Orig Life Evol Biosph (2017) 47:355–369
DOI 10.1007/s11084-017-9547-0

PREBIOTIC CHEMISTRY

Polycondensation of Asparagine-comprising Dipeptides in Aqueous Media-A Simulation of Polypeptide Formation in Primordial Earth Hydrosphere

Toratane Munegumi[1] · **Naoya Tanikawa**[2]

Received: 30 November 2016 / Accepted: 30 June 2017 /
Published online: 26 July 2017

Abstract Asparagine and aspartic acid might have mutually transformed in the primordial hydrosphere of the earth, if ammonia and aspartic acid had existed in equilibrium. These amino acids seem to contribute to polypeptides, while the simple amino acids glycine and alanine easily form cyclic dipeptides and do not achieve long peptide chains. Asparagine-comprising dipeptides contribute some kinds of activation forms of dipeptides because these can polymerize faster than asparagine only. The new finding of polypeptide formation suggests a pathway of sequential polypeptides to evolve a diversity of polypeptides.

Keywords Dipeptides · Condensation · Asparagine · Polypeptides

Introduction

Abiotic polypeptide formation pathways in prebiotic conditions have been discussed involving many scenarios (Huber et al. 2003; Huber and Wachtershäuser 2006; Danger et al. 2012), which explain the reaction pathways from amino acids in the primordial earth conditions. The estimated endogenous energy sources (Navarro-González et al. 1998) for the formation of organic compounds in the primordial earth may be ordered in the flux units of EJ/y (exajoule per year: 10^{18} J/ y) with two significant figures as follows: ultraviolet light (regions $\lambda < 270$ nm: 15,000 EJ/y; regions $\lambda < 200$ nm: 600 EJ/y), volcanic heat (5500 EJ/y), post-impact plumes (100 EJ/y), volcanic lightning (10 EJ/y), electrical activity in storms (lightening: 1.0 EJ/y; coronal: 0.50 EJ/y), and cosmic rays (0.23 EJ/y).

Dedicated to the memory of Jim Ferris

✉ Toratane Munegumi
tmunegumi@naruto-u.ac.jp

[1] Naruto University of Education, Naruto, Tokushima 772-8502, Japan

[2] Gifu Shotoku Gakuen University, Gifu-shi, Gifu 501-6194, Japan

Thermal energy sources including volcanic heating and hydrothermal vents (Corliss et al. 1979) would have contributed most to peptide formation (Imai et al. 1999; Kawamura et al. 2005) from amino acids at high temperatures and/or under high pressure, although each energy source might have played a role for the formation of amino acids (Miller 1953; Harada and Iwasaki 1974; Bada 2013; Munegumi 2014) and peptides.

Under the primordial earth conditions, the amino acids glycine (Gly), alanine (Ala), valine (Val), aspartic acid (Asp), and glutamic acid (Glu) may have been important for the formation of peptides because they are suggested as primitive. These amino acids have already been detected in the reactions using a Miller-type spark discharge (Miller 1953) in the gas phase and Harada glow discharge onto an aqueous phase (Harada and Iwasaki 1974). Moreover, these amino acids have been suggested as primitive amino acids by consideration of the genetic code (Sueoka 1961; Crick 1968; Woese 1973; Ishigami and Nagano 1975; Saetia et al. 1993). Analysis of extraterrestrial matter has also supported that these five amino acids are primitive because these have been detected in the Murchison meteorite (Kvenvolden et al. 1970; Cronin et al. 1979; Botta et al. 2002). Only glycine has been claimed to be observed in the interstellar medium (Kuan et al. 2003; Snyder et al. 2005; Majumdar et al. 2012), while according to the CDMS catalog (L Physikalisches Institut 2016), glycine has not been recorded.

Peptides have been found only as Gly-Gly and cyclo-(Gly-Gly) in the extraterrestrial matters: the Murchison meteorite and Yamato 791,198 (Shimoyama and Ogasawara 2002). Meanwhile, the simulation experiments have shown Ala-Ala formed by ultraviolet light in interstellar space conditions (Izumi and Nakagawa 2011) as well as several kinds of dipeptides (Gly-Gly, Ala-Gly, Ala-Ala, and Leu-Ala) formed using plausible interstellar ice mixtures (Kaiser et al. 2013). Abiotic condensations of glycine, alanine, and valine have reached only oligomers (Nagayama et al. 1990; Takaoka et al. 1991; Imai et al. 1999; Kawamura et al. 2005; Cleaves et al. 2009; Furukawa et al. 2012; Rodriguez-Garcia et al. 2015) except in ammonia (Oro and Guidry 1961), although much research on polypeptide syntheses using aspartic acid (Vegotsky et al. 1958), glutamic acid (Harada and Fox 1957), and lysine (Harada 1959a) has been published. Glycine and alanine easily form diketopiperazines, which had been 'considered to be an obstacle for the peptide elongation beyond dipeptide' (Nagayama et al. 1990). However, a programmed reaction condition gave an optimum yield of peptides (Rodriguez-Garcia et al. 2015).

One of the authors has recently reported an interesting pathway from alanine to asparagine (Munegumi 2014) as shown in Fig. 1. Alanine reacted with formamide to yield asparagine by glow discharge electrolysis onto the solution phase, which is a kind of simulation experiment of volcanic and stormy lightning on the primordial earth. The simulation experiments propose to change alanine residues in oligopeptides to asparagine residues, which have amide groups. Asparagine can self-polymerize (Kovacs and Nagy 1961; Bada and Miller 1968 Harada et al. 1978; Munegumi et al. 1994) in a heated aqueous solution and reach an equilibrium among ammonia, aspartic acid, 2-butenedioic acids, their mono-amides, and polypeptides (Munegumi et al. 1994). These results suggest a scenario that asparagine and aspartic acid could have mutually transformed in the primordial hydrosphere of the earth. Asparagine has also been considered to play an important role in the development of amino acid homochirality (Kojo et al. 2004). We wish to report the heating reactions of asparagine-comprising dipeptides to look at a new pathway of activation of oligopeptides.

The authors have briefly reported this topic before without an exact experimental description (Munegumi et al. 1992). The topic was reinvestigated from the viewpoint of the role of asparagine and oligopeptides in primordial polypeptide formation. The

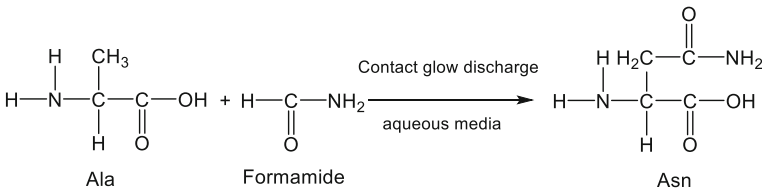

Fig. 1 Asparagine formation from alanine induced by contact glow discharge electrolysis (CGDE)

authors used the model compounds: glycyl-asparagine (Gly-Asn), alanyl-asparagine (Ala-Asn), and glycyl-glutamine (Gly-Gln) as similar amide-comprising compounds with the detailed experimental section.

Dipeptide substrates were prepared as shown in Fig. 2. Elemental analysis and other analyses showed pure compounds.

Experimental

Instrument

Physical data of the prepared compounds were measured with a MEL-TEMP (Mitamura Riken Kogyo, Tokyo, Japan) for melting point, A-3 (Jasco) for IR, and DIP-181 Digital Polarimeter (Jasco) for polarization. The molecular weights of polypeptides were estimated by comparing their retention times with a calibration curve of authentic compounds, in which the retention time of eluted samples from a G-50F column (420 mm × 10 mm) or a TSK gel G 3000 PW (300 mm × 7.5 mm) was determined with a Sic Chromatocorder II (System Instruments Co., Ltd., Tokyo, Japan).

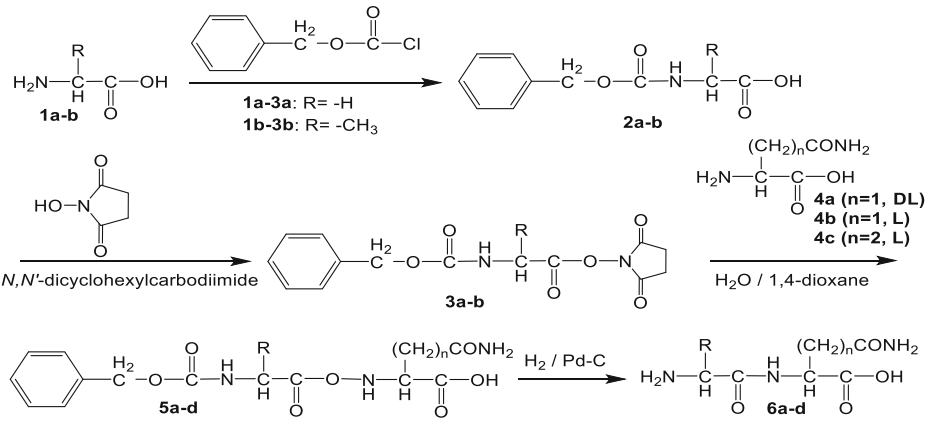

Fig. 2 Preparation of asparagine-comprising dipeptides and glycyl glutamine

Materials

Glycine (**1a**), L-alanine (**1b**), DL-asparagine (**4a**), L-asparagine (**4b**), and L-glutamine (**4c**) were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Z-Gly (2a):

To a cooled solution of glycine **1a** (7.52 g, 100 mmol) and 25 mL of 4 M–NaOH in an ice bath, was added dropwise benzyloxycarbonyl chloride (17.5 mL, 0.11 mol). The reaction mixture was stirred for about 1 h in an ice bath and washed with ether (20 mL). The remained water phase was acidified with 5 M–HCl to pH 2 and cooled in a refrigerator overnight. The filtered crystal was recrystallized with chloroform to yield *N*-benzyloxycarbonyl- glycine **2a**: 13.0 g (Yield: 62%, mp 121–123 °C (lit. 120 °C) (Greenstein and Winitz 1964).

Z-Gly-ONSu (3a):

The *N*-hydroxysuccinimide ester (Anderson et al. 1964) of *N*-benzyloxycarbonyl-glycine was prepared by the coupling of *N*-benzyloxycarbonyl-glycine (13.0 g, 62 mmol) and *N*-hydroxysuccinimide (7.14 g, 62 mmol) in the presence of *N*, *N*′-dicyclohexylcarbodiimide (DCC, 12.8 g, 62 mmol) in ethyl acetate (125 mL) in an ice-water bath. Yield: 86%, mp 114–115 °C.

Z-Gly-DL-Asn (5a):

The resulting *N*-hydroxysuccinimide ester (15.3 g, 60 mmol) dissolved in 1,4-dioxane (125 mL) was added to a solution containing DL-asparagine (9.0 g, 60 mmol), triethylamine (8.4 mL, 60 mmol), and water (125 mL). After adding 1,4-dioxane (125 mL) to the reaction solution, the resulting solution was stirred at room temperature overnight. The resulting solution was evaporated in vacuo to give ca 30 mL solution, which was cooled in an ice-water bath and then acidified and salted out by the addition of 50 mL of 10% tartaric acid saturated with sodium chloride. Adding ethyl acetate (100 mL) to the resulting mixture yielded a white precipitate, which was recrystallized to give 5.8 g (35%). mp 104–106 °C. Anal: Calcd for $C_{14}H_{17}N_3O_6$•0.2H_2O: C, 51.44; H, 5.36; N, 12.85%. Found: C, 51.77; H, 5.50; N, 12.33%.

Gly-DL-Asn (6a):

N-Benzyloxycarbonyl-glycyl-DL-asparagine (4.52 g, 15.0 mmol) dissolved in 100 mL of acetic acid was hydrogenated over 5% palladium on charcoal at room temperature for two days. The filtrate of the reaction mixture was lyophilized to give a white powder, which was recrystallized with water–ethanol to give 2.0 g (76%). mp 210–212 °C (decomposed). Anal: Calcd for $C_6H_{11}N_3O_4$•0.2H_2O: C, 37.38; H, 5.96; N, 21.80%. Found: C, 37.91; H, 6.02; N, 21.30%.

Z-Gly-L-Asn (5b):

N-Benzyloxycarbonyl-glycyl-L-asparagine was prepared by the coupling of L-asparagine monohydrate (L-Asn•H_2O) (7.23 g, 48.0 mmol) and *N*-hydroxysuccinimide (12.3 g, 40.0 mmol) in the presence of triethylamine (6.7 mL, 48 mmol) in a water–1,4-dioxane solution in a similar manner to the preparation of *N*-benzyloxycarbonyl-glycyl-DL-asparagine. The reaction solution was evaporated in vacuo to give an oily solution (30 mL), which was cooled and diluted with 50 mL water. After washing the resulting solution with ethyl acetate (80 mL × 2), the remaining aqueous layer was evaporated, cooled, and vigorously mixed with 30 g tartaric acid. The obtained precipitate after one week's cooling in a refrigerator was filtered to yield a crystalline product (7.63 g, 59%). mp 130–133 °C. $[\alpha]_D^{20}$ + 25.2° (c 2.0, ethanol). Anal: Calcd for $C_{14}H_{17}N_3O_6$•0.25H_2O: C, 51.29; H, 5.38; N, 12.81%. Found: C, 51.15; H, 5.19; N, 12.56%.

Gly-L-Asn (6b):

Glycyl-L-asparagine was prepared by hydrogenation of *N*-benzyloxycarbonyl-glycyl-L-asparagine (3.50 g, 11 mmol) over 5% palladium on charcoal (0.25 g) in acetic acid (50 mL) for 24 h. The solution of the lyophilized filtrate of the reaction solution in water (10 mL) was filtered again. The mixture of the filtrate with 20 mL ethanol was stood overnight in a refrigerator. The resulting crystal was 1.59 g (76%). mp 212–213 °C (decomposed). Anal: Calcd for $C_6H_{11}N_3O_4$•0.2H_2O: C, 37.38; H, 5.96; N, 21.80%. Found: C, 37.98; H, 5.91; N, 21.88%. $[\alpha]_D^{17}$–5.33° (c 1.3, H_2O).

Z-L-Ala-ONSu (3b):

The *N*-hydroxysuccinimide ester of *N*-benzyloxycarbonyl-L-alanine (Anderson et al. 1964) was prepared by the coupling of *N*-benzyloxycarbonyl-alanine (33.6 g, 150 mmol) and *N*-hydroxysuccinimide (7.14 g, 62 mmol) in the presence of DCC (30.95 g, 150 mmol) in ethyl acetate (250 mL) in an ice-water bath. Yield: 100%; mp 121–123 °C.

Z-L-Ala-L-Asn (5c):

L-Asparagine monohydrate (15.0 g, 100 mmol) was dissolved in water (200 mL) containing triethylamine (14 mL, 100 mmol). A 1,4-dioxane solution of *N*-benzyloxycarbonyl-L-alanine *N*-hydroxysuccinimide ester (31.7 g, 100 mmol) was added to the cooled solution. The reaction was carried out for 24 h. The resulting solution was evaporated in vacuo to give an oily product, which was cooled to room temperature and then acidified with 10% potassium hydrogen sulfate (100 mL) to give pH 2. Ethyl acetate was added to the resulting solution to give a white precipitate, which was filtered and washed with water to afford 23.35 g (69%). The precipitate was recrystallized from ethanol–petroleum ether to afford 20.47 g (61%). mp 173–175 °C. Anal: Calcd for $C_{15}H_{19}N_3O_6$: C, 53.41; H, 5.68; N, 12.46%. Found: C, 53.15; H, 5.67; N, 12.49%. $[\alpha]_D^{25}$ + 4.6° (c 0.26, ethanol).

L-Ala-L-Asn (6c):

Z-L-Ala-L-Asn (16.20 g, 45.6 mmol) dissolved in 100 mL acetic acid was hydrogenated over 5% palladium on charcoal (1.0 g) under a hydrogen atmosphere for 19 h. The filtrate of the resulting solution was lyophilized and then dissolved in distilled water. Lyophilization was repeated a total of three times to give a pale-yellow crystal, which was recrystallized from a mixture containing water (90 mL), ethanol (240 mL), and methanol (20 mL) to afford 7.22 g (78%). mp 210 °C (decomposed). Anal: Calcd for $C_7H_{13}N_3O_6$•H_2O: C, 38.01; H, 6.83; N, 19.00. Found: C, 37.70; H, 6.61; N, 18.81%. $[\alpha]_D^{25}$ + 0.39° (c 1.0, water).

Z-Gly-L-Gln (5d):

A solution containing Z-Gly-ONSu (14.54 g, 47.5 mmol) and tetrahydrofuran (100 mL) was added in 5 min to a solution of L-glutamine (8.07 g, 55.0 mmol) in 50 mL water in the presence of triethylamine (15.4 mL, 0.11 mL). The reaction was continued in an ice-water bath for 30 min. The reaction solution was evaporated in vacuo to give an oily product (ca 30 mL), which was extracted with ethyl acetate (100 mL × 2). A 10% citric acid solution saturated with sodium chloride (200 mL) was added to the remaining aqueous layer to give pH 2. The obtained solution was stood for four days in a refrigerator to give a white precipitate, which was recrystallized as colorless crystals (4.96 g, 31%). mp 155–158 °C (lit. 158.5–159 °C). Anal: Calcd for $C_{15}H_{19}N_3O_6$: C, 53.41; H, 5.68; N, 12.46. Found: C, 53.26; H, 5.65; N, 12.19%. $[\alpha]_D^{20}$ + 2.76° (c 0.5, methanol).

Gly-L-Gln (6d):

Z-Gly-Gln (4.96 g, 15.0 mmol) was hydrogenated in a mixture of methanol (50 mL) and acetic acid (0.5 mL) over 5% palladium on charcoal for a day. Palladium on charcoal (0.21 g) and methanol was added again to the reaction mixture. The hydrogenation was carried out for

three more days. Acetic acid (100 mL) was added to the reaction mixture to give a solution that was filtered and the resulting filtrate was lyophilized to give a precipitate. The precipitate was redissolved in 20 mL water and 30 mL ethanol was added to the resulting solution to afford colorless crystals (1.71 g, 56%). mp 210–211 °C (decomp) (198–200 °C). Anal: Calcd for $C_7H_{13}N_3O_4 \bullet H_2O$: C, 38.01; H, 6.83; N, 19.00. Found: C, 38.52; H, 6.84; 18.93%. $[\alpha]_D^{28}$ –1.88° (c 1.0, H_2O).

Heating reaction of peptide solutions

Dipeptides (1.5 mmol) were dissolved in 1.5 mL of distilled water in a glass tube (ID 8.0 mm), which was cooled at −40 °C under a reduced pressure (1 mmHg) and sealed. The sealed glass tube aliquots containing substrate L-Ala-L-Asn (**6c**) were separately heated at 80, 100, 110, and 120 °C. Sealed glass tube aliquots containing other substrates (**6a**, **6b**, and **6d**) were heated at 120 °C.

Amino acid analysis of heated reaction mixtures

The reaction mixtures after heating were analyzed with an amino acid analyzer (Hitachi 835, Hitachi Co. Ltd., Tokyo, Japan). The detection of the eluted compounds was carried out by means of visible absorption at 570 nm after a post-column derivatization method with ninhydrin.

Gel permeation chromatography of heated reaction mixture

Most of the reaction mixture was dissolved in 0.5 mL acetic acid and the solution was loaded onto a gel permeation glass column (G-25F, 100 cm × 2 cm ID). The eluted solution was collected as about 3 to 4 mL fractions. Ultraviolet absorption of each fraction was detected at 230 nm.

About 0.1 mg of the higher molecular weight fraction was dissolved in 0.1 M sodium phosphate buffer (pH 6.98, 0.100 mL), and a part (0.010 mL) of the resulting solution was injected into a TSK gel G3000 PW column (300 mm × 7.5 mm ID). The eluted solution was flowed with a HPLC Pump 576 at a flow rate of 0.6 mL/min to be detected with a Jasco 875-UV at 230 nm. The elution volume Vs (mL) was calculated from the retention time for each sample and then $K_{av} = (V_s - V_0) / (V_t - V_0)$ was obtained, where void volume V_0 = 11.088 (mL), total volume V_t = 16.558 (mL). K_{av} for each sample was compared with K_{av} values for known proteins and peptides. The calibration curve is shown in Fig. 3.

Chromatography conditions: Gel permeation column, TSK gel G 3000 PW; eluate, 0.1 M sodium phosphate buffer (pH 6.98, 0.100 mL) at a flow rate of 0.6 mL/min; detection, UV absorption at 230 nm. Kav = $(Vs - V_0) / (V_t - V_0)$; V_0 = 11.088 (mL); V_t = 16.558 (mL); Log MW: logarithm of molecular weight. (1) Blue dextran (MW: 2.0×10^6 Da); (2) ovalbumin (MW: 4.5×10^4Da); (3) carbonic anhydrase (MW: 36, 900 Da); (4) myoglobin (MW:17 , 200 Da); (5) cytochrome C (MW: 11,700 Da); (6) Insulin A (MW: 2400 Da); (7) $(Pro\text{-}Pro\text{-}Gly)_5$ (MW:1274 Da); (8) Ala-Asp (MW: 204 Da).

Gas chromatography

A Hitachi 163 gas chromatograph equipped with a capillary column Chirasil-Val (25 m × 0.25 mm ID) was used for analysis of *N*-trifluoroacetyl-amino acid 2-propyl ester. The analysis was carried out under a flow of nitrogen as the carrier gas. The detection was

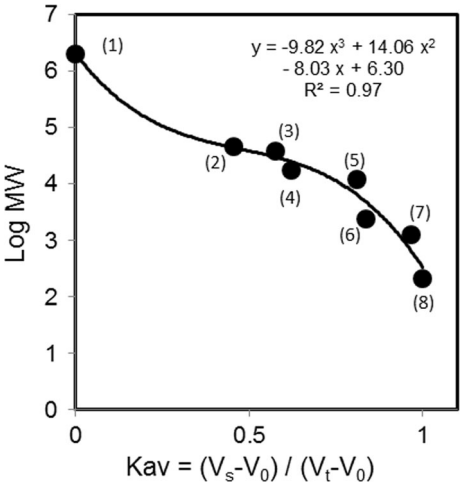

Fig. 3 Calibration curve of molecular weight using standard proteins and peptides

conducted by means of a flame ionization detector. The signals of the separated compounds were recorded and integrated with a Sic Chromatocorder II.

Results and Discussion

Time course study of heating reaction mixtures

Figure 4 shows a typical chromatogram of the reaction mixture of peptide L-Ala-L-Asn.

The chromatogram of the reaction mixture of L-Ala-L-Asn heated at 120 °C for 4 h shows a reparative decrease of the peak area for L-Ala-L-Asn and formation of ammonia.

Figure 5 shows the time course study of the heating reactions of substrate peptides **6a–d** at 120 °C in aqueous solutions in sealed tubes. The reaction mixtures were analyzed with an amino acid analyzer as described in the experimental section. Substrates **6a–c** decreased very rapidly to give a small amount of substrate recovery lower than 10% in 4 h, but a large amount of ammonia was released. The result indicates that the *N*-terminal amino group of the dipeptide would have attacked the amide carbonyl carbon of the asparagine residue to eliminate ammonia. In the reactions, free glycine and aspartic acid formed in a small amount, assumed to be hydrolysis of peptides. On the other hand, substrate **6d** (Gly-L-Gln) reacted rather slowly to release ammonia. Such slow reaction suggests that peptide formation is also slower than with asparagine-comprising dipeptides **6a–c**.

Higher molecular weight fraction

Each heated reaction mixture was loaded onto a gel permeation chromatograph (Sephadex G-25F). Figure 6 shows chromatograms for the reaction mixtures using a substrate peptide L-Ala-L-Asn (**6c**) at 120 °C in different reaction time (4, 8, 16, and 32 h). As the reaction time progressed, the higher molecular weight fractions showed higher UV absorption. The results show that polycondensation of dipeptides proceeded continuously. Similar results of the

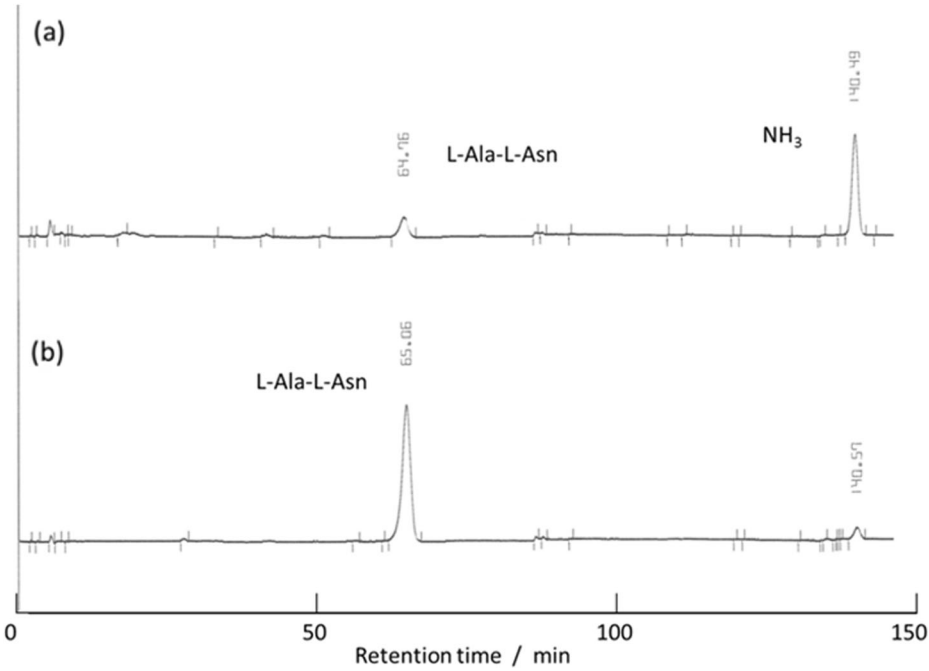

Fig. 4 A typical chromatogram of the reaction mixture of peptide L-Ala-L-Asn. **a** The reaction mixture of L-Ala-L-Asn heated at 120 °C for 4 h. **b** Standard of L-Ala-L-Asn

heating reaction were observed for other asparagine-comprising dipeptides Gly-DL-Asn (**6a**) and Gly-L-Asn (**6b**).

However, the chromatograms of the heated reaction solutions of Gly-L-Gln (**6d**) did not show absorption before tube number 40, suggesting that the reaction solutions did not give lower molecular weight fractions than the reaction solutions of **6a–c** (Fig. 7).

Ninhydrin-negative and UV absorption-positive higher molecular weight fractions, which eluted at a smaller elution volume than 130 mL, were collected and lyophilized to give an amorphous powder. It was analyzed with an infrared spectrometer to show typical absorbances (1720 cm^{-1} for –COOH; 1660 cm^{-1} for amide I, 1540 cm^{-1} for amide II) for peptides. The higher molecular weight fractions were analyzed with a standard gel permeation chromatograph, comparing their K_{av} values with those on the calibration curve prepared from proteins with known molecular weight as shown in Fig. 4. Moreover, the higher molecular weight fractions were hydrolyzed with 6 M HCl for 24 h and analyzed with an auto amino acid analyzer to reveal the same amino acid composition as that estimated from the substrate peptide composition. The data are shown in Table 1.

The estimated average molecular weight of the faster eluted fraction was in the range of 1300 to 5200 Da. The value for asparagine-comprising dipeptides (**6a–c**) depended not on the kinds of substrates but on the reaction temperature. The polymers made at shorter reaction times usually show lower molecular weight. The molecular weight 3500 Da is due to 40-residue sequential polypeptides: $(Gly\text{-}Asp)_{20}$, and 4300 Da is due to 50 residues: $(Gly\text{-}Asp)_{25}$. Amino acid analysis of the hydrolysates of polypeptides shows the same composition of the two amino acids. However, heating of Gly-L-Gln gave a very small amount of polypeptides, which were collected as a small number of fractions with higher molecular weight. Figure 7

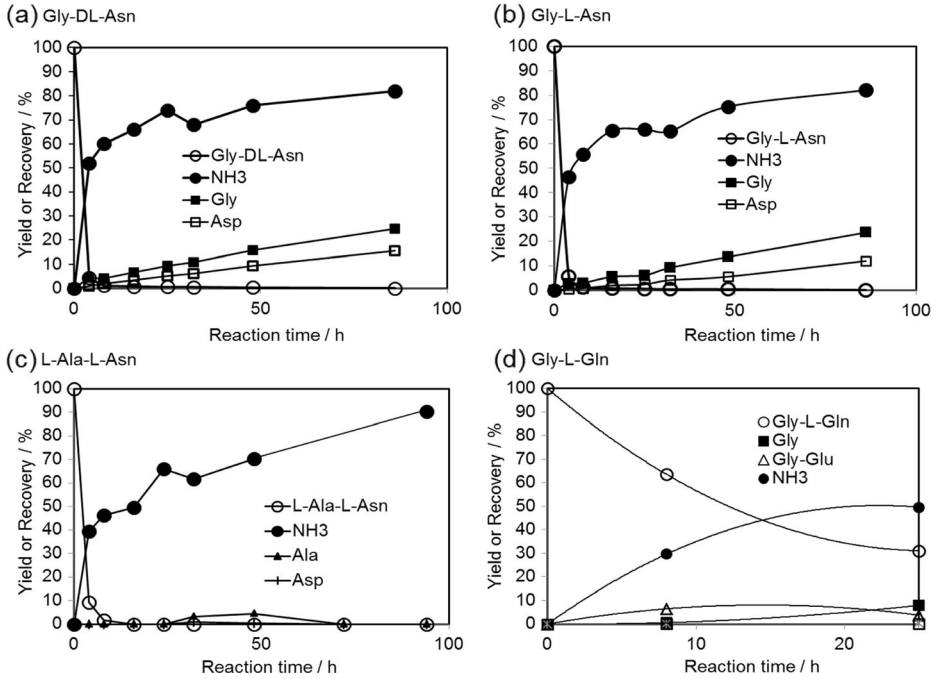

Fig. 5 The time course study of the heating reactions of AA-Asn and Gly-L-Gln peptides at 120 °C. Analyses of the reaction mixtures were carried out with an amino acid analyzer. **a** Gly-DL-Asn: **6a**; **b** Gly-L-Asn: **6b**; **c** L-Ala-L-Asn: **6c**; **d** Gly-L-Gln: **6d**

shows that gel permeation chromatography of the reaction mixtures of Gly-L-Gln revealed a lack of high molecular weight fractions. The results suggest that Gly-L-Gln reacts very slowly to yield lower molecular weight peptides.

However, there are some limits in estimation of molecular weight using gel filtration chromatography (or size exclusion chromatography), because 'a branched macromolecule will elute from the column later than a linear macromolecule of the same chemistry and equal molar mass' (Striegel et al. 2009a). The relative error in molecular weight depends on the molecular radius presumed by the degree of branching (Striegel et al. 2009b). If the higher molecular weight fractions obtained by heating dipeptides (**6a-6d**) are mainly composed of

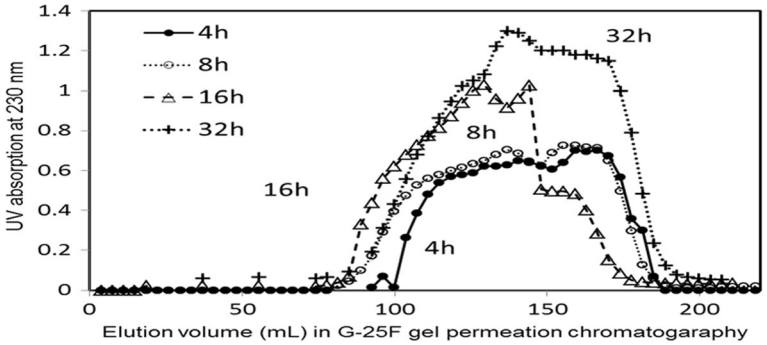

Fig. 6 Typical gel permeation chromatograms of the reaction mixtures using a peptide L-Ala-L-Asn (**6c**) at 120 °C in different reaction time

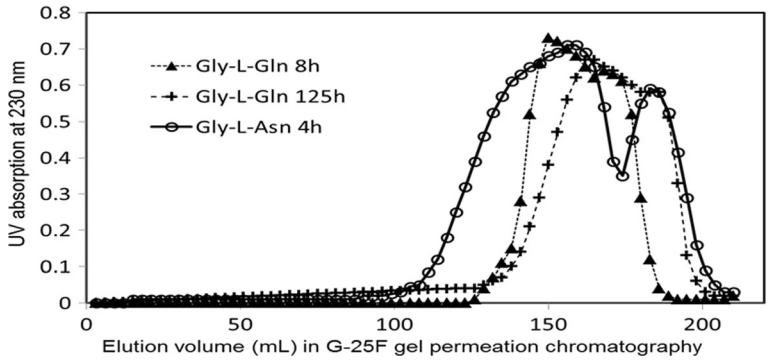

Fig. 7 Typical gel permeation chromatograms of the reaction mixtures at 120 °C using substrate peptides Gly-L-Asn (**6b**) for 4 h and Gly-L-Gln (**6c**) for 8 and 125 h

Table 1 Estimated molecular weight, amino acid composition, and D/L ratio for polypeptides

Substrate Dipeptide	Temperature /°C	Reaction Time/h	Molecular Weight/ Da (yield /%)[a]	Amino acid Composition (Asp/AA or Glu/AA)	D/L ratio of Asp	D/L ratio of Ala
6a	120	4	–	1.11	–	–
6a	120	8	–	1.05[b]	–	–
6a	120	16	–	1.06[b]	–	–
6a	120	25	–	1.09	–	–
6b	120	4	3500 (8.5)	1.05	0.53	–
6b	120	8	3900 (14)	1.08	0.70	–
6b	120	16	4400 (8.9)	1.08	0.86	–
6b	120	25	4300 (8.5)	1.09	0.88	–
6b	120	32	4300 (7.4)	1.12	0.88	–
6c	120	4	4400 (2.5)	0.91	0.21	0.03
6c	120	8	5200 (1.4)	0.93	0.43	0.04
6c	120	16	4400 (5.7)	0.94	0.68	0.07
6c	120	24	2500 (36)[c]	0.93	0.88	0.11
6c	120	32	2600 (22)[c)]	0.95	0.94	0.14
6c	120	48	4900 (15)	0.99	–	–
6c	120	72	4300 (17)	0.99	–	–
6c	120	94	4200 (16)	1.03	–	–
6c	110	4	1300	0.92	0.11	0.02
6c	110	8	2700	0.95	0.24	0.03
6c	110	16	3500	0.93	0.47	0.04
6c	110	24	3000	0.93	0.60	0.05
6c	110	48	2300	0.95	0.88	0.09
6c	110	72	2800	0.97	1.00	0.15
6c	100	4	2700 (3.6)	0.92	0.05	0.02
6c	100	12	4100 (5.4)	0.92	0.13	0.02
6c	100	36	3400	1.06	0.33	0.03
6c	80	32	2100 (3.6)	–	–	–
6c	80	123	5000 (5.4)	–	–	–
6d	120	8	3000 (0.7)	0.93[b]	–	–
6d	120	25	3000 (1.4)	1.02[b]	–	–

6a Gly-DL-Asn, *6b* Gly-L-Ala, *6c* L-Ala-L-Asn, *6d* Gly-L-Gln

[a] Yields were calculated on the base of the complete deamidation forms of substrates: -(Gly-Asp)$_n$- for **6a** and **6b**; −(Ala-Asp) $_n$- for **6c**; −(Gly-Glu) $_n$- for **6d**

[b] Reaction mixtures were directly hydrolyzed in 6 M HCl, evaporated and derivatized

[c] Some lower molecular weight fractions were included

branched polypeptides, the estimated molecular weight in Table 1 may give the relatively smaller values of molecular weight than the actual values. Therefore, the actual molecular weight must be higher than the estimated values in Table 1 as thousands Da of polypeptides. Future study of the polypeptides obtained will determine the structure of polypeptides using other techniques.

Epimerization of amino acid residues in polypeptides

A part of the acid-hydrolyzed samples was evaporated to give a solid, which was derivatized with 2-propanol containing 1.5 M HCl and then trifluoroacetic acid anhydride to afford an *N*-trifluoroacetyl-amino acid 2-propyl ester. The gas chromatographic analyses of the derivatives with a chiral glass capillary column are shown in Fig. 8 and Table 1. Comparing with reactions at different temperatures at the same reaction time, the higher reaction time showed higher epimerization. Aspartic acid residues epimerized faster than alanine residues. The reason can be explained by the imide formation as well as the electron-withdrawing effect of the two carboxy groups of the aspartic acid residue.

Postulated polycondensation mechanism

A proposed mechanism of polycondensation of dipeptides is shown in Fig. 9. An amino group of the dipeptide attacks an amide bond of another dipeptide to release an ammonia molecule and form a new peptide bond. The reaction proceeds very rapidly to give polypeptides. The mechanism is supported by the very rapid diminishment of dipeptides and ammonia release. Free aspartic acids cannot release water molecules easily to make peptide bonds in heated

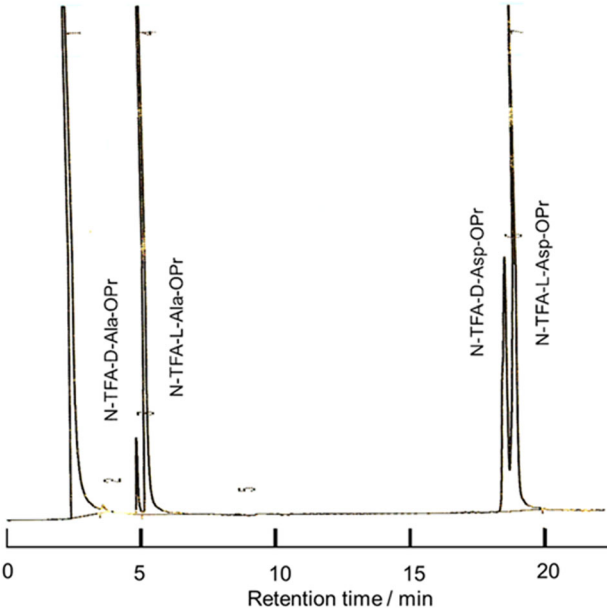

Fig. 8 A typical gas chromatogram of the *N*-trifluoroacetyl-DL-alanine 2-propyl ester and *N*-trifluoroacetyl-DL-aspartic acid di-2-propyl ester, which was derived from the hydrolysate of the higher molecular weight fraction obtained from the reaction mixture of L-Ala-L-Asn at 120 °C

aqueous solutions, while heating solid aspartic acid can release water molecules (Vegotsky et al. 1958). The elimination rate of an ammonia molecule from an amide group is much faster than the elimination rate of a water molecule from a carboxyl group. Superior proton catchers, which are stronger bases, are generally superior leaving groups. The value of pKa for ammonium (protonated ammonia) is 9.25 and the value of pKa for hydronium (protonated water) is −1.74. These values clearly show that ammonia is much superior leaving group than water and support that the sequential polypeptide formation by deamination.

However, the polypeptides make imide bonds, which are in equilibrium between α- and β-aspartate residues. The imide formation mechanism is supported by some reports (Harada et al. 1978; Harada 1959b; Radkiewicz et al. 1996, 2001). The imide formation also suggests dipeptide imides from substrates and the ring-opening reactions of imides with substrate (AA^1-AA^2) as shown in Fig. 9. Although the polypeptides may have complexed structures, the amino acids composition of resulting polypeptides was almost same as those of the starting dipeptides.

Comparison of dipeptides with free asparagine in aqueous media

Asparagines in high concentrations in aqueous media react with each other to produce polypeptides (Kovacs and Nagy 1961; Harada et al. 1978; Munegumi et al. 1994) under heating. However, asparagine, aspartate, ammonia, and other organic compounds achieve equilibrium between them (Munegumi et al. 1994). The equilibrium between aspartate, ammonia, and fumaric acid has been reported (Bada and Miller 1968; Bada and Miller 1970). These reports showed that asparagine and aspartic acid release ammonia from their α-carbons as shown in Fig. 10.

Elimination of ammonia from the α-carbon would decrease the concentration of asparagine that condense with each other to reduce the condensation rate. Although asparagine and aspartic acid can release ammonia, peptides having an asparagine residue at the *C*-terminal cannot release amino acid instead of ammonia. Therefore, the peptides having an asparagine

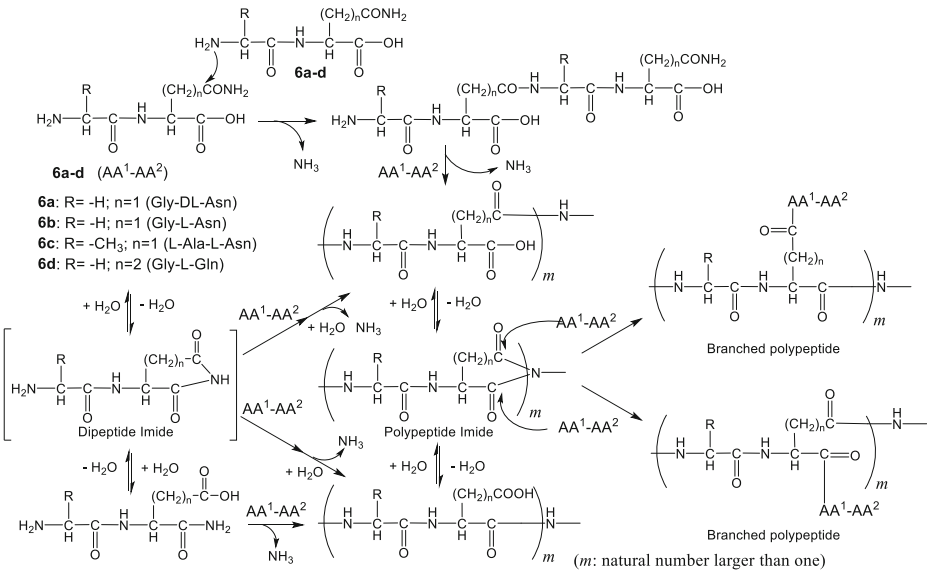

Fig. 9 A postulated reaction mechanism of polypeptide formation from AA^1-AA^2 (**6a–d**)

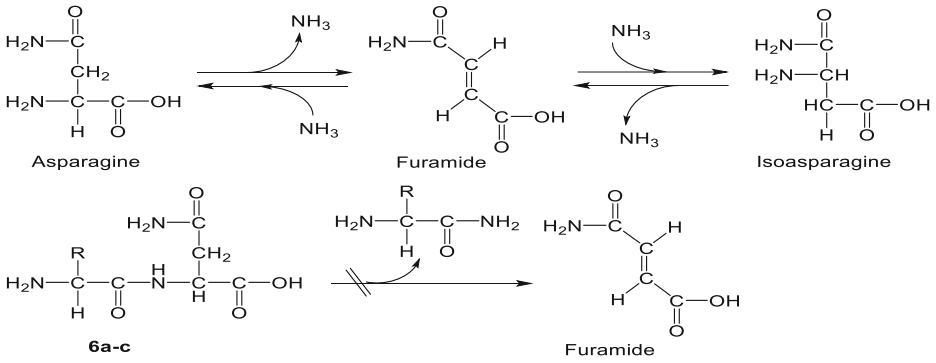

Fig. 10 Elimination of ammonia from the α-carbon of asparagine

residue at the *C*-terminal retain their concentration for condensation to afford polypeptides. The increase of ammonia in the reaction solutions during the earlier stage may be caused by the release of ammonia not from the α-carbon but the side-chain amide carbon. At the same time as the ammonia release, peptides having an asparagine at the *C*-terminal may condense with each other to afford polypeptides.

If substrate dipeptides (**6a–d**) would be hydrolyzed to amino acids during the reactions, aspartic acid and glutamic acid may be discussed in the viewpoint of polypeptide formation. As shown in Fig. 5, near hundred hour's reaction of substrates gave aspartic acid up to 0.15 M (ca 15%), which supposes to be obtained by hydrolysis of substrates and polypeptides. Even if aspartic acid existed in the reaction mixture, it could be in the equilibrium among other compounds: ammonia, asparagine, polypeptides etc. Polypeptide formation depends on the concentration of asparagine. Heating reaction of aspartic acid (1 M) in ammonia solution for 94 h at 120 °C gave aspartic acid (86%), asparagine (9%), isoasparagine (0.3%), and polypeptide (0%) possessing higher than 1500 Da (Munegumi et al. 1994). Glutamic acid itself forms pyroglutamic acid which does not polymerize. Harada et al. have already reported this reaction (Harada and Fox 1957). And Table 1 shows the compositions of aspartic acid and another amino acid are even in the hydrolysates of higher molecular weight polypeptides. Therefore, aspartic acid and glutamic acid would not contribute to form higher molecular weight polypeptide.

Estimated dipeptides comprising asparagine at the *N*-terminal can be discussed by using the results of the reaction mixture of asparagine and other amino acids because in that case both the substitution agent and the substituted position are in the same asparagine residue. However, this type of reaction cannot make sequential peptides, in which asparagine (or aspartyl) residue and another amino acid residue exist in a line.

Conclusions

Asparagine can be produced by coupling (Munegumi 2014) of a simple compound, formamide, with glycine and alanine, which have been suggested to make oligopeptides in the primordial earth conditions. Therefore, Gly-Asn and Ala-Asn would have been produced in the primordial conditions. Gly-Asn and Ala-Asn condensed with each other very rapidly compared with Gly-Gln to make polypeptides. These results mean that the evolution of Ala to Asn in the dipeptides fosters the polycondensation and a kind of divergence of polypeptides

like a kind of sequential polypeptide. Although the polypeptides may have complexed structures which could include imide structures, α- and β-carboxy groups, and many braches, the amino acids composition of resulting polypeptides was almost same as those of the starting dipeptides. This study suggests a possible polypeptide formation pathway from asparagine-comprising dipeptides. Further characterization of the polypeptides obtained in this study will be carried out using other techniques to clarify the detailed reaction mechanism.

Acknowledgements The authors wish to thank the late Emeritus Professor Kaoru Harada and Naoko Suzuki who was a student of the University of Tsukuba for fruitful assistance with amino acid analysis and other experimental work.

References

Anderson GW, Zimmerman JE, Callahan FM (1964) The use of esters of *N*-hydroxysuccinimide in peptide synthesis. J Am Chem Soc 83:1839–1842

Bada JL (2013) New insights into prebiotic chemistry from Stanley Miller's spark discharge experiments. Chem Soc Rev 42:2186–2196

Bada JL, Miller SL (1968) Equilibrium constant for the reversible deamination of aspartic acid. Biochemistry 7: 3403–3408

Bada JL, Miller SL (1970) Kinetics and mechanism of reversible nonenzymatic deamination of aspartic acid. J Am Chem Soc 92:2774–2782

Botta O, Glavin DP, Kminek G, Bada JL (2002) Relative amino acid concentrations as a signature for parent body processes of carbonaceous chondrites. Orig Life Evol Biosph 32:143–163

Cleaves HJ, Aubrey AD, Bada JL (2009) An evaluation of the critical parameters for abiotic peptides synthesis in submarine hydrothermal systems. Orig Life Evol Biosph 39:109–126

Corliss JB, Dymond J, Gordon LI, Edmond JM, von Herzen RP, Ballard RD, Green K, Williams D, Bainbridge A, Crane K, van Andel TH (1979) Submarine thermal springs on the Galapagos rift. Science 2196803:1073–1083

Crick FHC (1968) The origin of the genetic code. J Mol Biol 38:367–379

Cronin JR, Pizzarello S, Moore CB (1979) Amino acids in an Antarctic carbonaceous chondrite. Science 206: 335–337

Danger G, Plasson R, Pascal R (2012) Pathways for the formation and evolution of peptides in prebiotic environments. Chem Soc Rev 41:5416–5429

Furukawa Y, Otake T, Ishiguro T, Nakazawa H, Kakegawa T (2012) Abiotic formation of valine peptides under conditions of high temperature and high pressure. Orig Life Evol Biosph 42:519–531

Greenstein JP, Winitz M (1964) "Chemistry of the Amino Acids," Vol. 2: p.891, John Willey & Sons

Harada K (1959a) Thermal homopolymerization of lysine and copolymerization with natural and acidic amino acids. Bull Chem Soc Jpn 32:1007–1008

Harada K (1959b) Polycondensation of thermal precursors of aspartic acid. J Org Chem 24:1662–1666

Harada K, Fox SW (1957) The thermal condensation of glutamic acid and glycine to linear peptides. J Am Chem Soc 80:2694–2697

Harada K, Iwasaki T (1974) Syntheses of amino acids from aliphatic carboxylic acid by glow discharge electrolysis. Nature 250:426–428

Harada K, Matsuyama M, Kokufuta E (1978) The aqueous thermal polycondensation of asparagine and isoasparagine and the structure of polyaspartic acid. Polymer Bull 1:177–180

Huber C, Wachtershäuser G (2006) α-Hydroxy and α-amino acids under possible Hadean, volcanic origin-of-life conditions. Science 314:630–632

Huber C, Eisenreich W, Hecht S, Wachtershäuser G (2003) A possible primordial peptide cycle. Science 301: 938–940

Imai E, Honda H, Hatori K, Brack A, Matsuno K (1999) Elongation of oligopeptides in a simulated submarine hydrothermal system. Science 283:831–833

Ishigami M, Nagano K (1975) The origin of the genetic code. Orig Life Evol Biosph 6:551–560

Izumi Y, Nakagawa K (2011) Quantum yields of decomposition and homo-dimerization of solid L-alanine induced by 7.2 eV vacuum ultraviolet light irradiation: an estimate of the half-life of L-alanine on the surface of space objects. Orig Life Evol Biosph 41:385–395

Kaiser RI, Stockton AM, Kim YS, Jensen EC, Mathies RA (2013) On the formation of dipeptides in interstellar model ices. Astrophys J 765:111 (p.9)
Kawamura K, Nishi T, Sakiyama T (2005) Consecutive elongation of alanine oligopeptides at the second time range under hydrothermal conditions using a microflow reactor system. J Am Chem Soc 127:522–523
Kojo S, Uchino H, Yoshimura M, Tanaka K (2004) Racemic D,L-asparagine causes enantiomeric excess of other coexisting racemic D,L-amino acids during recrystallization: a hypothesis accounting for the origin of L-amino acids in the biosphere. Chem Commun 2004:2146–2147
Kovacs J, Nagy H (1961) Polypeptide formation from asparagine under hypothetically primitive conditions. Nature 190:531–532
Kuan Y, Charnley SB, Huang H, Tseng W, Kisiel Z (2003) Interstellar glycine. Astro Phys J 593:848–867
Kvenvolden K, Lawless J, Pering K, Peterson E, Flores J, Ponnamperuma C, Kaplan IR, Moore C (1970) Evidence for extraterrestrial amino-acids and hydrocarbons in the Murchison meteorite. Nature 228:923–926
L Physikalisches Institut (2016) http://www.astro.uni-koeln.de/cdms/molecules
Majumdar L, Das A, Chakrabarti SK, Chakrabarti S (2012) Hydro-chemical study of the evolution of interstellar pre-biotic molecules during the collapse of molecular clouds. Res Astron Astrophys 12:1613–1624
Miller SL (1953) A production of amino acids under possible primitive earth conditions. Science 117:528–529
Munegumi T (2014) Chemical evolution of simple amino acids to asparagine under discharge onto primitive hydrosphere: simulation experiments using contact glow discharge. Bull Chem Soc Jpn 87:1208–1215
Munegumi T, Suzuki N, Tanikawa N, Harada K (1992) Polypeptide formation from asparagine-containing dipeptides in aqueous solution upon heating. Chem Lett 21:1679–1682
Munegumi T, Tanikawa N, Mita H, Harada K (1994) Peptide formation by heating an aqueous solution containing asparagine. Viva Origino 22:111–125
Nagayama M, Takaoka O, Inomata K, Yamagata Y (1990) Diketopiperazine-mediated peptide formation in aqueous solution. Orig Life Evol Biosph 20:249–257
Navarro-González R, Molina MJ, Molina LT (1998) The chemistry of Archean volcanic lightning, 121–141. In "The role of radiation in the origins of life and evolution of life," Ed. by Akobayashi M, Fujii N, Navarro-González R, Kyoto University Press
Oro J, Guidry CL (1961) Direct synthesis of polypeptides: I. Polycondensation of glycine in aqueous ammonia. Arch Biochem Biophys 93:166–171
Radkiewicz JL, Zipse H, Clarke S, Houk KN (1996) Accelerated racemization of aspartic acid and asparagine residues via succinimide intermediates: an ab initio theoretical exploration of the mechanism. J Am Chem Soc 118:9148–9155
Radkiewicz JL, Zipse H, Clarke S, Houk KN (2001) Neighboring side chain effects on asparaginyl and aspartyl degradation: an ab initio study of the relationship between peptide conformation and backbone NH acidity. J Am Chem Soc 123:3499–3506
Rodriguez-Garcia M, Surman AL, Cooper GJT, Suárez-Marina I, Hosni Z, Lee MP, Cronin L (2015) Formation of oligopeptides in high yield under simple programmable conditions. Nat Commun 6:8385. doi:10.1038/ncomms9385
Saetia S, Liedl K, Eder AH, Rode BM (1993) Evaporation cycle experiments—a simulation of salt-induced peptide synthesis under possible prebiotic conditions. Org Life Evol Biosph 23:167–176
Shimoyama A, Ogasawara R (2002) Dipeptides and diketopiperazines in the Yamato-791198 and Murchison carbonaceous chondrites. Orig Life Evol Biosph 32:165–179
Snyder LE, Lovas FJ, Hollis JM, Friedel DN, Jewell PR, Remijan A (2005) A rigorous attempt to verify interstellar glycine. Astro Phys J 619:914–930
Striegel AM, Yau WW, Kirkland JJ, Bly DD (2009a) Modern size-exclusion liquid chromatography 2nd Ed. John Wiley & Sons, Inc, USA, p 294
Striegel AM, Yau WW, Kirkland JJ, Bly DD (2009b) Modern size-exclusion liquid chromatography 2nd Ed. John Wiley & Sons, Inc, USA, pp 292–321
Sueoka N (1961) Correlation between base composition of deoxyribonucleic acid and amino acid composition of protein. Proc Natl Acad Sci U S A 47:1141–1149
Takaoka O, Yamagata Y, Inomata K (1991) Diketopiperazine-mediated peptide formation in aqueous solution II. Catalytic effect of phosphate. Orig Life Evol Biosph 21:113–118
Vegotsky A, Harada K, Fox SW (1958) The characterization of polyaspartic acid and some related compounds. J Am Chem Soc 80:3361–3366
Woese CR (1973) Evolution of the genetic code. Naturwissenschaften 60:447–459