetic Acid

The optimum conditions for the polycondensation of glycine[2] were also adopted for other amino acids, that is, 0.0056 mole of water or 0.005 mole of acetic acid was added to each 0.0133 mole of amino acid and the mixture was heated in a sealed tube at 150–160°C for 20 hrs. The results of the reaction are summarized in Table 1. It would be concluded from the table that it was alanine, aspartic acid and lysine which produced a respective oligomer giving positive biuret reaction, and acetic acid was more effective than water as catalyst. It has been previously reported that anhydropolyaspartic acid[5] and its hydrate[6] are formed when the DL-form of aspartic acid is heated alone at 180–200°C, and that, when DL-lysine is heated at 180–230°C lysine homopolymer is formed via its liquid lactam.[7] However, nothing has been reported about the thermal polycondensation of alanine. Serine and threonine also gave positive biuret reaction after treated as described above. However, this is not always a definitive evidence for polypeptide formation, because the amino acids themselves also give positive biuret reaction. So separation of both the reaction mixtures was attempted by paper-chromatography. But the chromatogram gave only one spot identical with that of the starting amino acid itself, in both cases. From these results the authors deduced a conclusion that no polycondensation took place in the case of these two amino acids.

In the next step, the experiments were repeated on more drastic conditions in the hope that other sorts of amino acids might cause polycondensation: the reaction temperature was raised to 200°C and maintained here for 3 hrs, using acetic acid as catalyst. As a result, not only the products from alanine, aspartic acid and lysine, but also from valine and phenylalanine gave positive biuret reaction, though very weak. At the same time some amino acids gave diketopiperazines on this condition. The results are shown in Table 2. It is noteworthy that the yield of the diketopiperazine was fairly good with alanine and valine. With the other amino acids the yield was rather poor, but probably it might be improved by properly selecting the reaction conditions. It is also noticeable that

Table 1. Thermal Polycondensation of Amino Acids in the Presence of Water or Acetic Acid. Amino Acid: 0.0133 mol, Water: 0.0056 mol (0.1 g), Acetic Acid: 0.005 mol (0.3 g), Temperature: 150–160°C, Time: 20 hr.

Amino Acid	Reaction Products in the Presence of			
	Water		Acetic Acid	
	Appearance	Biuret Reaction*)	Appearance	Biuret Reaction*)
Alanine (L,DL)	Brownish yellow semi-solid	++	Brownish yellow semi-solid	++
Valine (L,DL)	White	−	Light yellow semi-solid	−
Leucine (L,DL)	Light brown	−	Dark brown	−
Isoleucine (L,DL)	Light yellow	−	Brownish yellow semi-solid	−
Phenylalanine (L,DL)	Yellow	−	Yellow semi-solid	−
Serine (L,DL)	Brownish semi-solid	+	Brown taffy-like	+
Threonine (L,DL)	Brown semi-solid	+	Brown melt-like	+
Tyrosine (L)	Dark brown	−	Dark brown	−
Tryptophane (L)	Brown	−	Reddish brown	−
Cystine (L)	Brown taffy-like	−	Brown taffy-like	−
Methionine (L)	Light yellow	−	Brownish yellow semi-solid	−
Proline (L)	Dark brown	−	Brown taffy-like	−
Aspartic acid (L,DL)	Brown taffy-like	+	Dark brown	++
Glutamic acid (L,DL)	Brown melt-like	−	Light yellow melt-like	−
Ornicine (L)	Reddish orange melt	−	Brown liquid	−
Lysine (L,DL)	Brown taffy-like	+	Brownish yellow melt-like	++
Arginine (L)	Gray	−	Gray	−
Histidine (L)	Dark brown semi-solid	−	Brownish yellow taffy-like	−

*) ++: strongly positive +: weakly positive −: negative

Table 2. Preparation of Diketopiperazines from Amino Acids in the Presence of Acetic Acid. Amino Acid: 0.01 mol, Acetic Acid: 0.005 mol, Temperature: 200±1°C, Time: 3 hr.

Amino acid	Diketopiperazine							Biuret reaction of the residue**)
	Yield %	m.p.°C (lit.)*)	Elemental analysis					
			found			calcd.		
			C%	H%	N%	C%	H%	N%	
L-Alanine	50.1	270 (277–278)	50.69	7.14	19.79	50.69	7.09	19.70	++
DL-Alanine	48.8	269–270 (271)	50.84	7.14	19.85	50.69	7.09	19.70	++
L-Valine	42.1	292–294 (295)	60.68	9.26	14.36	60.58	9.15	14.13	+
L-Leucine	16.3	270–271 (270)	63.81	9.92	12.31	63.69	9.80	12.38	−
L-Isoleucine	18.6	275–277 (280–281)	63.71	9.99	12.40	63.69	9.80	12.38	−
L-Phenylalanine	21.4	282–283 (280)	72.98	6.30	9.28	73.45	6.16	9.52	+

*) Beilstains Handbuch der organischen Chemie.

**) Biuret reaction of the solution after the removal of diketopiperazine.
++: strongly positive, +: weakly positive, −: negative.

even on this drastic condition glycine gave no diketopiperazine, though plenty of the oligomer was obtained.

As seen in Table 2, in the thermal condensation, alanine is very marked both in the yield of the diketopiperazine and the intensity of biuret reaction of the oligomer. So we decided to study further in detail the reaction of this amino acid.

III.2. The Thermal Polycondensation of Alanine in the Presence of Acetic Acid

III.2.1. The Effects of the Amount of Acetic Acid and Reaction Temperature

A mixture of 0.00166–0.02 mole of acetic acid and 0.0122 mole (1 g) of DL-alanine was treated as above at a temperature ranging from 160 to 220°C. The results are shown in Fig. 1. Apparently the amounts of both 2,5-diketo-3,6-dimethylpiperazine (alanine anhydride) and DL-alanine oligomer increase, with the increase of the amount of acetic acid, pass through a respective maximum, and then decrease. Such tendency is marked especially as for the oligomer formation. The higher the reaction temperature within 220°C, the more the yield of alanine anhydride, while the yield of the oligomer reaches its maximum at 200°C in the presence of 0.005 mole of acetic acid. The effect of the amount of acetic acid on the molecular weight and the nitrogen contents of the oligomer, obtained by heating for 4 hrs at 200°C, are shown in Table 3. The molecular weights of the oligomer calculated from the determination of the *N*-terminal and the *C*-terminal did not coincide

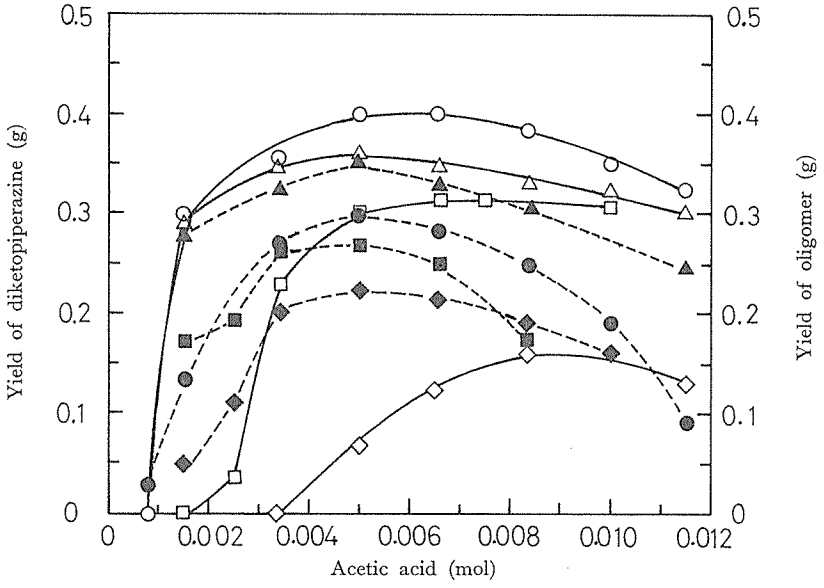

Fig. 1. Effect of the amount of acetic acid on the thermal condensation of DL-alanine.
DL-Alanine: 0.0112 mol (1g)
——: Yield of diketopiperazine, – – –: Yield of oligomer
◇, ◆: 160±1°C, 20 hr
□, ■: 180±1°C, 8 hr
△, ▲: 200±1°C, 4 hr
○, ●: 220±1°C, 2 hr

Table 3. Effect of the Amount of Acetic Acid on the Yield, the Molecular Weight and the Nitrogen Contents of Alanine-Oligomer. DL-Alanine: 0.0112 mol (1 g), Temperature: 200±1°C, Time: 4 hr.

Acetic acid		Oligomer						
		Yield		Molecular weight (Polymerization degree) by the method of		N %		Polymerization degree calcd. from N-contents
g	mol	g	%	*C*-terminal determination	*N*-terminal determination	found	calcd.	
0.1	0.0016	0.28	30.5	455 (5.6)	890 (11.7)	17.28	17.11	6.0
0.2	0.0033	0.33	35.8	442 (5.4)	1070 (14.2)	17.12	17.03	5.6
0.3	0.0050	0.35	37.8	434 (5.3)	1560 (21.1)	16.79	16.98	4.9
0.5	0.0080	0.31	32.9	390 (4.7)	1660 (22.8)	16.65	16.69	4.7
0.7	0.0116	0.25	24.8	287 (3.2)	1830 (25.0)	15.42	15.58	3.0
1.0	0.0166	0.15	14.4	260 (2.8)	2260 (31.0)	15.00	15.13	2.7
1.5	0.0250	0	—	—	—	—		—

quite as in the case of glycine, although the discrepancy was less remarkable. The discrepancy increases with the increase of the amount of acetic acid, while the nitrogen contents approximately agree with the calculated value on the assumption that the oligomer is acetylated. The degree of polymerization calculated from the nitrogen contents also agrees approximately with the one calculated from the *C*-terminal determination when this assumption is adopted. These facts support that the terminal amino group of the oligomer is also acetylated in this case, as in the case of glycine.

III.2.2. The Effect of Reaction Temperature

The effect of reaction temperatures of 180, 200 and 220°C was investigated on the above-mentioned optimum conditions, *i.e.*, adding 0.005 mole (0.3 g) of acetic acid to 0.0112 mole (1 g) of DL-alainne. The yields of the alanine anhydride and the oligomer are shown in Fig. 2. The molecular weight and the nitrogen contents of the oligomer are shown in Table 4. As seen in Fig. 2, the higher the reaction temperature, the more rapid the formation of the anhydride, and for the synthetic purposes the reaction at 220°C for 2 hrs is the most desirable affording the yield of 48%. In a series of the reactions the maximum yields of the oligomer were attained, at 200°C for 1.5 hrs, and at 220°C for 1 hr, but a higher yield was observed on the former condition. The yields decreased rapidly with further elongation of the reaction time. Such decreases in the yields would be probably due to an irreversible decomposition of the oligomer, because in such cases the contents of the sealed tube turned brown and some pressure was produced in the tube owing to a large amount of gas evolved during the reaction.

The higher the reaction temperature and the longer the reaction time, the more the molecular weight and the nitrogen contents of the oligomer, as shown in Table 4. Thus the optimum conditions for obtaining the oligomer are, at 200°C for 1.5–2 hrs or 220°C for 1 hr. By these methods the oligomer with polymerization degree of 5–6 is obtainable in a yield of 40–43%. The oligomer obtained in this way is light yellow powder, which shows thermoplasticity at 90–95°C and melts at 200–210°C. It is soluble in water, methanol or

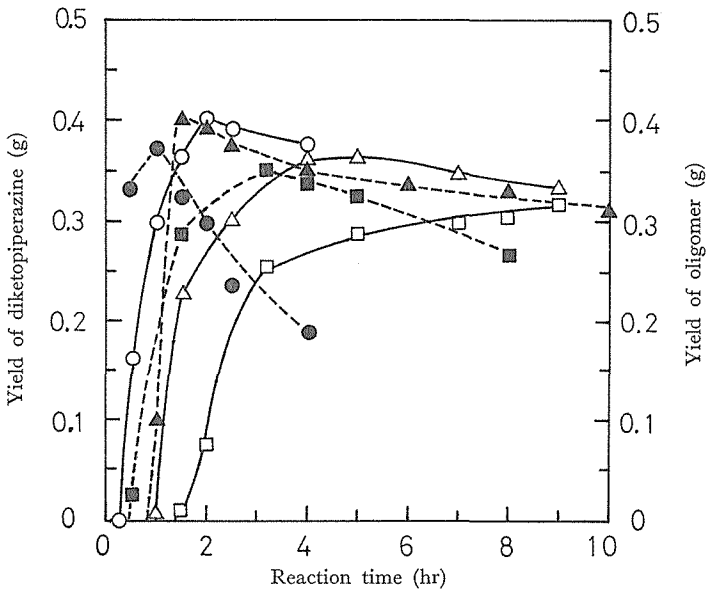

Fig. 2. Effect of reaction time on the thermal condensation of DL-alanine in the presence of acetic acid.

DL-Alanine: 0.0112 mol (1g), Acetic acid: 0.005 mol (0.3g)
——: Yield of diketopiperazine, - - -: Yield of oligomer
□, □: 180±1°C
△, ▲: 200±1°C
○, ●: 220±1°C

Table 4. Effects of the Reaction Temperature and Time on the Thermal Polycondensation of DL-Alanine in the Presence of Acetic Acid.
DL-Alanine: 0.0112 mol (1 g), Acetic acid: 0.005 mol (0.3 g).

Reaction conditions		Oligomer						
Temp.	Time	Yield		Molecular weight (Polymerisation degree) by the method of		N %		Polymerization degree calcd. from N-contents
°C	hr	g	%	*C*-terminal determination	*N*-terminal determination	found	calcd.	
160	20	0.07	6.8	254 (2.7)	860 (11.3)	15.45	15.00	3.1
180	3.5	0.35	36.3	309 (3.5)	980 (12.9)	15.90	15.86	3.6
180	8	0.27	27.5	320 (3.7)	1130 (15.0)	16.21	16.03	4.0
200	1.5	0.40	42.7	402 (4.8)	1350 (18.1)	16.70	16.74	4.7
200	4	0.35	37.8	434 (5.3)	1560 (21.1)	16.79	16.98	4.9
200	8	0.33	35.9	455 (5.6)	1680 (22.8)	17.05	17.11	5.4
220	1	0.37	40.9	506 (6.3)	1470 (19.8)	17.24	17.36	5.9
220	2	0.30	33.4	536 (6.7)	1640 (22.3)	17.30	17.49	6.1
220	4	0.19	21.4	607 (7.7)	2030 (27.7)	17.48	17.74	6.7

ethanol, but insoluble in acetone, ether, petroleum ether, benzene, dioxane or dimethylformamide.

In one case, L-alanine was used instead of DL-alanine, and the oligomer obtained was hydrolyzed with 6*N*-HCl, but the recovered amino acid showed no optical activity at all.

III.2.3. The Thermal Polycondensation of Alanine in the Presence of Various other Carboxylic Acids than Acetic Acid

The catalysts used in this experiment were saturated carboxylic acids including propionic, *n*-valeric, *n*-caprylic and *n*-lauric acid, and unsaturated carboxylic acids including acrylic, methacrylic and oleic acid. The results are shown in Table 5. Apparently the yields of alanine anhydride and the oligomer decrease with the increase of the carbon number of the saturated carboxylic acid. In the case of lauric acid, the anhydride is formed at 200°C, but no oligomer is formed. A similar phenomenon is observed in an unsaturated carboxylic acid series.

The *N*-terminal of the oligomer obtained here is recognized to be acylated, as in the case with acetic acid which shows that the effect of acetic acid is common to all the carboxylic acids.

Table 5. Thermal Condensation of DL-Alanine in the Presence of Higher Carboxylic Acids. DL-Alanine: 0.0112 mol (1 g), Carboxylic Acid: 0.005 mol, Temperature: 200±1°C, Time: 2 hr.

Carboxylic acid	Diketopiperazine Yield		Oligomer Yield		Molecular weight (Polymerization degree) by the method of		N %	
	g	%	g	%	*C*-terminal determination	*N*-terminal determination	found	calcd.
Propionic acid	0.34	41.5	0.36	37.2	420 (4.9)	1460 (19.5)	16.30	16.24
n-Valeric acid	0.29	35.4	0.24	23.1	438 (4.7)	1510 (19.8)	15.11	15.08
Caprylic acid	0.26	31.7	0.22	18.8	455 (4.4)	1500 (19.1)	13.40	13.48
Capric acid	0.24	29.3	0.13	10.3	464 (4.1)	1440 (17.9)	12.42	12.38
Lauric acid	0.15	18.3	0	—	—	—	—	
Acrylic acid	0.13	15.9	0.54	56.1	416 (4.9)	1670 (22.5)	16.25	16.31
Methacrylic acid	0.12	14.6	0.58	57.3	393 (4.5)	1710 (22.7)	15.40	15.51
Oleic acid	0.08	9.8	0	—	—	—	—	

III.3. The Discussion of Reaction Mechanism

When alanine is heated with acetic acid, the most probable reaction is acetylation into acetylalanine with a melting point, unlike amphoteric alanine, and molten acetylalanine may dissolve alanine. The dissolved alanine molecule reacts (1) with acetylalanine producing acetyldipeptide, which reacts further with another alanine molecule giving acetyltripeptide, thus the acetylated oligopeptides would be produced, or (2) with another molecule of alanine producing a cyclic diketopiperazine.

According to this assumption, acetic anhydride is more effective than acetic acid itself,

Table 6. Thermal Condensation of Alanine in the Presence of Acetic Anhydride or Acetylalanine. Temperature: 200±1°C, Time: 2 hr.

Reaction conditions			Diketopiperazine		Oligomer						
	Reagent	Alanine	Yield		Yield		Molecular weight (Polymerization degree) by the method of		N %		Polymerization degree calcd. from N-contens
	g	g	g	%	g	%	*C*-terminal determination	*N*-terminal determination	found	calcd.	
Acetic anhydride	0.2	1.0	0.30	37.5	0.42	44.7	400 (4.8)	1660 (22.6)	16.58	16.74	4.5
Acetic anhydride	0.3	1.0	0.31	38.8	0.40	42.2	380 (4.5)	1820 (24.8)	16.39	16.58	4.2
Acetyl-alanine	1.0	0	0	—	0	0	—	—	—		—
Acetyl-alanine	0.3	0.7	0.15	26.9	0.28	41.7	410 (4.9)	1980 (27.0)	16.58	16.79	4.5
Acetyl-alanine	0.3	1.0	0.13	16.3	0.33	35.5	420 (5.1)	1760 (24.0)	16.69	16.89	4.7

and acetylalanine must be also effective. Thus the thermal condensation of alanine was carried out in the presence of acetic anhydride and acetylalanine. From the results, shown in Table 6, it would be seen both the compounds are effective for the thermal condensation of alanine giving the same oligomer as above. It is noteworthy that no oligomer was obtained by heating acetylalanine alone, but when alanine was added, plenty of the oligomer was produced. This fact may emphasize the importance of acetylalanine as a solvent. In this sense acetylalanine may be replaceable with another acetylated amino acid, *e.g.*, acetylglycine. When alanine was heated in the presence of acetylglycine, alanine anhydride and alanine oligomer were obtained. Hydrolyzing the diketopiperazine and the oligomer obtained here, and analyzing the hydrolyzates by paper-chromatography, no glycine was found in the hydrolyzate of the diketopiperazine, while it was detected in the hydrolyzate of the oligomer. This fact indicates that the diketopiperazine never originates, at least, in acetylglycylalanine, the first condensation product.

The increase of the acyl carbon number of acylated alanine increases the lipophilicity of the compound, which makes the compound unfavourable to dissolve amino acid, therefore both the yields of the diketopiperazine and the oligomer would decrease. The increase of the molecular weight of acylalanine gives no influence upon the chance of effective collision between the alanine molecules dissolved in it, so no marked decrease of the yield of diketopiperazine occurs. But it gives remarkable influence upon the one between molecules of acylalanine and alanine. The greater the molecule of acylated alanine, the less the chance, that is, the slower the reaction between acylalanine and alanine, in accordance with which the yield of the oligomer decreases markedly.

Another mechanisms may also be possible. The diketopiperazine may be produced by degradation of the oligomer, or the oligomer may be produced by the ring-opening polymerization of the diketopiperazine. But when 2,5-diketo-3,6-dimethylpiperazine or alanine oligomer was treated on the same conditions as above, no oligomer or no diketopiperazine was obtained in either case.

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