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Studies on the Synthesis of Protein Analogus (Part VII)

The Synthesis of Protein Analogus having periodic arrangement of each Amino acid. (Poly-glycyl-DL-alanine, poly-glycyl-DL-phenylalanine, poly-glycyl-L-leucine and copoly-(glycyl-DL-phenylalanine and glycyl-L-leucine))

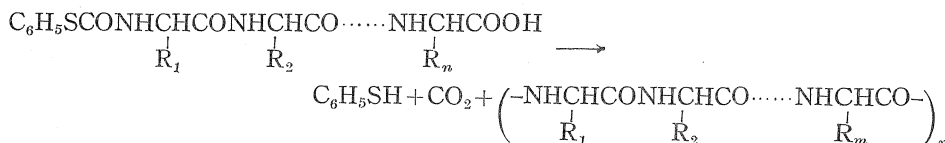
By

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In the previous reports,¹⁾ we showed that a carboxylic group and a N-carbothiophenyl group react easily to make a peptide bond (-CONH-) by heating and that by using this reaction, we could easily synthesize polypeptides by fusing or heating the solution of N-carbothiophenylamino acids. Further, we showed that this reaction was a general synthetic method of protein analogus having the same molecular weight as natural protein, of scores of thousand order, by which we could polymerize β -alanine, ϵ -aminocaproic acid and other ω -amino acids.

The employment of this method which has replaced N-carboxylic acid anhydride method²⁾ has made it possible to synthesize the protein models of high molecular weight in which each amino acid is periodically arranged. We believe that this would contribute much to the studies on the natural protein structures.



The polymerisation of glycyl-DL-alanine, glycyl-DL-phenylalanine and glycyl-L-leucine, and the copolymerisation of glycyl-L-leucine and glycyl-DL-phenylalanine will be reported in this paper. The later copolymer is periodic for glycyl residue.

Poly-glycyl-DL-alanine—As the result of the preliminary polymerisation of N-carbothiophenyl-glycyl-DL-alanine under several conditions, dioxane was the most suitable solvent for its polymerisation as shown in Table I.

N-carbothiophenyl-glycyl-DL-alanine was polymerized in dioxane containing a small amount of pyridine at 60°C for 1000 hours. It was only about 5% yield of the

* It was published in the Jan. of the Chem. Soc. of Japan **75**, 849—853 (1954) ; J. Noguchi, T. Hayakawa, J. Am. Chem. Soc., **76**, 2846 (1954).

1) J. Noguchi, Jan. of the Chem. Soc. of Japan **74**, 961 (1953); *ibid.* **75**, 639—647 (1954).

2) H. Leuchs, Ber. **39**, 857 (1906); R. B. Woodward, C. H. Schramm, J. Am. Chem. Soc., **69**, 1551 (1947).

Table I

Temp. 60 °C ; Time 500 hrs. ; Cps- : Carbothiophenyl.

Exp. No.	1	2	3	4
Reaction Components of solution	Cps-glycylalanine 0.1 g pyridine 0.28 g dioxane 3 cc	Cps-glycylalalanine 0.1 g pyridine 0.28 g tetrahydrofurane 5 cc	Cps-glycylalanine 0.1 g pyridine 0.28 g dimethylformamide 3 cc	Cps-glycylalanine 0.1 g dioxane 3 cc HgCl ₂ 0.001 g
Reference	Protein-like ppt. after one day. best.	a little ppt. on the glass-wall	clear soln. non-polymerized	a little powder ppt. polymer adsorpted salt. orange color soln.

theoretical, the molecular weight $M=34,813$, $(-G-A-)_n=272$ (G : glycyl, A : Alanyl) and the intrinsic viscosity $[\eta]=0.0528$. It was insoluble in ether, petrol-ether, chloroform, dioxane and acetone, soluble in methanol, ethanol and water, and easily soluble in acetic acid, formic acid and dichlor acetic acid. It showed clear Biuret's reaction and contains some water which could hardly be eliminated by the vacuum dryness at about 100 °C. In spite of the high molecular weight, the poly-glycyl-DL-alanine was soluble in water and it corresponded to the fact that the polymer, $(-A-G-G-)_n$, which Pacsu etc. prepared by the method of ester polymerisation from DL-alanyl-glycyl-glycine methylester, was also soluble in cold water. Therefore, the solubility of protein in water is considered to have much to do with the main chain structure of protein molecule as well as the polarities of its amino residues. The results of infra-red absorption spectra of the poly-glycyl-DL-alanine resemble the crystal part of silk fibroin which was prepared by Narita, but the x-ray analysis showed rather different results. So we are now preparing the poly-glycyl-L-alanine which will be an important model to study the structure of silk fibroin. The details will be reported in other papers.

Poly-glycyl-DL-phenylalanine—N-carbothiophenyl-DL-phenylalanine (m. p. 178–179 °C) was dissolved in benzene containing pyridine and polymerized at 60 °C for 1000 hours in a sealed tube. The yield was about 17% of the theoretical, the molecular weight $M=30,603$, $(-G-ph-)_n=150$. (ph : phenylalanine), and the intrinsic viscosity $[\eta]=0.1180$. It was insoluble in water, dioxane, pyridine, benzene, acetone, methanol, ethanol and ether, hardly soluble in formic acid and acetic acid, soluble in monochlor acetic acid and acetic acid (1 : 1) and easily soluble in dichlor acetic acid. It did not show any clear Biuret's reaction.

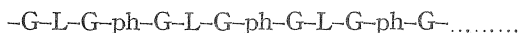
Poly-glycyl-L-leucine—N-carbothiophenyl-glycyl-L-leucine (m. p. 155 °C) was polymerized in benzene containing pyridine or in dioxane at 60 °C for 1000 hours. The yield of the polymer in benzene containing pyridine was about 5%, but the yield in dioxane was about 30%. There was not so much difference between these two polymerisation degrees, and the molecular weight was rather high in dioxane than in benzene having pyridine. The polymerisation of dipeptides in benzene containing pyridine was of very lower yield of the polymer and not easy to purify it in the elimination of the

adsorpted pyridine. But, when dioxane was used as solvent in the solution polymerisation of N-carbothiophenyl method, the more concentrated solution of the monomer gave the better result both in the yield and the molecular weight.

	in benzene containing pyridine	in dioxane
rate of polymerisation	5%	27.6%
molecular weight	36,091	37,911
intrinsic viscosity	0.178	0.121

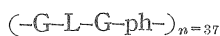
It seems it is not because of the errors of measurments that both intrinsic voscocities are reverse to these molecular weights. It might explain the different distribution of the polymerisation degrees or the difference of the polymer structure. It was insoluble in ether, petrol-ether, acetone, chloroform, benzene, methanol, ethanol, ethyl-acetate, water and dioxane, sparengly soluble in hot water and acetic acid, soluble in hot acetic acid and easily soluble in monochloroacetic acid and acetic acid (1 : 1) and in dichloroacetic acid. A slight Biuret's reaction was observed in cobalt colour.

Copolymerisation of glycyl-DL-phenylalanine and glycyl-L-leucine—N-carbothiophenyl-glycyl-DL-phenylalanine (m. p. 178–179 °C) and N-carbothiophenyl-glycyl-L-leucine (m. p. 155 °C) were mixed in equal moles ratio and copolymerized at 60 °C for 1000 hours in benzene containing pyridine. Yield 8.4%. The analytical data corresponded to the copolymer having 1 : 1 ratio of glycyl-DL-phenylalanyl and, glycyl-L-leucyl. There must naturally be periodic arrangement of glycine and between the glycyl residues, DL-phenylalanyl or L-leucyl residue must be bonded at the rate of 1 : 1 as a whole.



molecular weight $M = 13,702$,

intrinsic viscosity $[\eta] = 0.1080$



It is insoluble in cold water, alcohol, ether, acetone, benzene, alkali and hydrochloric acid, sparengly soluble in hot water and acetic acid, and soluble in acetic acid and monochloroacetic acid (1 : 1) and in dichloroacetic acid. Biuret's reaction was not clearly observed.

Experimental

*N-carbothiophenyl-glycyl-DL-alanine methylester*³⁾—A ether solution of DL-alanine methylester was prepared by the general synthetic method. DL-alanine methylester hydrochloride 35 g was dissolved in 20 cc of water and covered with ether, the mixture was cooled at -10 °C and stirred on, and dropped into the 50% solution of sodium hydroxide 10 g and then anhydrous K₂CO₃ is added immediately.

The sample 2 cc was taken up from the solution, titrated with 0.1 N-HCl with

3) A. Lindenmann, N. H. Khan, K. Hofmann, J. Am. Chem. Soc. **74**, 476 (1952) prepared DL-alanine methylester from it's HCl salt and diethylamine, and carbothiophenyl-glycyl-chloride by PCl₅.

methyl-red indicator and the total DL-alanine methyl ester in the ether solution was calculated. Yield 19.6 g (76% of the theoretical).

On the other hand chloroform 40 cc containing thionylchloride 13.5 g was added to carbothiophenyl-glycine 20 g which was dried and powdered perfectly. The mixture was completely dissolved in solution with gradual blister in about one hour.⁴⁾ After the evaporation of the chloroform in vaccum, 10 cc of ether was added and removed again in vaccum. When the residual thionylchloride was completely evaporated, carbothiophenyl-glycylchloride became crystal. It was dissolved in chloroform 60 cc and used in the following reaction.

The ether solution of DL-alanine methylester was cooled at -10°C and stired and the chloroform solution of carbothiophenylglycylchloride was dropped into it. The reaction mixture was stirred in the same temperature for 30 minuts and then kept in room temperature. Carbothiophenyl-glycyl-DL-alanine ester precipitates as brown syrup with DL-alanine methylester hydrochloride, and the syrup easily crystalized by rubbing. After leaving at alone overnight, it was filtered off and washed out with water containing a small amount of hydrochloric acid. DL-alanine methylester hydrochloride was easily soluble and carbothiophenyl-glycyl-DL-alanine methylester insoluble in the acidified water. The crystal was dried in a vaccum decicator. Yield 20 g (71.4% of the theoretical). m. p. $122-123^{\circ}\text{C}$ (Literatur $121-123^{\circ}\text{C}$).

N-carbothiophenyl-glycyl-DL-alanine—The mixture of N-HCl 25 cc and acetic acid 25 cc was added to N-carbothiophenylglycyl-DL-alanine methylester. It was heated for just 30 minuts with shaking on boiling water after all was dissolved, and it was cooled immediately. When it was concentrated in vaccum at below 35°C , it became crystal. It was evaporated to dryness, had a small amount of water added, evaporated again, washed well with acidified water having dil. HCl and dried in a vaccum decicator. Crude 8.6 g (90.3% of the theoretical). m. p. $154-155^{\circ}\text{C}$. It was purified by refluxing with benzene, which dissolves carbothiophenyl-glycyl-DL-alanine methylester, and then with ether 20 cc, which dissolves carbothiophenyl-glycine. Yield 8 g (84% of the theoretical) m. p. 156°C .

N analysis found N=9.78%

for $\text{C}_{12}\text{H}_{14}\text{O}_4\text{N}_2\text{S}$ calc. N=9.93%

N-carbothiophenyl-glycine—The condition (of which A. Lindemann and others write), on which N-carbothiophenyl-glycine ethylester was hydrolized into N-carbothiophenyl-glycine, was not suitable for the treatment of a large amount of sample in acid concentration and reaction time. We got the better results on the following condition.

The mixture of N-HCl 75 cc and acetic acid 75 cc was added to N-carbothiophenyl-glycine ethylester 30 g, and boiled for just 30 minuts after all was dissolved, and it

4) SOCl_2 must not be used on over-excess. If it is used on too excess, the reaction gives insoluble matter to separate carbothiophenyl group.

was cooled immediately. It became crystal, was filtered off, washed well with HCl-acidified water and dried in vaccum.

The mother liquor was concentrated and the residue was washed with acidified water. The more crystal was obtained. Total Yield 24 g. (90.6% of the theoretical). m. p. 152—153°C (Literature 153°C).

N-carbothiophenyl-glycyl-DL-alanine ethylester—It was synthesized in the same way as carbothiophenyl-glycyl-DL-alanine methylester. m. p. 109—110°C.

N analysis found N=8.91%
for C₁₄H₁₈O₄N₂S calc. N=9.03%

It could be hydrolysed into N-carbohtiophenyl-glycyl-DL-alanine on the same condition as its methylester.

Poly-glycyl-DL-alanine—5.64 g (0.02 moles) of N-carbothiophenyl-glycyl-DL-alanine was dissolved in the hot mixture of dioxane 60 cc and pyridine 9.5 g (0.12 moles) and filtered off the impurity. The clear solution was polymerized in a sealed tube at 60°C for 1000 hours. Gelatinous precipitation was formed. After separating with centrifuge and washing with acetone several times, the polymer was refluxed with acetone, centrifuged, and washed with acetone. It gives no thiophenol smell. It was washed with ether and dried in vaccum. Yield 121.5 mg. (4.7% of the theoretical).

N analysis found N = 17.03%
for (C₅H₈O₂N₂.2H₂O)_n calc. N = 17.07%

Further, it was dried at 135—140°C for ten hours with Abderhalden's apparatus by xylene.

N analysis found N=21.74%
for (C₅H₈O₂N₂)_n calc. N=21.88%

Measurement of the viscosity and molecular weight of poly-glycyl-DL-alanine : Sample was dissolved in water and measured the viscosity at 30 ± 0.1°C with Ostwald's viscosimeter.

concentration C=0.7300 g/100cc, $\eta_{sp}/c=0.0418$

C=0.4867 g/100cc, $\eta_{sp}/c=0.0453$

C=0.3244 g/100cc, $\eta_{sp}/c=0.0475$

intrinsic viscosity $[\eta]=[\eta_{sp}/c]_{c \rightarrow 0}=0.0528$

It was dissolved in water and its molecular weight was measured by the osmotic method using cellophan as semipermeable membrane.

temp. 30 ± 0.1°C, concentration C=4.38 g/L, specific gravity $\rho=1.004$,

osmotic press. $\pi=3.258$ cm=3.126 × 10⁻³ atm., molecular weight M=34,813

polymerisation degree n=272

N-carbothiophenyl-glycyl-DL-phenylalanine ethylester—The chloroform solution of N-carbothiophenyl-glycylchloride, which was prepared from N-carbothiophenyl-glycine 9 g and thionylchloride 6 g, was dropped at -7—-10°C with stirring into the ether solution of DL-phenyl-alanine ethylester, which was prepared in the same way

as *N*-carbothiophenyl-glycyl-alanine methylester, and then the mixture was left alone overnight at room temperature. DL-phenylalanine ethylester hydrochloride separated out gradually from the clear solution. The crystal was filtered off and washed with ether. The ether solution was combined with the mother liquor, washed with HCl water and dried with Na_2SO_4 . When it was concentrated in vacuum, it became syrup. After drying in a vacuum desiccator overnight, it was rubbed with a small amount of ether. It became crystal. It was filtered off and dried in a vacuum desiccator. When the mother liquor was concentrated, more crystal was obtained. Crude 10 g (61% of the theoretical) m. p. 88–93 °C. It was recrystallized with equal volume of alcohol. Yield 8 g (48.8% of the theoretical). m. p. 107–108 °C.

N analysis found N=7.21%
for $\text{C}_{20}\text{H}_{22}\text{O}_4\text{N}_2\text{S}$ calc. N=7.25%

N-carbothiophenyl-glycyl-DL-phenylalanine—The mixture of 8 N-HCl 16 cc and acetone 38 cc was added to *N*-carbothiophenyl-glycyl-DL-phenylalanine ethylester 5.5 g and heated in a boiling water bath for just 30 minutes and cooled immediately. Acetone and hydrochloric acid was evaporated in vacuum at below 35 °C. The residual syrup was washed with HCl water and dried in a vacuum desiccator. When it was rubbed with a small amount of ether, it became crystal. It was taken out with ether, filtered and dried. Washing with alcohol and ethylacetate, pure crystal was obtained. Yield 25 g (50% of the theoretical). m. p. 178–179 °C.

N analysis found N=7.66%
for $\text{C}_{18}\text{H}_{18}\text{O}_4\text{N}_2\text{S}$ calc. N=7.82%

Poly-glycyl-DL-phenylalanine—*N*-carbothiophenyl-glycyl-DL-phenylalanine 1.79 g (0.005 moles) was dissolved in a hot mixture of pyridine 3.85 g (0.05 moles) and benzene 25 cc, and the impurity was filtered off. The clear solution was polymerized in a sealed tube at 60 °C for 1000 hours. Much white polymer separates out from the solution. It was centrifuged, washed with benzene and acetone, and then distilled with steam until no thiophenol smell remained. It was centrifuged, washed with acetone and ether, dried in vacuum. It became white powder. It was dried for several hours in vacuum under about 1 mm Hg with Abderhalden's apparatus of xylene. Yield 0.174 g (17.1% of the theoretical).

N analysis found N=13.71%
for $\text{C}_{11}\text{H}_{12}\text{O}_2\text{N}_2$ calc. N=13.73%

Measurement of the molecular weight and viscosity of poly-glycyl-DL-phenylalanine : Sample was dissolved in dichloroacetic acid and the viscosity was measured with Ostwald's viscosimeter and the molecular weight by osmotic method. Temp. 30 ± 0.1 °C

concentration $C=0.7750$ g/100cc, $\eta_{sp}/c=0.1052$

$C=0.5166$ g/100cc, $\eta_{sp}/c=0.1093$

$C=0.3444$ g/100cc, $\eta_{sp}/c=0.1127$

intrinsic viscosity $[\eta]=[\eta_{sp}/c]_{c \rightarrow 0}=0.1180$

The osmotic pressure was measured with semipermeable membrane, whose plasticizer was extracted with acetone.

concentration $C=3.875$ g/L, specific gravity $\rho=1.559$,

osmotic press. $\pi=2.086$ cm = 3.146×10^{-3} atm.,

molecular weight $M=30,603$, polymerisation degree $n=150$

N-carbothiophenyl-glycyl-L-leucine ethylester—The ether solution of L-leucine ethylester, which was prepared from L-leucine ethylester hydrochloride 50 g and NaOH 10 g and contains L-leucine ethylester 35 g (0.22 moles), and the chloroform solution of N-carbothiophenylglycylchloride, which was prepared from carbothiophenyl-glycine 23.3 g (0.11 moles) and thionylchloride 15.8 g (0.13 moles), were reacted at -7 — -10°C on cooling in the same way as the N-carbothiophenyl-glycyl-DL-alanine mentioned above. The produced L-leucine ethylester hydrochloride was filtered and the mother liquor was washed with HCl water, dried with Na_2SO_4 and evaporated to dryness. The residue was syrup and did not crystalize. Yield 36 g (93% of the theoretical).

N-carbothiophenyl-glycyl-L-leucine—To the syrupous N-carbothiophenyl-glycyl-L-leucine ethylester 28 g was added acetone 196 cc and 8 N-HCl 84 cc, and after heating for just 30 minutes in a boiling water bath, it was cooled immediately. When it was concentrated in vacuum at below 40°C , carbothiophenyl-glycyl-L-leucine crystalized. The crystal was filtered, washed with HCl-water and ether. After drying, it was purified by refluxing with a small amount of ether. Yield 12 g (46%) m. p. 155°C $[\alpha]_D^{25} = -15.0^\circ$ (in absolute alcohol).

N analysis found N=8.69%

for $\text{C}_{15}\text{H}_{20}\text{O}_4\text{N}_2\text{S}$ calc. N=8.64%

Poly-glycyl-L-leucine—(A) Benzene 75 cc and pyridine 11.85 g was added to carbothiophenyl-glycyl-L-leucine 4.860 g (0.015 moles) and dissolved in complete solution with warming. It was polymerized in a sealed tube at 60°C for 1000 hours. Protein-like precipitate was separated. It was centrifuged, washed with acetone, and refluxed several times with acetone. It became white powder. After expelling the adsorbed thiophenol and pyridine with steam distration, it was centrifuged, washed twice with acetone by refluxing and washed with ether. It was dried in about 1 mm Hg vacuum with Abderhalden's apparatus of toluene. Yield 83.1 mg. (5% of the theoretical).

N analysis found N=16.42%

for $(\text{C}_8\text{H}_{14}\text{O}_2\text{N}_2)_n$ calc. N=16.47%

Viscosity : It was measured at $30 \pm 0.1^\circ\text{C}$ in dichlor acetic acid.

concentration $C=0.7675$ g/100cc, $\eta_{sp}/c=0.1321$

$C=0.5116$ g/100cc, $\eta_{sp}/c=0.1452$

$C=0.3410$ g/100cc, $\eta_{sp}/c=0.1574$

intrinsic viscosity $[\eta]=[\eta_{sp}/c]_{c \rightarrow 0}=0.178$

Molecular weight : It was measured at $30 \pm 0.01^\circ\text{C}$ in dichloracetic acid using the

semipermeable membrane of vinylchloride.

concentration $C = 4.62$ g/L, specific gravity $\rho = 1.5492$,

osmotic pressure $\pi = 2.122$ cm = 3.181×10^{-3} atm.,

Molecular weight $M = 36,091$

polymerisation degree $n = 212$

(B) N-carbothiophenyl-glycyl-L-leucine 6.7 g was dissolved in hot dioxane 20 cc, filtered and the clear solution was put in a sealed tube. It was polymerized at 60 °C for 1000 hours. After two weeks, it became viscous and then precipitated gelatinous polymer and at last almost solidified in orange color. The sealed tube had a considerable pressure on it for the evolution of CO₂ gas. It was centrifuged with a small amount of acetone, washed with acetone several times. It became semigelatinous powder and was refluxed with acetone several times, until it became white powder. It was centrifuged, washed with ether and dried. The white powder of the polymer was dried for several hours in a vacuum of about 1 mm Hg with Abderhalden's apparatus of xylene. Yield 1.06 g (27.6% of the theoretical).

N analysis found N = 16.52%

for (C₈H₁₄O₂N₂)_n calc. N = 16.47%

Viscosity : It was measured as same as (A)

$C = 0.7850$ g/100cc, $\eta_{sp}/c = 0.0870$

$C = 0.5234$ g/100cc, $\eta_{sp}/c = 0.0976$

$C = 0.3483$ g/100cc, $\eta_{sp}/c = 0.1055$

intrinsic viscosity $[\eta] = [\eta_{sp}/c]_{c \rightarrow 0} = 0.121$

Molecular weight :

$C = 5.233$ g/L, $\rho = 1.5503$, $\pi = 2.290$ cm = 3.43×10^{-3} atm.,

molecular weight $M = 37,906$

polymerisation degree $n = 223$

Copolymerisation of glycyl-DL-phenylalanine and glycyl-L-leucine—N-carbothiophenyl-glycyl-DL-phenylalanine 0.71 g (0.002 moles) and N-carbothiophenyl-glycyl-L-leucine 0.65 g (0.002 moles) were dissolved in the mixture of benzene 20 cc and pyridine 3.16 g (0.04 moles). It was polymerized in a sealed tube at 60 °C for 1000 hours. After several weeks, the mixture became viscous and some precipitate separated out. After the complete reaction, the polymer was centrifuged and washed with acetone several times. It became white powder. It was refluxed with acetone, centrifuged and expelled the thiophenol smell with steam distration. It was concentrated, refluxed again with acetone, centrifuged and washed with acetone and ether. It was white powder. It was dried for several hours in a vacuum of about 1 mm Hg with Abderhalden's apparatus of xylene.

N analysis found N = 14.89%

for (C₈H₁₄O₂N₂ : C₁₁H₁₂O₂N₂ = 1:1)_n calc. N = 14.97%

Viscosity : It was measured at 30 ± 0.1 °C in dichlor acetic acid.

$$C = 0.6350 \text{ g/100cc, } \eta_{sp}/c = 0.0911$$

$$C = 0.4234 \text{ g/100cc, } \eta_{sp}/c = 0.0995$$

$$C = 0.2822 \text{ g/100cc, } \eta_{sp}/c = 0.0988$$

$$C = 0.1822 \text{ g/100cc, } \eta_{sp}/c = 0.1034$$

$$\text{intrinsic viscosity } [\eta] = [\eta_{sp}/c]_{c \rightarrow 0} = 0.1080$$

Molecular weight : It was measured in dichloro acetic acid using the semipermeable membrane of vinylchloride at $30 \pm 0.01^\circ\text{C}$.

$$C = 2.02 \text{ g/L, } \rho = 1.5502,$$

$$\pi = 2.443 \text{ cm} = 3.663 \times 10^{-3} \text{ atm.},$$

$$\text{molecular weight } M = 13,702$$

