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Resolution of Amino Acids. IX. Studies on the Preparation of β-Hydroxyasparagines and Configuration of Natural Hydroxyasparagine[1—3]

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Crystalline *threo*- and *erythro*-β-hydroxy-L-asparagine were prepared *via* asymmetric hydrolysis of the corresponding hydroxy-L-aspartic acid diamide by leucine aminopeptidase. The isomers of hydroxy-L-asparagine obtained were compared with the natural hydroxyasparagine isolated from human urine with respect to optical rotation and chromatography. From the results it is concluded that the spacial configuration of the natural product is that of *erythro*-β-hydroxy-L-asparagine. Additional studies were also carried out to prepare *threo*- and *erythro*-β-hydroxy-DL-asparagine from the corresponding hydroxy-DL-aspartic acid diamide without the enzyme.

Tominaga and his colleagues isolated 116 mg of a crystalline new amino acid from 200 *l* of human urine of normal male adults.[6] They presumed it to be β-hydroxyasparagine since it had produced β-hydroxyaspartic acid by acid hydrolysis. However, its spacial configuration has not been determined. Recently the four optical isomers of β-hydroxyaspartic acid were synthesized,[1,7] and their spacial configurations were established.[7] If the natural compound from human urine is one of possible four optical isomers of β-hydroxyasparagine, the compound may produce one of the four optical isomers of β-hydroxyaspartic acid by acid hydrolysis. However, it has been reported that the heating of either *threo*- or *erythro*-β-hydroxyaspartic acid in hydrochloric acid resulted in an interconversion of the diastereomers.[8] Therefore, we attempted

1) Part VIII of this series: H. Okai, N. Imamura and N. Izumiya, This Bulletin, **40**, 2154 (1967).

2) Presented at the 21st Annual Meeting of the Chemical Society of Japan, Osaka, April, 1968; Preprints of the Meeting, Vol. 3 (1968) p. 2226.

3) The following abbreviations are used: LAPase, leucine aminopeptidase; Z, benzyloxycarbonyl; Hyasp or Hya, β-hydroxyaspartic acid; Hyasp-diNH_2, β-hydroxyaspartic acid diamide; Hyasn, β-hydroxyasparagine; *t*, *threo*; *e*, *erythro*.

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6) F. Tominaga, C. Hiwaki, T. Maekawa and H. Yoshida, *J. Biochem.*, **53**, 227 (1963).

7) K. Kaneko and H. Katsura, This Bulletin, **36**, 899 (1963).

to determine the spacial configuration of the natural product by the comparison with synthetic *threo*- and *erythro*-β-hydroxy-L-asparagine.

This paper describes the preparation of *threo*- and *erythro*-β-hydroxy-L-asparagine *via* asymmetric hydrolysis of the corresponding hydroxy-L-aspartic acid diamide by LAPase and the comparison of the natural product with the isomers of hydroxy-L-asparagine in respect to optical rotation and column chromatography. Attempts were made to prepare *threo*- and *erythro*-hydroxy-DL-asparagine without using the enzyme.

Several isomers (L-, DL-*threo* and L-, DL-*erythro*) of hydroxyaspartic acid diamide were synthesized in the conventional manner. As an example, the reaction sequence for the synthesis of *erythro*-hydroxy-L-aspartic acid diamide is shown in Fig. 1.

Preparation of *threo*- and *erythro*-hydroxy-L-asparagine was attempted by LAPase treatment on the diamides synthesized. As an example, the two schemes for the preparation of *erythro*-hydroxy-L-asparagine are presented in Fig. 2. Prior to preparation of optical active hydroxyasparagines by LAPase, the rates of hydrolysis were determined to estimate the susceptibility of the diamides for the enzyme, the results being shown in Table 2. It was reported that L-cysteic acid amide was hydrolyzed especially slowly by LAPase among the substrates tested.[9] However, it was observed that the diamides of hydroxyaspartic acid were hydrolyzed more poorly (Table 2). The low susceptibility of these diamides is considered to be due to their β-hydroxy group. It was observed that

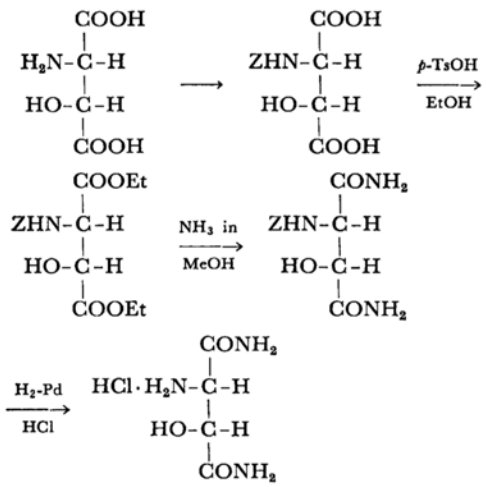

Fig. 1. Synthesis of *erythro*-hydroxy-L-aspartic acid diamide.

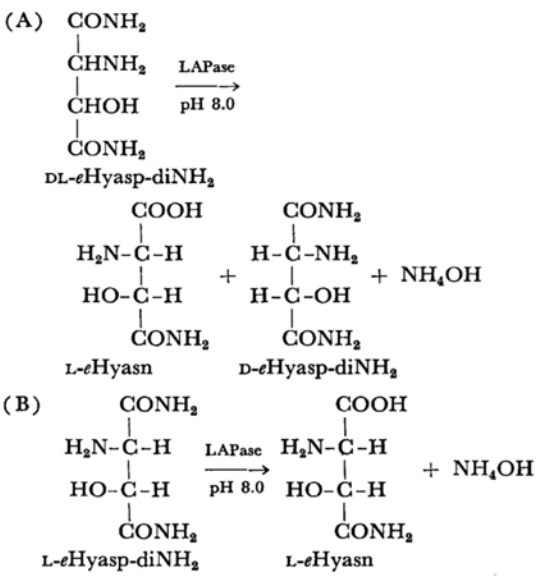

Fig. 2. Two schemes for preparation of *erythro*-hydroxy-L-asparagine.

the susceptibility of the *erythro*-L-diamide was slightly lower than that of the *threo*-L-diamide (Table 2). Thus a very high activity of LAPase was necessary for the effective hydrolysis of the diamides, and the highly purified enzyme preparation[10] was employed in this experiment.

For the determination of the rates of hydrolysis of *threo*- and *erythro*-DL-diamide, the course of hydrolysis in an incubation mixture of the substrate with the enzyme at pH 8.0 was followed by measuring the ammonia evolved. Contrary to expectation, it was observed that the amount of ammonia measured was greater than the theoretical 50% after 15—20 hr. The analysis of the mixture at 15—20 hr incubation by an amino acid analyzer and paper chromatography revealed the presence of hydroxyaspartic acid and other by-products in small amounts besides the major products, hydroxy-L-asparagine and hydroxy-D-aspartic acid diamide. The hydroxyaspartic acid and the by-products might be produced through the spontaneous degradation of the diamide and hydroxyasparagine at pH 8.0. The reason for the ammonia evolution more than the theoretical amount was further clarified by the observation that the diamide at pH 8.0 without the enzyme evolved ammonia gradually as shown in Fig. 5-b.

For the preparation of pure *threo*- and *erythro*-hydroxy-L-asparagine in preparative scale, the corresponding L-diamide was incubated with a large amount of the highly purified enzyme. This

8) T. Wieland, *Helv. Chim. Acta*, **44**, 919 (1961); M. L. Kornguth and H. J. Sallach, *Arch. Biochem. Biophys.*, **104**, 79 (1964).

9) S. Ouchi, A. Tanaka and N. Izumiya, *J. Biochem.*, **46**, 185 (1959).

10) E. L. Smith and D. H. Spackman, *J. Biol. Chem.*, **212**, 271 (1955); E. L. Smith, "Methods in Enzymology," Vol. II, ed. by S. E. Colowick and N. O. Kaplan, Academic Press, New York (1956), p. 88.

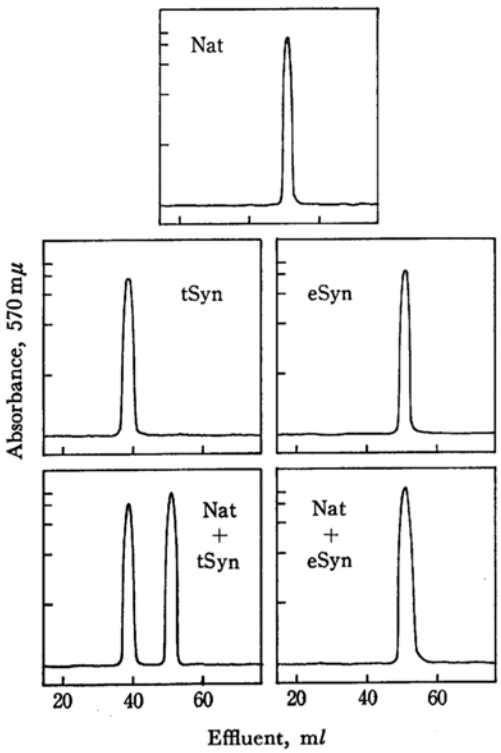

Fig. 3. Elution patterns of several hydroxyasparagine preparations by amino acid analyzer. Nat, the natural product; tSyn, L-tHyasn(synthetic); eSyn, L-eHyasn (synthetic).

Table 1. Specific rotations at 20°C of hydroxyasparagines

Compound	in H_2O[a)]	in 5 N HCl[b)]
L-*t*Hyasn (synthetic)	−29.6°	−28.0°
L-*e*Hyasn (synthetic)	+48.4	+63.0
Hyasn (natural)	+48.0	+61.6

a) *c* 0.5. b) *c* 1.0.

time, it was found that occurrence of the by-products was negligible even after the substrate was hydrolyzed completely. The crystalline *threo*- and *erythro*-hydroxy-L-asparagine were isolated from the incubation mixture in good yields by the use of Dowex 50 and 1 columns.

The synthetic hydroxyasparagines were compared with the natural product isolated from human urine. The values of specific rotation of *erythro*-hydroxy-L-asparagine and the natural product concurred within experimental error as shown in Table 1. A mixture of both the compounds afforded a single peak on co-chromatography by the amino acid analyzer as shown in Fig. 3. These data permit the conclusion that the spacial configuration of the natural product is that of *erythro*-β-hydroxy-L-asparagine. In this connection, it would be of interest to note that *erythro*-β-hydroxy-L-aspartic acid has been obtained by the action of a transaminase on dihydroxyfumaric acid and L-glutamic acid.[11)]

We attempted to prepare hydroxyasparagine through spontaneous degradation of the diamides in acid and alkaline mediums because it had been found that the diamides degraded spontaneously in the buffer solution at pH 8.0. Treatment of the diamides in dilute hydrochloric acid for 24 hr at 38°C afforded a mixture of many products including hydroxyasparagine. The chromatographic pattern by the amino acid analyzer are shown in Fig. 6-B. However, isolation of hydroxyasparagine from the reaction mixture was not attempted. In an alkaline treatment of the diamide for 5 hr at 38°C, the chromatographic patterns of degradation products in *threo*- and *erythro*-DL-diamide were different; *threo*-hydroxyasparagine was produced from the corresponding DL-diamide in considerable amount as shown in Fig. 6-C. The crystalline *threo*-hydroxy-DL-asparagine was isolated from the reaction mixture in a yield of 32%.

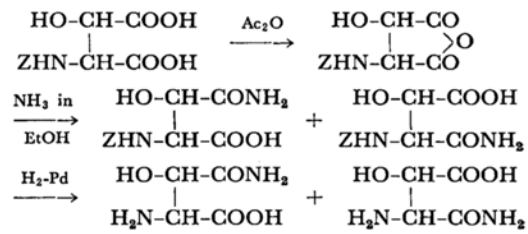

Fig. 4. Scheme for preparation of a mixture of hydroxyasparagine and hydroxyisoasparagine.

We attempted to prepare hydroxyasparagine *via* ammonolysis of benzyloxycarbonyl-hydroxyaspartic acid anhydride as shown in Fig. 4. It should be noted that Ressler and her colleagues prepared pure L-asparagine and L-isoasparagine from benzyloxycarbonyl-L-aspartic acid in a similar reaction sequence as shown in Fig. 4.[12)] The chromatographic patterns of the hydrogenated material are given in Fig. 6-D in which *erythro*-hydroxyasparagine is eluted as a single peak while *threo*-hydroxyasparagine is overlapped with other by-product. The crystalline *erythro*-hydroxy-DL-asparagine was isolated from the hydrogenated material derived from benzyloxycarbonyl-*erythro*-hydroxy-DL-aspartic acid in a yield of 33%. It seems that the preparation of the hydroxy-L-asparagines with the use of LAPase is preferable to that without the enzyme which is described in this paper.

11) H. J. Sallach and T. H. Peterson, *J. Biol. Chem.*, **223**, 629 (1956); H. J. Sallach and M. L. Kornguth, *Biochim. Biophys. Acta*, **34**, 582 (1959).

12) C. Ressler, H. Malodeczky and D. V. Kashelicker, "Biochemical Preparation," Vol. 10, ed. by C. B. Brown, John Wiley & Sons, New York (1963), p. 83.

Experimental

Synthesis of Compounds. *N-Benzyloxycarbonyl-threo-hydroxy-DL-aspartic Acid.* To a solution of DL-*t*Hyasp[1] (1.49 g, 10 mmol) and sodium bicarbonate (3.18 g, 36 mmol) in water (30 ml) was added a solution of benzyloxycarbonyl chloride (2.22 g, 13 mmol) in ether, and the reaction mixture was stirred for 4 hr at room temperature. After the reaction mixture was extracted with ether, the aqueous layer was acidified with 5 N hydrochloric acid and then extracted with ethyl acetate thrice (40 ml each). The extract was dried over sodium sulfate and evaporated *in vacuo*. The residual oil solidified after the addition of petroleum ether. It was recrystallized from ethanol-ether-petroleum ether. Yield, 2.12 g (75%); mp 186—187°C.

Found: C, 50.93; H, 4.74; N, 4.92%. Calcd for $C_{12}H_{13}O_7N$: C, 50.88; H, 4.63; N, 4.95%.

N-Benzyloxycarbonyl-threo-hydroxy-L-aspartic Acid. This was prepared from L-*t*Hyasp[1] as has been described for the preparation of Z-DL-*t*Hyasp. Yield, 86%; mp 106—107°C; $[\alpha]_D^{17}$ −8.0° (*c* 1, DMF).

Found: C, 50.93; H, 4.78; N, 4.83%.

N-Benzyloxycarbonyl-erythro-hydroxy-DL-aspartic Acid. This was prepared from DL-*e*Hyasp[1] as described for the preparation of Z-DL-*t*Hyasp. Yield, 85%; mp 155—156°C.

Found: C, 50.85; H, 4.85; N, 4.86%.

N-Benzyloxycarbonyl-erythro-hydroxy-L-aspartic Acid. This was prepared from L-*e*Hyasp.[1] Yield, 83%; mp 143—144°C; $[\alpha]_D^{17}$ +17.5° (*c* 1, DMF).

Found: C, 50.90; H, 4.86; N, 4.91%.

N-Benzyloxycarbonyl-threo-hydroxy-DL-aspartic Acid Diethyl Ester (Z-DL-tHyasp-diOEt). This compound was prepared according to the procedure of Kato *et al.*[13] for the preparation of benzoyl-DL-serine ethyl ester. A solution of Z-DL-*t*Hyasp (2.83 g, 10 mmol) and *p*-toluenesulfonic acid monohydrate (0.02 g) in a mixture of ethanol (20 ml) and carbon tetrachloride (100 ml) was refluxed for 64 hr, and the water liberated was removed as an azeotropic mixture. The reaction mixture was concentrated *in vacuo*, and the residual oil was dissolved in ethyl acetate (40 ml). The solution was washed with 4% sodium bicarbonate, 2% hydrochloric acid and water, and dried over sodium sulfate. The filtrate was evaporated *in vacuo*, and an oily residue was obtained. Yield, 3.36 g (99%); R_f 0.85.[14]

Z-L-tHyasp-diOEt, Z-DL-eHyasp-diOEt and Z-L-eHyasp-diOEt. These esters were also obtained as oily substance in nearly quantitative yields as described above.

N-Benzyloxycarbonyl-threo-hydroxy-DL-aspartic Acid Diamide. Z-DL-*t*Hyasp-diOEt (1.62 g, 4.7 mmol) was dissolved in methanol (80 ml) previously saturated with dry ammonia at 0°C, and the solution was kept at room temperature for 3 days and then concentrated *in vacuo* to dryness. The residual crystals were collected with the aid of a mixture of methanol and acetone. It was recrystallized from hot methanol. Yield, 1.01 g (77%); mp 209—211°C; R_f 0.66.[14]

Found: C, 51.25; H, 5.44; N, 14.71%. Calcd for $C_{12}H_{14}O_5N_3$: C, 51.42; H, 5.03; N, 14.99%.

N-Benzyloxycarbonyl-threo-hydroxy-L-aspartic Acid Diamide. This was prepared from Z-L-*t*Hyasp-diOEt as desbribed above. Yield, 66%; mp 194—196°C; $[\alpha]_D^{17}$ −25.5° (*c* 1, DMF).

Found: C, 51.15; H, 5.36; N, 14.65%.

N-Benzyloxycarbonyl-erythro-hydroxy-DL-aspartic Acid Diamide. This was prepared from Z-DL-*e*Hyasp-diOEt. Yield, 86%; mp 204—205°C.

Found: C, 51.38; H, 5.00; N, 14.61%.

N-Benzyloxycarbonyl-erythro-hydroxy-L-aspartic Acid Diamide. This was prepared from Z-L-*e*Hyasp-diOEt. Yield, 75%; mp 198—199°C; $[\alpha]_D^{17}$ +42.0° (*c* 0.5, DMF).

Found: C, 51.48; H, 5.48; N, 14.72%.

threo-Hydroxy-DL-aspartic Acid Diamide Hydrochloride. A solution of Z-DL-*t*Hyasp-diNH_2 (1.12 g, 4 mmol) in acetic acid (80 ml) was treated with hydrogen in the presence of palladium black. The catalyst was removed by filtration, and the filtrate was evaporated *in vacuo*. The residual oil was dissolved in N hydrochloric acid (4.2 ml) and the solution was evaporated *in vacuo* to dryness. The residue was recrystallized from methanol-ether. Yield, 0.86 g (93%); mp 190—192°C (decomp.).

Found: C, 26.36; H, 5.40; N; 22.84%. Calcd for $C_4H_9O_3N_3 \cdot HCl$: C, 26.17; H, 5.49; N, 22.90%.

threo-Hydroxy-L-aspartic Acid Diamide Hydrochloride. This was obtained by the hydrogenolysis of Z-L-*t*Hyasp-diNH_2 as described above. Yield, 91%; mp 190—192°C (decomp.); $[\alpha]_D^{17}$ −20.5° (*c* 1, H_2O).

Found: C, 25.96; 5.57; N, 22.82%.

erythro-Hydroxy-DL-aspartic Acid Diamide Hydrochloride. This was obtained by the hydrogenolysis of Z-DL-*e*Hyasp-diNH_2. Yield, 92%; mp 195—196°C (decomp.).

Found: C, 26.07; H, 5.47; N, 22.73%.

erythro-Hydroxy-L-aspartic Acid Diamide Hydrochloride. This was obtained by the hydrogenolysis of Z-L-*e*Hyasp-diNH_2. Yield, 90%; mp 193—194°C (decomp.); $[\alpha]_D^{17}$ +87.0° (*c* 0.5, H_2O).

Found: C, 25.80; H, 5.50; N, 22.86%.

Hydrolysis of Hydroxyaspartic Acid Diamide by LAPase. *Hydrolysis of DL-tHyasp-diNH_2 and DL-eHyasp-diNH_2.* A highly purified LAPase solution of step 6 by Smith was prepared following the method given in literature.[10] The enzyme solution was made to contain 4 mg protein per ml. One milliliter of the enzyme solution was dissolved in 0.4 M Tris buffer (0.5 ml) at pH

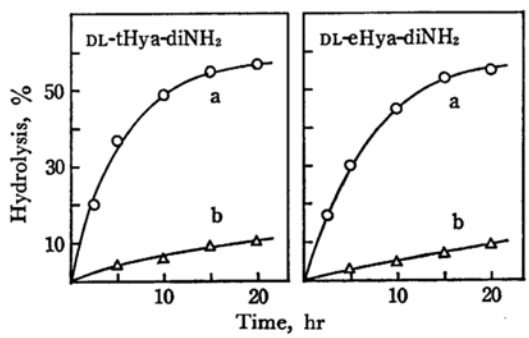

Fig. 5. Time-course of hydrolysis of *threo-* and *erythro*-hydroxy-DL-aspartic acid diamide with (a) without (b) LAPase.

13) T. Kato, S. Makisumi, M. Ohno and N. Izumiya, *Nippon Kagaku Zasshi (J. Chem. Soc. Japan, Pure Chem. Sect.)* **83**, 1151 (1962).

14) The R_f value refers to the thin layer chromatography with Merck silica gel G and to the *n*-butanol-acetic acid-pyridine-water (4:1:1:2, v/v) system.

8.0 and 0.12 M manganese chloride (0.5 ml). The solution was preincubated for 1 hr at 38°C to obtain maximal activation. This was named as an activated enzyme solution. A solution of DL-*t*(or *e*)Hyasp-diNH$_2$·HCl(0.1 mmol) in the activated enzyme solution (0.5 ml) and 0.1 M Tris buffer (1.5 ml) at pH 8.0 was incubated at 38°C for 20 hr. The process of hydrolysis was followed by means of Conway's microdiffusion method[15] to determine ammonia (Fig. 5-a) and by paper chromatography. Analysis by an amino acid analyzer[16] of an aliquot after 20 hr incubation indicated the presence of hydroxyaspartic acid and other by-products as minor products. As a control experiment, a solution of DL-*t* (or *e*) Hyasp-diNH$_2$·HCl (0.1 mmol) in 0.1 M Tris buffer (2 ml) at pH 8.0 without the enzyme was incubated at 38°C and the amount of ammonia was determined. The results are given in Fig. 5-b.

Table 2. Susceptibility of amino acid amides by lapase

Compound	C_1	Relative rate
L-Leu-NH$_2$	63	100
L-Asp-diNH$_2$	1.8	2.8
L-cysteic acid diamide		0.1[a]
L-*t*Hyasp-diNH$_2$	0.0069	0.011
L-*e*Hyasp-diNH$_2$	0.0049	0.0078

a) The datum is cited from literature.[9]

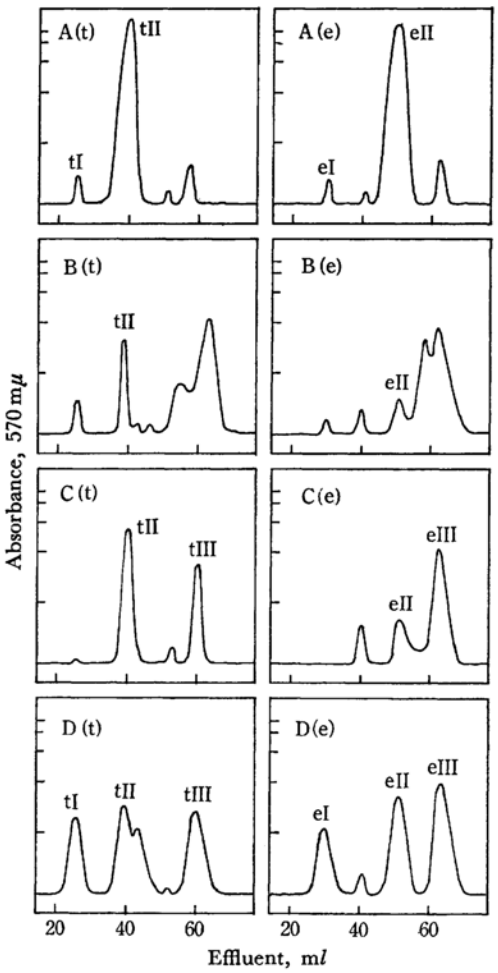

Fig. 6. Elution patterns of mixtures derived from hydroxyaspartic acid diamide and Z-hydroxyaspartic acid. (t), *threo*; (e), *erythro*; A, L-Hyasp-diNH$_2$ with LAPase; B, DL-Hyasp-diNH$_2$ with HCl; C, DL-Hyasp-diNH$_2$ with NaOH; D, hydrogenated material from Z-DL-Hyasp; I, Hyasp; II, Hyasn; III, Hyisoasn.

Hydrolysis of L-tHyasp-diNH$_2$ and L-eHyasp-diNH$_2$. For determination of the accurate rate of hydrolysis of the L-Hyasp-diNH$_2$, an increased amount of LAPase was used. The occurrence of the by-products through spontaneous degradation was found to be negligible. A solution of L-*t*(or *e*)Hyasp-diNH$_2$·HCl (0.05 mmol) in the activated enzyme solution (1 ml) and 0.1 M Tris buffer (1 ml) at pH 8.0 was incubated 38°C. The patterns given by the amino acid analyzer with the samples of 10 hr incubation are shown in Fig. 6-A. The proteolytic coefficients (C_1) of the diamides are shown in Table 2, the C_1 of amides of L-leucine, L-asparagine and L-cysteic acid[9] being included for comparison. The C_1 values are calculated from $C_1=k/e$, where $k=(1/\text{min})\log[100/(100-\%\text{ hydrolysis})]$ and e is the protein concentration in mg of protein N per ml of test solution.

Preparation of L-*t*Hyasn and L-*e*Hyasn by LAPase. *L-tHyasn.* L-*t*Hyasp-diNH$_2$·HCl (92 mg, 0.5 mmol) was dissolved in a mixture of the activated enzyme solution (10 ml) and water (30 ml), the pH of the solution being adjusted to 8.0 with N sodium hydroxide. After being kept 8 hr at 38°C, the incubation mixture indicated complete hydrolysis of the diamide producing hydroxyasparagine through the assays by the amino acid analyzer and paper chromatography. The mixture was adjusted to pH 5 with acetic acid and warmed with a small amount of charcoal to remove protein. The filtrate was put on a column (0.9×20 cm) of Dowex 1×8 (OH^- form), and the column was washed with water and eluted with 0.5 N acetic acid. Ninhydrin positive fractions were collected and evaporated *in vacuo*. The residual crystals were collected with the aid of ethanol and recrystallized from water-ethanol. Yield, 53 mg (71%); mp 246—248°C (decomp.); R_f 0.15.[17]

Found: C, 32.10; H, 5.45; N, 18.57%. Calcd for $C_4H_8O_4N_2$: C, 32.44; H, 5.44; N, 18.79%.

L-eHyasn. This was obtained from L-*e*Hyasp-diNH$_2$·HCl (0.5 mmol) in the same way as has been described for the preparation of L-*t*Hyasn except for the incubation time being 12 hr. Yield, 50 mg (68%); mp 230—

15) R. B. Johnston, M. J. Mycek and J. S. Fruton, *J. Biol. Chem.*, **185**, 629 (1950).

16) The analyzer used was a Hitachi amino acid analyzer (Model KLA-3B) with spherical resin. The conditions were as follows: 0.9×50 cm column; 0.2 M standard citrate buffer of pH 3.25; flow rate, 60 ml/hr; jacket temperature, 55°C.

17) The R_f value refers to the paper chromatography with the solvent system of *n*-butanol - acetic acid - pyridine - water (4 : 1 : 1 : 2, v/v). It was observed that R_f for L-*t*Hyasp and L-*t*Hyasp-diNH$_2$·HCl are 0.13 and 0.22 respectively.

232°C(decomp.);[18] R_f 0.15.[17]

Found: C, 32.08; H, 5.44; N, 18.63%.

Comparison of Synthetic L-Hydroxyasparagines and the Natural Product. The three samples were employed for the measurements of optical rotation, using water and 5 N hydrochloric acid as solvent. As recognized from Table 1, the values of specific rotation of synthetic L-*e*Hyasn and the natural product are identical within experimental error. The three samples afforded the same R_f values in paper chromatography.[17] However, the L-*e*Hyasn and the natural product gave a peak at the same place while the L-*t*Hyasn gave one at a different place according to the amino acid analyzer (Fig. 3).[19] Furthermore, a mixture of the L-*e*Hyasn and the natural product afforded a single peak on the co-chromatography (Fig. 3). Thus, it is concluded that the spacial configuration of the natural product is that of L-*e*Hyasn.

Spontaneous Degradation of DL-Hyasp-diNH$_2$ in Acid Solution. DL-*t*(or *e*)Hyasp-diNH$_2$·HCl (18.4 mg, 0.1 mmol) was dissolved in 0.1 N hydrochloric acid (2 m*l*). After the solution had been allowed to stand for 24 hr at 38°C, an aliquot was analyzed by the amino acid analyzer, the patterns being shown in Fig. 6-B. Although *t*(or *e*)Hyasn was produced in a small amount, its isolation from the solution was not attempted.

Spontaneous Degradation of DL-Hyasp-diNH$_2$ in Alkaline Solution and Preparation of DL-tHyasn. *Preliminary Studies.* DL-*t*(or *e*)Hyasp-diNH$_2$·HCl (0.1 mmol) in 0.1 N sodium hydroxide (2 m*l*) was treated in the same way as above for 5 hr at 38°C. The patterns given by the amino acid analyzer are shown in Fig. 6-C. It was observed that the amount of DL-*t*Hyasn produced was much greater than that of DL-*e*Hyasn as shown in Fig. 6-C. Thus the separation of DL-*t*Hyasn from the reaction mixture was attempted as follows.

DL-tHyasn. DL-*t*Hyasp-diNH$_2$·HCl (368 mg, 2 mmol) in 0.1 N sodium hydroxide (40 m*l*) was allowed to stand for 5 hr at 38°C. After the solution was neutralized to pH 7, it was evaporated *in vacuo* to dryness. The residue was dissolved in a small amount of dilute acetic acid, and was applied on a column (1.8×150 cm) of Amberlite CG-120 (type III, Na^+ form). The column was eluted with 0.2 M sodium citrate buffer of pH 3.25 at a flow rate of 60 m*l*/hr; the fractions of 220—380 m*l* were confirmed to contain DL-*t*Hyasn. The fractions combined were put on a column of Dowex 1 (OH^- form), and the column was washed with water and eluted with 0.5 N acetic acid. After the eluate was evaporated *in vacuo*, the crystals were collected with the aid of ethanol and recrystallized from water-ethanol. Yield, 95 mg (32%); mp 228—232°C (decomp.); R_f 0.15.[17]

Found: C, 32.16; H, 5.62; N, 18.89%. Calcd for $C_4H_8O_4N_2$: C, 32.44; H, 5.44; N, 18.79%.

Preparation of DL-*e*Hyasn through Z-DL-*e*Hyasp Anhydride. *Preliminary Studies.* A solution of Z-DL-*t*(or *e*)Hyasp (142 mg, 0.5 mmol) in acetic anhydride (2 m*l*) was allowed to stand for 6 hr at 50°C. After evaporation *in vacuo* to dryness, the residue was dissolved in N ethanolic ammonia (5 m*l*) at 0°C. The solution was left for 30 min at 0°C, then 2 hr at room temperature, and evaporated to dryness. The residue was dissolved in acetic acid (3 m*l*) and hydrogenated in the presence of palladium black. An aliquot of the filtrate was analyzed by the amino acid analyzer (Fig. 6-D). It was assumed that a compound in peak *t*III or *e*III in Fig. 6 was β-hydroxyisoasparagine, however, conclusive evidence could not be obtained.[20] As shown in Fig. 6-D, it appeared that the *e*Hyasn might be isolated from the reaction mixture. The separation of DL-*e*Hyasn was attempted as follows.

DL-eHyasn. Two millimoles (566 mg) of Z-DL-*e*Hyasp were treated as described avove. Acetic acid in the hydrogenated material was removed by evaporation, and the residue was dissolved in a small amount of water. It was treated with a Amberlite CG-120 column (1.8×50 cm) and 0.2 M sodium citrate buffer of pH 3.25 as had been described for the separation of DL-*t*Hyasn. The fractions of 120—155 m*l* were treated with a Dowex 1 column as described and yielded crystalline DL-*e*Hyasn. Yield, 98 mg (33%); mp 232—235°C(decomp.); R_f 0.15.[17]

Found: C, 32.51; H, 5.38; N, 18.62%. Calcd for $C_4H_8O_4N_2$: C, 32.44; H, 5.44; N, 18.79%.

We wish to express our thanks to Dr. Fumio Tominaga of Takeda Chemical Industries, Ltd. for supplying the natural hydroxyasparagine from human urine.

18) The mp of the natural hydroxyasparagine was determined to be 231—234°C (decomp.).

19) A sample each of 0.5 μmol with respect to one component was subjected to the analyzer.

20) The fractions of a peak *t*III or *e*III were collected in an experiment of preparative scale, however, the evaporation procedure *in vacuo* yielded Hyasp instead of Hyisoasn because of unstability of a compound (presumably, Hyisoasn) in *t*III or *e*III.