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THERMODYNAMICS OF ENZYMIC SYNTHESIS OF SOLID-PHASE PEPTIDES

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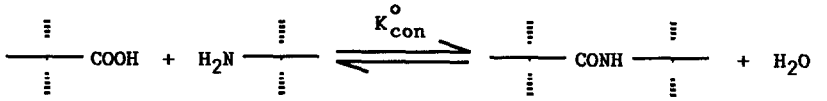
ABSTRACT The Gibbs free energy changes of the individual and net synthetic equilibrium of solid-phase tert-butyloxycarbonyl-Phe-Gly p-substituted anilides are calculated from the HPLC analysis data for the equilibrium concentrations A linear free energy relationship is observed for the net synthetic equilibrium and precipitation equilibrium, suggesting that the latter provides the driving force for the chymotryptic synthesis of these insoluble peptides This conclusion is strongly supported by a linear correlation between the synthetic yield and the square root of the product solubility

Synthesis involving precipitation of the product(s) during the reaction is a method of choice in enzymic peptide synthesis since it avoids the laborious downstream processes[1,2] The thermodynamics of such reactions is not well studied or is limited to simple application of the of law of mass action[3-5] Actually, the formation of a solid phase is a separate favourable equilibrium that can affect the direction of the net reaction[6] Here we report the effect of the changes of the nucleophile structure on the individual and net synthetic equilibrium during the chymotryptic synthesis of solid-phase tert-butyloxycarbonyl-Phe-Gly p-substituted anilides under physiological

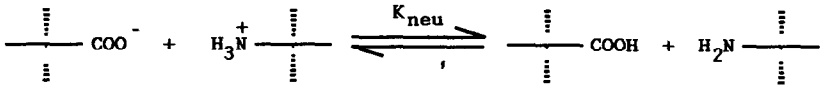
conditions (pH 7 0)

In neutral aqueous solutions the peptide bond formation



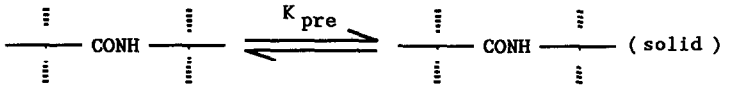
1

is coupled with the neutralization reaction[7]



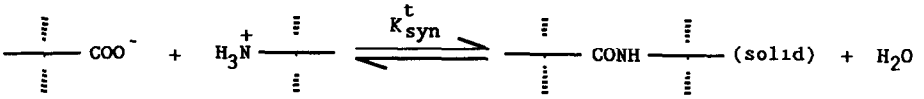
2

A precipitation of the peptide after the condensation reaction 1. suggests its coupling with the precipitation equilibrium



3

The sum of these three equilibrium is the net equilibrium



4

The meaning of the equilibrium constants and the corresponding free energy changes follow from the Scheme 1 -4. Furthermore, according to the law of mass action

$$K^{o}_{con} = \frac{K^{o}_{syn}}{K_{neu}} = \frac{a_{+CONH+}}{a_{+COOH}\, a_{H_2N+}} = \frac{[+CONH+]_e}{[+COOH]_e\,[H_2N+]_e} \qquad 5$$

where K^{o}_{syn} is the pH-independent equilibrium constant of the net reaction of the condensation 1 and neutralization 2., and a - the activity equal to the equilibrium concentration in diluted solutions

$$K_{neu} = \frac{a_{+COOH}\, a_{H_2N+}}{a_{+COO^-}\, a_{H_3\overset{+}{N}+}} = \frac{K_a^{H_3\overset{+}{N}+}}{K_a^{+COOH}} = 10^{(pK_a^{+COOH} - pK_a^{H_3\overset{+}{N}+})} \qquad 6$$

where K_a are the acid dissociation constants,

$$K_{pre} = \frac{a_{+CONH+(s)}}{a_{+CONH+}} = \frac{1}{[+CONH+]_e} = \frac{1}{K_s} \qquad 7$$

where K_s is the solubility equilibrium constant and $a_{+CONH+(s)}=1$ for the homogeneous solid-phase peptide,

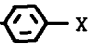
$$K^t_{syn} = \frac{a_{+CONH+(s)}}{a_{+COO^-}\ a_{H_3\overset{+}{N}+}} = \frac{K_{pre}\,[+CONH+]_e}{[+COO^-]_e\ [H_3\overset{+}{N}+]_e} \qquad 8$$

and

$$K^t_{syn} = K^o_{con}\,K_{neu}\,K_{pre} = K^o_{syn}\,K_{pre} \qquad 9$$

$$\Delta G^t_{syn} = \Delta G^o_{con} + \Delta G_{neu} + \Delta G_{pre} = \Delta G^o_{syn} + \Delta G_{pre} \qquad 10$$

The rate-limiting step in the net synthetic reaction is the condensation step 1 To reduce the time spent attaining equilibrium this step needs catalysis Taking into consideration the electrophile structure, we used chymotrypsin in the case of synthesis of the insoluble Boc-Phe-Gly-NHPh(pX) from Boc-Phe-OH and H-Gly-NHPh(pX) The HPLC analyses of the equilibrium reaction mixtures provide data for the calculation of the equilibrium constants, summarized in Table 1

Table 1 Equilibrium Parameters and Synthetic Yield of the Chymotryptic Synthesis of Boc-Phe-Gly-NH-  X , pH=7 , μ=1 , 25°C

X	$10^2K^o_{syn}$ M^{-1}	$10^{-4}K^o_{con}$ M^{-1}	pK_a [a,b]	10^6K_{neu}	10^6K_s M	Yield %
Ac	9 23	1 08	8 56	8 51	18 90	27 50
OMe	10 25	1 15	8 54	8 91	15 70	37 40
H	9 40	1 03	8 53	9 12	8 90	50 30
Cl	10 00	1 07	8 52	9 33	7 20	56 90
NO_2	9 20	0 94	8 50	9 70	4 20	65 70

a) The pK_a-values of H-Gly-NHPh(pX) are measured at 25°C and μ=1 (1M KCl)

b) The pK_a-value of Boc-Phe-OH is 3 49 (measured at 25°C and μ=1 (1M KCl))

Using the relationship

$$\Delta G = - RT\ln K \qquad (11)$$

the Gibbs free energy changes ΔG corresponding to the different individual steps of the peptide synthetic reaction are calculated and summarized in **Table 2.**

Table 2. Gibbs Free Energy Changes of the Individual Steps of the Chymotryptic Synthesis of Boc-Phe-Gly-NH-C$_6$H$_4$-X, pH=7, 25°C

pX	ΔG^{o}_{con}	ΔG_{neu}	ΔG^{o}_{syn}	ΔG_{pre}	ΔG^{t}_{syn}
			kcal mol^{-1}		
Ac	-5 50	6 91	1 41	-6 44	-5 03
OMe	-5 54	6 89	1 35	-6 55	-5 20
H	-5 47	6 87	1 40	-6 89	-5 49
Cl	-5 49	6 86	1 37	-7 01	-5 64
NO_2	-5 42	6 83	1 41	-7 33	-5 92

When the free energy change of the net synthetic reaction 4., ΔG^{t}_{syn}, is plotted against the free energy change of the precipitation reaction 3., a straight line is observed **(Fig.1)** This linear free-energy relationship suggests that the factors controlling the precipitation equilibrium dominate the net synthetic equilibrium 4 as well This conclusion is strongly supported by the effect of the nucleophile structure on the individual free energy changes of the condensation reaction 1 , ΔG^{o}_{con}, and the neutralization reaction 2 , ΔG_{neu} **(Table 2)**

The ΔG^{o}_{con} and ΔG_{neu} -values are practically insensitive to electronic and steric effects of the p-substituents in the anilide moiety ΔG^{o}_{con}'s vary around 5 5 kcal/mole **(Table 2)** They differ from that (5 9 kcal/mole) obtained for acetyl-Phe-Gly-NH_2 by Fersht[8] suggesting a noticeable effect of the nature of C- and N- blocking groups This is not the case with the p-substitution in the anilide moiety due to the remoteness of this modification from the reaction center For the same reason, the substituent effect on the ΔG_{neu} is negligible

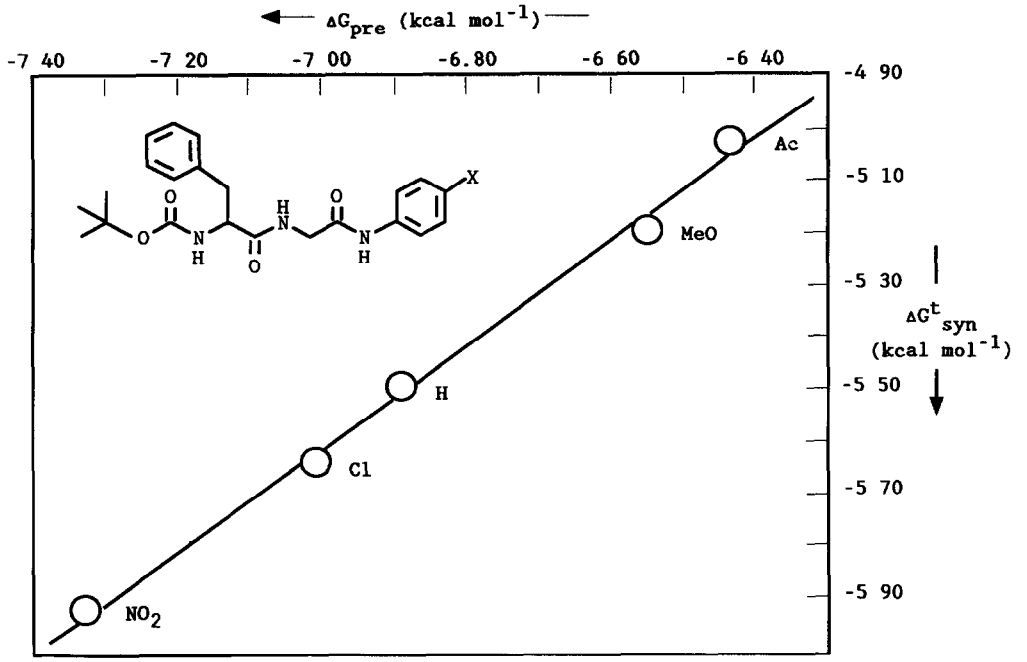


FIG. 1

The free-energy changes ΔG^{o}_{con} and ΔG_{neu} are opposite and their sum ΔG^{o}_{syn} is positive suggesting that the coupling of the condensation and neutralisation reaction 1 and 2 is a net endergonic process The net synthetic reaction 4 can then be exergonic, resulting in the net formation of solid phase peptide, if the free - energy change for the precipitation reaction is sufficiently exergonic to overcome the unfavourable free-energy change for the coupled neutralization 2 and condensation reaction 1 Actually, as can be seen from **Table 2** this is exactly the case Therefore, the three reaction 1 , 2 , and 3 are coupled and the precipitation reaction provides the driving force for the coupled neutralization and condensation reactions

At equal initial concentrations of the electrophile, $+COO^{-}$, and nucleophile, $H_3N^{+}+$, in the net reaction 4 , the following expression for the synthetic yield, Y, of the peptide +CONH+ can be derived using equation **8**

$$Y(\%) = \left\{\frac{\{[+COOH] + [+COO^-]\}_e}{\{[+COOH] + [+COO^-]\}_o}\right\} 100 = 100\left[1 - \frac{\left[\frac{[+CONH+]_e\left(1 + \frac{[H^+]}{K_a^{+COOH}}\right)\left(1 + \frac{K_a^{H_3\overset{+}{N}+}}{[H^+]}\right)}{K^o_{syn}}\right]^{1/2}}{\{[+COOH] + [+COO^-]\}_o}\right] \quad 12$$

Since K^o_{syn} is insensitive to the nature of the p-substituents (**Table 1**), this equation predicts a linear correlation between the synthetic yield and the square root of the solubility, $(+CONH+)_e = 1/K_{pre}$, of the product This is observed (**Fig 2**) supporting once more the domination of the precipitation equilibrium in the thermodynamics of the enzymic synthesis of insoluble peptides

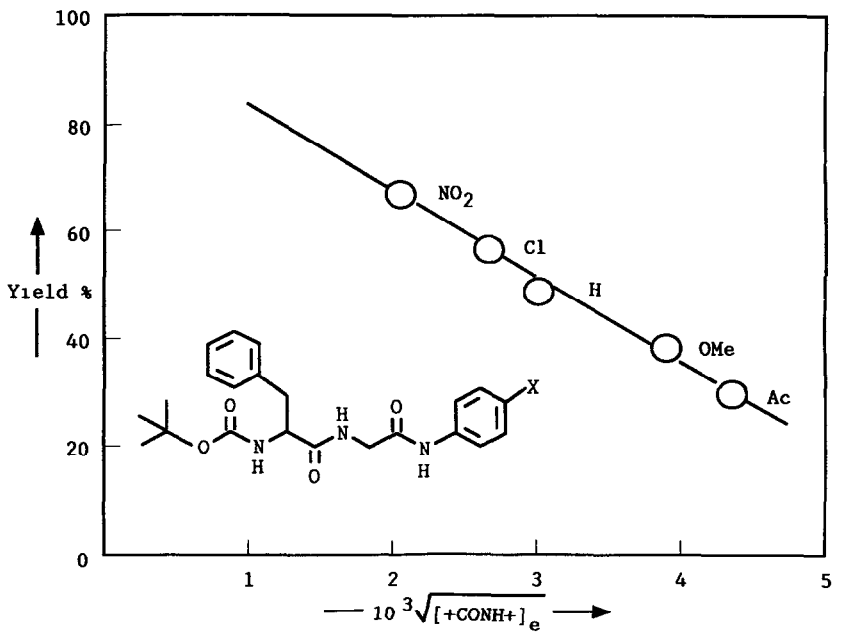


FIG 2

Known values for the free-energy change of the individual equilibrium (**Table 2**)

allow to draw the reaction profile diagram (Fig 3) It represents graphically that the precipitation equilibrium 3 controls the position of the net equilibrium 4 being more exergonic than the couple reactions 1 and 2 are endergonic

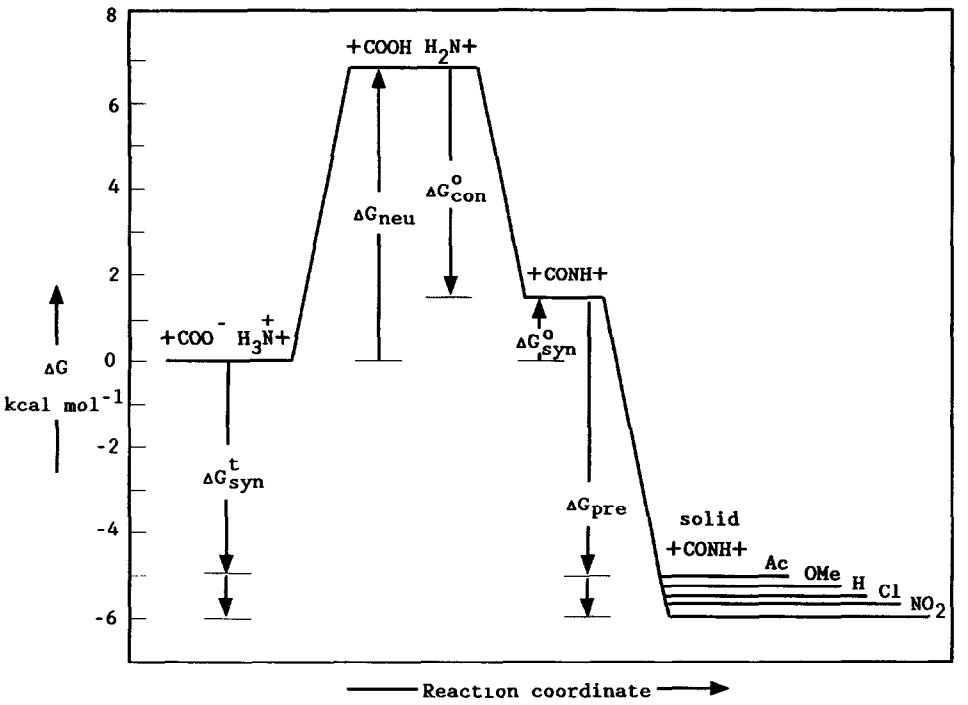


FIG 3.

EXPERIMENTAL

Enzyme Bovine pancreatic α-chymotrypsin was obtained from Sigma and used without further purification

Substrates Boc-Phe-OH was a product of Riedel de Haen H-Gly p-substituted anilides were obtained from the corresponding Z- or Boc-derivatives, by the method described by

Kasafirek[9] for H-Gly p-nitroanilide Boc-Phe-Gly p-substituted anilides were synthesized both by a chemical method (method A) and by thermodynamically controlled chymotrypsin-catalyzed coupling of Boc-Phe-OH and H-Gly p-substituted anilides (method B)

Method A Boc-Phe-OH (530 mg, 2 mmol), H-Gly p-substituted anilide (2 mmol), 1-hydroxybenzotriazole (105 mg, 3 mmol) and 4-methylmorpholine (220 μl, 2 mmol) were dissolved in 40 ml anhydrous tetrahydrofuran Dicyclohexylcarbodiimide (112 mg, 2 mmol) was then added to the stirred solution After 3 hours the precipitate was filtered off and the filtrate evaporated in vacuo The residue was dissolved in ethyl acetate (75 ml) and the solution extracted with 0 5 N Na_2CO_3 (2 x 20 ml), water (2 x 30 ml), 10% solution of citric acid (2 x 20 ml), water (2 x 30 ml) and saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated to dryness in vacuo The residue was recrystallized from ethyl acetate/n-hexane

Method B Boc-Phe-OH (0 04 mmol) and H-Gly p-substituted anilide (0 04 mmol) were dissolved in 2 ml of 0 43 M phosphate buffer pH 7 0, μ=1 and reaction was initiated by 2 5 mg of α-chymotrypsin Every 24 hours the addition of this portion of the enzyme was repeated until no change of the reagents concentrations was observed (usually several days) The precipitated product was collected by filtration and dried under vacuo

The physical constants, elemental analyses, yields and ^1H NMR data of the obtained Boc-Phe-Gly p-substituted anilides are given below

Boc-Phe-Gly-NH—⟨⟩—**Ac** yield (method A/B) 75%/27%, mp 195-198 °C, $[\alpha]_D$ -8° (c 0 5, DMF), ^1H NMR (DMSO-d_6, 200 MHz) δ 10 22 (s, 1H, NH-anilide), 8 39 (t, J = 5 6 Hz, 1H, NH-Gly), 7 94 (d, J = 8 7 Hz, 2H), 7 74 (d, J = 8 8 Hz, 2H), 7 23 (m, 5H), 7 05 (d, J = 8 2 Hz, 1H, NH-Phe), 4 21 (m, 1H, αCH), 3 95 (d, J = 5 6 Hz, 2H, αCH_2), 3 04 (m, 1H, βCH_2), 2 76 (m, 1H, βCH_2), 2 51 (s, 3H), 1 29 & 1 21 (s, 9H, *cis & trans*), Anal calc for $C_{24}H_{29}N_3O_5$ C 65 59, H 6 65, N 9 56, Found C 65 50, H 6 47, N 9 40

Boc-Phe-Gly-NH—⟨⟩—**OMe** yield (method A/B) 85%/37%, mp 171-173 °C, $[\alpha]_D$ +0 3° (c 0 5, DMF), ^1H NMR (DMSO-d_6, 200 MHz) δ 9 68 (s, 1H, NH-anilide), 8 33 (t, J = 5 6 Hz, 1H, NH-Gly), 7 51 (d, J = 8 9 Hz, 2H), 7 23 (m, 5H), 7 05 (d, J = 8 2 Hz, 1H, NH-Phe), 6 88 (d, J = 9 0 Hz, 2H), 4 20 (m, 1H, αCH), 3 85 (d, J = 5 3 Hz, 2H, αCH_2), 3 72 (s, 3H), 3 03 (m, 1H, βCH_2), 2 75 (m, 1H, βCH_2), 1 29 & 1 21 (s, 9H, *cis & trans*), Anal calc for

$C_{23}H_{29}N_3O_5$ C 64 62, H 6 13, N 9 83, Found C 64 61, H 6 28, N 9 72

Boc-Phe-Gly-NH—⟨⟩—**H** yield (method A/B) 80%/50%, mp 178-180 °C, $[\alpha]_D$ -15° (c 0 5, DMF), ^1H NMR (DMSO-d_6, 200 MHz) δ 9 83 (s, 1H, NH-anilide), 8 35 (t, J = 5 5 Hz, 1H, NH-Gly), 7 27 (m, 5H), 7 07 (d, J = 7 0 Hz, 1H, NH-Phe), 4 21 (m, 1H, αCH), 3 91 (d, J = 5 6 Hz, 2H, αCH_2), 3 03 (m, 1H, βCH_2), 2 76 (m, 1H, βCH_2), 1 29 & 1 21 (s, 9H, *cis & trans*), Anal calc for $C_{22}H_{27}N_3O_4$, C 66 48, H 6 59, N 10 56, Found C 66 32, H 6 38, N 10 50

Boc-Phe-Gly-NH—⟨⟩—**Cl** yield (method A/B) 82%/57%, mp 146-148 °C, $[\alpha]_D$ -0 2° (c 0 5, DMF), ^1H NMR (DMSO-d_6, 200 MHz) δ 10 00 (s, 1H, NH-anilide), 8 36 (t, J = 5 6 Hz, 1H, NH-Gly), 7 64 (d, J = 8 8 Hz, 2H), 7 37 (d, J = 8 8 Hz, 2H), 7 24 (m, 5H), 7 05 (d, J = 8 2 Hz, 1H, NH-Phe), 4 21 (m, 1H, αCH), 3 91 (d, J = 5 6 Hz, 2H, αCH_2), 3 03 (m, 1H, βCH_2), 2 75 (m, 1H, βCH_2), 1 29 & 1 21 (s, 9H, *cis & trans*), Anal calc for $C_{22}H_{26}ClN_3O_4$ C 61 18, H 6 07, Cl 8 21, N 9 73, , Found C 61 27, H 5 95, Cl 7 91, N 9 67

Boc-Phe-Gly-NH—⟨⟩—**NO_2** yield (method A/B) 91%/66%, mp 170-172 °C, $[\alpha]_D$ -0 1° (c 0 5, DMF), ^1H NMR (DMSO-d_6, 200 MHz) δ 10 54 (s, 1H, NH-anilide), 8 41 (t, J = 5 5 Hz, 1H, NH-Gly), 8 24 (d, J = 10 2 Hz, 2H), 7 86 (d, J = 10 3 Hz, 2H), 7 25 (m, 5H), 7 04 (d, J = 8 4 Hz, 1H, NH-Phe),), 4 23 (m, 1H, αCH), 3 98 (d, J = 5 4 Hz, 2H, αCH_2), 3 05 (m, 1H, βCH_2), 2 75 (m, 1H, βCH_2), 1 29 & 1 21 (s, 9H, *cis & trans*), Anal calc for $C_{22}H_{26}N_4O_6$ C 64 62, H 6 13, N 9 83, Found C 64 61, H 6 28, N 9 72

HPLC analyses They were performed using WATERS 510 solvent delivery system, Radial-PAK™ RP μ-Bondapak C_{18} cartridge 8x10, tunable absorbance detector WATERS 484, automated gradient controller, data module WATERS 745 and isocratic elution with MeOH/H_2O/TFA (65 35 0 1 v/v %), flow rate 1 5 ml/min

Aliquots (20 μl) were withdrawn from the reaction mixture, filtered off, diluted with 1 ml eluent buffer and then 10 μl aliquots were subjected to analysis 10 μl aliquots without diluting are used for determination of the product solubility

pK_a determination were performed potentiometrycally using Radiometer pH-stat assembly 0 005 m Boc-Phe-OH or H-Gly p-substituted anilides hydrohalogenides in 1 M KCl (μ=1) was titrated with 0 001 M KOH

Thermodynamic studies. The equilibrium of the synthetic reaction 4. was reached by **Method B** When no change of the reagents concentrations was observed after addition of 2 5 mg of α-chymotrypsin, they were considered as equilibrium concentrations Then K^{o}_{syn} at pH 7 0 was calculated using the expression for pH-dependent equilibrium constant $K^{o'}_{syn}$ of the net reaction of condensatoin 1 and neutralization 2.

$$K^{o'}_{syn} = \frac{[+CONH+]_e}{\{[+COOH] + [+COO^-]\}_e\{[H_3\overset{+}{N}+] + [H_2N+]\}_e} = \frac{K^{o}_{syn}}{\left(1 + \frac{10^{-7}}{K_a^{+COOH}}\right)\left(1 + \frac{K_a^{H_3\overset{+}{N}+}}{10^{-7}}\right)} \quad 13$$

Moreover, after separate determination of K_{neu} according to eq 6, the equilibrium constant of the condensation reaction 1 K^{o}_{con} was calculated using equation 5 The equilibrium constant of the precipitation reaction 3 K_{pre} is reciprocal to the peptide product solubility $[+CONH+]_e$ (eq 7)

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