Syntheses of Eisenin and Its Amide by the *N*-Carboxy α-Amino Acid Anhydride Method

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Received July 13, 1973

Eisenin (L-pyroglutamyl-L-glutaminyl-L-alanine) and its amide derivative were synthesized in high yields by a new method using the controlled reaction of *N*-carboxy α-amino acid anhydride (NCA) in the heterogeneous reaction system acetonitrile–water. A tripeptide precursor, γ-methyl-L-glutamyl-γ-methyl-L-glutamyl-L-alanine, and the amide were successfully prepared by two successive reactions of γ-methyl L-glutamate NCA with L-alanine and the amide. The tripeptides were converted into eisenin and the amide through cyclization of the N-terminal γ-methyl ester and simultaneous amidation of the internal L-glutamic acid residue by the action of ammonia.

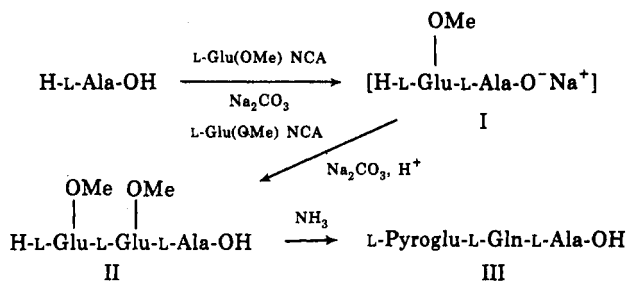
Eisenin is a naturally occurring tripeptide isolated from *Eisenia bicyclis* (Kjellm.) Stetchell by Oohira.[1] The primary structure of the peptide was determined as L-pyroglutamyl-L-glutaminyl-L-alanine.[2] Recently, a peptide with biological activity having an N-terminal pyroglutamyl residue was found. The peptide was named thyroid-stimulating hormone releasing factor (TRF) and has the sequence L-pyroglutamyl-L-histidyl-L-prolinamide.[3] It is of interest that the N-terminal amino acid and the chain length of the newly obtained TRF resemble those of eisenin. The synthesis of TRF has been carried out by conventional[4] and solid-state[5] methods for peptide synthesis and recently by a modified solid-state method involving the simplest strategy.[6]

This paper describes the synthesis of eisenin[7] and its amide derivative by a simple procedure using the controlled reaction of *N*-carboxy α-amino acid anhydride (NCA) in the heterogeneous system acetonitrile (ACN)–water.[8-10]

Synthesis of Eisenin. The NCA method of peptide synthesis is the simplest procedure for the preparation of free peptides because it involves direct acylation of an amino acid or a peptide by the NCA.[8,11-13] As the reaction of the NCA proceeds almost quantitatively at optimal conditions,[8,11,14] small peptides can be prepared by successive addition of NCA's to the reaction system without isolation and purification of intermediate peptides.[8,15]

In the synthesis of eisenin, a synthetic scheme using two reaction steps, first, the preparation of a tripeptide precursor of eisenin, γ-methyl-L-glutamyl-γ-methyl-L-glutamyl-L-alanine, by the NCA method, and second, the treatment of the precursor with ammonia, was considered as the simplest route to eisenin. The latter treatment could convert the tripeptide precursor into eisenin through the cyclization[16] of the N-terminal and the simultaneous amidation[17] of the internal γ-methyl L-glutamyl residue.

The eisenin precursor (II) was prepared by two successive reactions of γ-methyl L-glutamate NCA with L-alanine. The intermediate dipeptide, γ-methyl L-glutamyl-L-alanine (I), as a sodium salt resulting from the initial reaction of the NCA was not isolated, but thin layer chromatography revealed no appreciable by-products. After the second reaction of the NCA with the crude dipeptide, the tripeptide II was isolated from the reaction system, purified by recrystallization from aqueous methanol (86% yield), and dissolved in methanol saturated by ammonia. Subsequently, the product III was isolated and purified by the method reported by Oohira[1] (89% yield).



The synthetic product (III) was demonstrated to be identical with naturally occurring eisenin, kindly supplied by Dr. Oohira, by measurement of physical properties including ir and nmr spectra. This shows that cyclization and amidation of the γ-methyl ester of the glutamyl residues was achieved at the same time expected.

Eisenin may be synthesized by the NCA method through an alternative simplified scheme that involves two successive reactions of L-glutamine NCA[18] with L-alanine followed by heating the aqueous solution of the product. However, L-glutamine NCA is not easily obtained in high yield compared with those NCA's having nonpolar and protected polar side chains, as is shown by a reported synthesis[18] involving treatment of the benzyloxycarbonyl derivative of L-glutamine with PBr_3 followed by column chromatography. Thus the synthetic scheme adopted in this study has a practical merit in obtaining L-glutaminyl peptide in high yield because of very easy preparation of γ-methyl L-glutamate NCA from the amino acid by phosgenation.[19]

Synthesis of Eiseninamide. Eiseninamide was synthesized by the same procedure as that for eisenin except for the use of L-alaninamide. Prior to the synthesis, it was demonstrated that there is no hydrolysis of the amide group during reaction with NCA in the heterogeneous system.[20] For this purpose, NCA's were treated with L-glutamine and L-asparagine, which have the amide group in the side chains, at optimal conditions for the NCA method in this system. Subsequently the aqueous solution of the heterogeneous system was analyzed by paper chromatography. The chromatogram contained a single spot of the dipeptide with C-terminal glutamine and asparagine different from spots of dipeptides containing C-terminal glutamic acid and aspartic acid and the amino acids com-

Table I
Results of Syntheses of Dipeptides with C-Terminal Glutamine and Asparagine

Registry No.	Dipeptide[a]	Yield, %	[α]D, deg	Calcd, % C	Calcd, % H	Calcd, % N	Found, % C	Found, % H	Found, % N
42854-54-6	H-Val-Gln-OH	80	15.7 (*c* 3.2, 1 *N* HCl)[b]	48.96	7.81	17.13	49.20	7.90	17.05
38062-70-3	H-Leu-Gln-OH	82	13.6 (*c* 2.9, 1 *N* HCl)[c]	50.95	8.16	16.21	50.63	8.26	16.37
39537-24-1	H-Phe-Gln-OH	88	18.7 (*c* 2.4, 1 *N* HCl)[d]	57.32	6.53	14.33	57.95	6.60	14.45
14608-81-2	H-Leu-Asn-OH	82	14.4 (*c* 2.1, 80% MeOH)[e]	48.96	7.81	17.13	48.67	8.01	17.20
39537-20-7	H-Phe-Asn-OH	78	4.9 (*c* 3.1, 1 *N* HCl)	55.90	6.14	15.05	55.87	6.20	15.21

[a] All amino acid residues have the L configuration. [b] 15.8° (*c* 3.08, 1 *N* HCl): Y. Shimonishi, *Bull. Chem. Soc. Jap.*, **37**, 200 (1964). [c] 14.2° (*c* 3.57, 1 *N* HCl); see footnote *b*. [d] 17.3° (*c* 3.42, 1 *N* HCl); see footnote *b*. [e] 15.7° (*c* 5.0, H_2O): A. Miller, A. Neidle, and H. Waelsch, *Arch. Biochem. Biophys.*, **56**, 11 (1955).

ing from the starting material and hydrolysis of the NCA. For example, the system containing the product resulting from the reaction of L-leucine NCA with L-glutamine gave a spot with R_f[21] 0.55 while L-leucyl-L-glutamic acid showed a spot with R_f 0.63. This suggests that no side reactions such as hydrolysis of the amide group occur during the reaction carried out in the heterogeneous system and is in accord with results by methods using aqueous systems.[13,20] Results of the syntheses are shown in Table I.

On the basis of these results, eiseninamide was synthesized by the NCA method in the heterogeneous system. L-Alaninamide, which was prepared by treatment of the free benzyl ester of L-alanine with ammonia, was dissolved in the heterogeneous system of ACN–water which had been cooled to −10° in order to avoid hydrolysis of the amide. A solution of γ-methyl L-glutamate NCA in cooled ACN was added to the system and allowed to react. The resulting dipeptide amide was not isolated but was again treated with NCA as before.

When an amino acid amide is allowed to react in a heterogeneous system, attention must be paid to the solubility of the amide in the ACN layer. Since an appreciable quantity of L-alaninamide appears to be present in the ACN layer as well as in the aqueous one, higher temperatures may result in undesirable polymerization of the NCA initiated by the amide in the ACN layer. Indeed, the controlled reaction of γ-methyl L-glutamate NCA with L-alaninamide carried out at −7° gave not only the dipeptide amide but a small portion of by-products, which were identified as the tripeptide amide, γ-methyl-L-glutamyl-γ-methyl-L-glutamyl-L-alaninamide and the higher tetrapeptide amide by tlc. However, this is not true at −10°. Therefore the temperature of the reaction with amide should be carefully kept below −10°.

After the second reaction of the NCA and nautralization of the aqueous solution of the heterogeneous system with dilute sulfuric acid, the tripeptide amide was carefully isolated and purified by recrystallization from methanol (87% yield from L-alaninamide). The product was treated with anhydrous ammonia to yield eiseninamide (91% yield).

Experimental Section

γ-Methyl L-Glutamate NCA.[19] Into a suspension of 20 g of γ-methyl L-glutamate in 400 ml of tetrahydrofuran was bubbled dry phosgene at 40° with stirring. After the amino acid derivative had dissolved to give a clear solution, the solvent was removed at reduced pressure to yield an oil. The oily product was crystallized by addition of 200 ml of *n*-hexane followed by cooling. The crystals of NCA were twice recrystallized from ethyl acetate to give 20.5 g (88%) of pure NCA, mp 100°, [α]D −20.2° (*c* 1.8, ACN). *Anal.* Calcd for $C_7H_9NO_5$: C, 44.92; H, 4.85; N, 7.48. Found: C, 44.76; H, 4.83; N, 7.50.

γ-Methyl-L-glutamyl-γ-methyl-L-glutamyl-L-alanine. To a solution of 0.89 g (0.01 mol) of L-alanine and 1.06 g (0.01 mol) of anhydrous sodium carbonate in 50 ml of 0.2 *N* sodium hydroxide was added 40 ml of ACN to yield a heterogeneous system, which was cooled to −10°. A solution of 2.07 g (0.011 mol) of γ-methyl L-glutamate NCA in 20 ml of ACN was added to the heterogeneous system and allowed to react for 2 hr at −10° with magnetic stirring. After 2 hr, the system was taken into a separating funnel. The upper layer of the heterogeneous system was removed and the aqueous layer was washed with 50 ml of ethyl acetate to remove unreacted NCA. The resulting aqueous solution was analyzed by tlc. Chromatography on silica gel plate developing 1-butanol–acetic acid–water (4:1:2) showed a single spot with R_f 0.11. Then the aqueous solution was diluted to 60 ml of water and 40 ml of ACN was added. The heterogeneous system was cooled again to −10°. After addition of 2.07 g of γ-methyl L-glutamate NCA in 20 ml of ACN, the system was stirred for 2 hr at −10°. Removal of the ACN layer and washing the aqueous solution by ethyl acetate was followed by neutralization of the aqueous solution with 6 *N* sulfuric acid. The sodium sulfate resulting from the neutralization was precipitated by addition of 200 ml of ethanol and removed by filtration. The filtrate was concentrated at reduced pressure to yield a syrup, which was crystallized by addition of 100 ml of ethanol and 100 ml of diethyl ether. The product was recrystallized from 80% methanol to give 3.2 g (86%) of crystalline γ-methyl-L-glutamyl-γ-methyl-L-glutamyl-L-alanine, mp 97°, [α]D −21.1° (*c* 2.0 water). *Anal.* Calcd for $C_{15}N_{25}N_3O_8 \cdot 4H_2O$: C, 40.26; H, 7.43; N, 9.39. Found: C, 40.41; H, 7.38; N, 9.26.

The tripeptide (5 g) was dissolved in 150 ml of dry methanol saturated with ammonia and the solution was allowed to stand for 3 days at room temperature. Then the solvent was removed at reduced pressure at 25° to give a residue, which was dissolved in 100 ml of methanol, and the solution was concentrated again. The resulting product was recrystallized from aqueous methanol, 3.27 g (89%), mp 223–227° dec, [α]D −52.8° (*c* 6.5, water). *Anal.* Calcd for $C_{13}H_{20}N_4O_6$: C, 47.55; H, 6.14; N, 17.07. Found: C, 47.68; H, 6.23; N, 17.01.

The sample of eisenin which had been isolated by Dr. Oohira gave mp 224–226° dec and [α]D −53.5° (*c* 6.5, water).

General Procedure for the Synthesis of Dipeptides with C-Terminal Glutamine and Asparagine. An aqueous solution of 1.06 g (0.01 (0.01 mol) of sodium carbonate in 50 ml of 0.2 *N* sodium hydroxide was cooled to −5°. To the solution was added 0.01 mol of the amino acid and then 40 ml of ACN was added to form the heterogeneous system. After the addition of a solution of 0.011 mol of NCA in 20 ml of ACN, the reaction was allowed to proceed in a usual manner. Isolation of the dipeptide from the aqueous solution gave a white, crystalline product, which was recrystallized from aqueous ethanol.

Synthesis of Eiseninamide. L-Alaninamide. L-Alanine benzyl ester *p*-toluenesulfonate (17.5 g, 0.05 mol) was dissolved in 50 ml of water and the solution was neutralized carefully to pH 7.5 by addition of 0.1 *N* sodium hydroxide. Then the solution was immediately extracted by one 150-ml portion of diethyl ether and two 75-ml portions of ether. The combined extract was dried overnight with anhydrous sodium sulfate at −20°. The ether solution was concentrated at reduced pressure at 10° to give an oily residue of the free benzyl ester of L-alanine, 7.3 g (82%). The ester was dissolved in 220 ml of methanol saturated with ammonia and the solution was allowed to stand for 3 days at room temperature. Solvent was removed *in vacuo* at 15° to yield an oil, which was crystallized by addition of 50 ml of diisopropyl ether and 100 ml of *n*-hexane. Recrystallization from methanol gave plate crystals of L-alaninamide, 2.4 g (78%), mp 72°, [α]D 6.5° (*c* 2.0, water). *Anal.* Calcd for $C_3H_8N_2O \cdot H_2O$: C, 33.95; H, 9.50; N, 26.40. Found: C, 34.14; H, 9.65; N, 26.68.

γ-Methyl-L-glutamyl-γ-methyl-L-glutamyl-L-alaninamide. To the heterogeneous system of 50 ml of ACN and 50 ml of an aqueous solution of 1.06 g (0.01 mol) of anhydrous sodium carbon-

ate, which had been cooled to −10°, was dissolved 0.88 g (0.01 mol) of L-alaninamide. A solution of 2.07 g of γ-methyl L-glutamate NCA in 20 ml of ACN was added to the system and allowed to react for 2 hr under −10° with stirring. After the reaction, the aqueous layer was washed with 50 ml of ethyl acetate. The resulting aqueous solution was demonstrated to contain only one component which showed a single spot having an R_f of 0.26 on silica gel tlc. Then the solution was diluted to 60 ml by addition of fresh ice and 50 ml of ACN was added. The system was cooled to −10°. After the addition of 2.07 g of γ-methyl L-glutamate NCA and reaction by the same procedure as that for eisenin, the tripeptide amide was isolated from the alcoholic solution free from sodium sulfate resulting from the neutralization, by concentrating *in vacuo* at 20° followed by addition of 50 ml of ethanol and 150 ml of diethyl ether. Recrystallization from 150 ml of methanol gave a crystalline tripeptide amide, 3.3 g (87% yield from L-alaninamide), mp 126–127°, $[\alpha]_D$ −25.8° (*c* 2.0, water). *Anal.* Calcd for $C_{15}H_{26}N_4O_7 \cdot 4H_2O$: C, 40.35; H, 7.68; N, 12.55. Found: C, 40.42; H, 7.62; N, 12.78.

The tripeptide amide (3.74 g, 0.01 mol) was dissolved in 100 ml of dry methanol saturated with ammonia and the solution was allowed to stand for 3 days. The solution was concentrated at 20° to give a residue, which was redissolved in 50 ml of methanol. The solution was concentrated again to give a white residue. The product was recrystallized from water, 3.0 g (91%), mp 251–254° dec, $[\alpha]_D$ −46.0° (*c* 2.0, water). *Anal.* Calcd for $C_{13}H_{21}N_5O_5$: C, 47.70; H, 6.47; N, 21.40. Found: C, 47.86; H, 6.56; N, 21.26.

Acknowledgment. The authors wish to express their gratitude to Dr. T. Oohira, Professor Emeritus of Shinshyu University, for a generous gift of eisenin and his suggestive discussion. This work was supported partly by the Scientific Grant of the Japanese Ministry of Education.

Registry No. γ-Methyl L-glutamate NCA, 1663-47-4; γ-methyl L-glutamate, 1499-55-4; γ-methyl-L-glutamyl-γ-methyl-L-glutamyl-L-alanine, 42854-60-4; L-alanine, 56-41-7; eisenin, 21477-57-6; L-alaninamide, 7324-05-2; L-alanine benzyl ester *p*-toluenesulfonate, 42854-62-6; γ-methyl-L-glutamyl-γ-methyl-L-glutamyl-L-alanine amide, 42854-63-7; eiseninamide, 38357-81-2.

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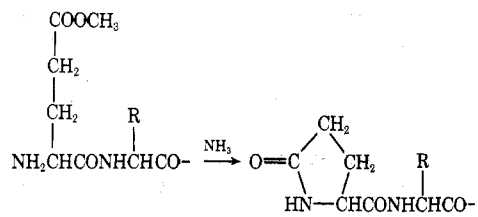

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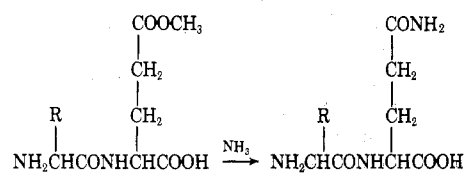

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(20) Though a dipeptide amide has been synthesized by the NCA method in an aqueous system involving the reaction of NCA with an amino acid amide [R. Hirschmann, R. G. Strachan, H. Schwam, E. F. Schoenewaldt, H. Joshua, B. Barkemeyer, D. F. Veber, W. J. Paleveda, Jr., T. A. Jacob, T. E. Beesley, and R. G. Denkewalter, *J. Org. Chem.*, **32**, 3415 (1967)], the possibility that the amide group of the amino acid amide is partially hydrolyzed during the reaction with NCA in the heterogeneous system is present, because the heterogeneous system requires a higher pH (>11) and longer reaction time (2 hr) for optimal condition than the homogeneous one (pH 10.2 and a few minutes).
(21) A solvent system of 1-butanol–acetic acid–water (4:1:2) was used as a developer.