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α-Pyrone-6-carboxylic Acid Derivatives. IV.[1] Optical Resolution and Configuration of Stizolobic Acid, Stizolobinic Acid and β-(6-Carboxy-α'-pyron-5-yl)alanine Lactam

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The optical resolution of three isomers of DL-β-(6-carboxy-α'-pyronyl)alanine was carried out by chemical as well as enzymatic methods and six optically active isomers were obtained. The configuration of each aspartic acid, derived from the ozonolysis of these optical isomers in aqueous solution, was analyzed enzymatically using glutamic-oxaloacetic transaminase and malate dehydrogenase. By the same procedure, the configuration of the two natural amino acids, stizolobic acid and stizolobinic acid, was confirmed and both were assigned the L-form. Thus the absolute configuration of six optical active isomers of β-(6-carboxy-α'-pyronyl)alanine and the two natural amino acids is assigned unequivocally, and the relationship between the synthetic and the natural amino acids is established.

In previous communications,[2,3] it was reported that the structure of stizolobinic acid (I) and stizolobic acid (II), isolated from the epicotyls of *stizolobium hassjoo*, was determined as a α-pyrone-6-carboxylic acid derivative with the alanyl side chain in the 3 and 4-position, respectively (Fig. 1).

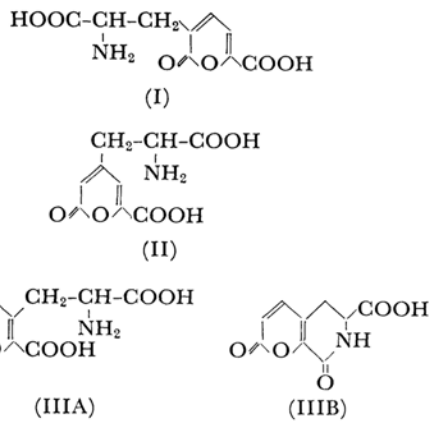

Fig. 1. Three isomers of β-(6-carboxy-α'-pyronyl)-alanine.

This paper describes the optical resolution of three isomers of DL-β-(6-carboxy-α'-pyronyl)-alanine and determination of the configuration of each optical active isomers and of the two natural amino acids.[4]

Results and Discussion

The synthetic amino acids were resolved by chemical as well as enzymatic methods. The results are summarized in Table 1.

DL-Stizolobinic acid (I) was resolved into the pure optical isomers by fractional recrystallization of the equimolar mixture of DL-amino acid and L-lysine from aqueous alcohol. The (+)-amino acid, $[\alpha]_D^{25}$ +19.5° (3 N HCl), was obtained from the less soluble salt. The (−)-isomer, $[\alpha]_D^{25}$ −20.5° (3 N HCl), was obtained from the more soluble fraction.

The salt of acetyl-DL-stizolobinic acid was resolved by repeated recrystallization of the strychnine salt from ethanol. The (+)-amino acid was obtained after hydrolysis of (+)-acetylstizolobinic acid isolated from the less soluble salt.

Bergmann and Fraenkel-Conrat developed the

1) Preceding paper in this series: S. Imamoto, Y. Maeno, S. Senoh, T. Tokuyama and T. Sakan, *Nippon Kagaku Zasshi*, (*J. Chem. Soc. Japan, Pure Chem. Sect.*), **87**, 1230 (1966).

2) S. Senoh, S. Imamoto, Y. Maeno, T. Tokuyama, T. Sakan, A. Komamine and S. Hattori, *Tetrahedron Letters*, **1964**, 3431.

3) S. Senoh, S. Imamoto, Y. Maeno, K. Yamashita, M. Matsui, T. Tokuyama, S. Sakan, A. Komamine and S. Hattori, *ibid.*, **1964**, 3439.

4) Partially reported by S. Senoh, S. Imamoto and Y. Maeno, IUPAC International Symposium on the Chemistry of Natural Products, Kyoto (1964), Abstracts of Papers, p. 169; S. Senoh, *Nippon Kagaku Zasshi* (*J. Chem. Soc. Japan, Pure Chem. Sect.*), **86**, 1087 (1965).

TABLE 1. OPTICAL RESOLUTION OF THE SYNTHETIC AMINO ACIDS

Racemic compound	Reagent	Optical antipode	
		$[\alpha]_D^{T}$	Isolated from
Stizolobinic acid	L-Lysine	+19.5°[a]	Less soluble fraction
		−20.5°	Soluble fraction
Acetylstizolobinic acid	Strychnine	+18.5°	Less soluble fraction
Cbz.-stizolobinic acid	Papain, aniline	−19.5°	Anilide fraction
		+20.0°	Unreacted fraction
Chloroacetylstizolobic acid	Aminoacylase	+16.0°[b]	Hydrolysis product
		− 4.5°	Unreacted fraction
β-(6-Carboxy-α'-pyron-5-yl)alanine lactam	Brucine	−30.0°[c]	Less soluble fraction
		+28.5°	Soluble fraction

a) Measured with 1% of the isomer in 3N aqueous hydrochloric acid solution at 25°C.
b) 1% in 1N aqueous hydrochloric acid solution at 25°C.
c) 1% in water solution at 25°C.

TABLE 2. OZONOLYSIS OF THE NATURAL AND SYNTHETIC AMINO ACIDS

Amino acid	Sample used	Aspartic acid	
		Found	Yield
	mg	γ	%
Synthetic stizolobinic acid	2	780—920	59—69
Natural stizolobinic acid	0.9	260	49
Synthetic stizolobic acid	2	200—260	17—22
Natural stizolobic acid	1.1	240	37
β-(6-Carboxy-α'-pyron-5-yl)alanine lactam	2	400—650	31—51

TABLE 3. ENZYMATIC DETERMINATION OF THE ABSOLUTE CONFIGURATION OF THE NATURAL AND SYNTHETIC AMINO ACIDS

Amino acid	$[\alpha]_D^{T}$	Enzymatic assay			Configuration
		Aspartic acid Calcd.	L-Aspartic acid found		
	in 3N HCl	γ	γ	%	
DL-Aspartic acid	0	79.8	39.5	49.5	
L-Aspartic acid	+25.5	39.9	40.0	100.2	
D-Aspartic acid	−25.5	53.2	0	0	
	in 3N HCl				
Synthetic stizolobinic acid	0	88.0	43.7	49.6	DL
	−20.5	39.0	37.5	96.1	L
	+19.5	50.0	2.0	4.0	D
Natural	unknown	32.5	32.0	98.5	L
	in N HCl				
Synthetic stizolobic acid	0	36.4	17.8	48.0	DL
	+16.0	44.0	39.2	89.0	L
	− 4.5	74.5	29.5	39.5	D
Natural	unknown	35.1	29.5	84.0	L
	in H_2O				
β-(6-Carboxy-α'-pyron-5-yl)alanine lactam	0	60.0	30.5	50.8	DL
	−30.0	35.0	34.5	98.5	L
	+28.5	55.0	0.5	0.9	D

method of enzymatic resolution of DL-α-acylamino acids with papain.[5] The anilide of the acylamino acid was precipitated from the incubation mixture of carbobenzyloxy-DL-stizolobinic acid and aniline in the presence of papain. Hydrolysis of the anilide gave the (−)-amino acid. The (+)-isomer was obtained from the hydrolysis of unreacted carbobenzyloxystizolobinic acid recovered from the incubation mixture.

The resolution of DL-stizolobic acid (II) posed a problem. By the chemical and enzymatic methods as described above, DL-stizolobic acid was not resolved at all or only partially into the optical isomers. Finally, it was found that the chloroacetyl derivative was slowly hydrolyzed by amino acylase extracted from takadiastase.[6] After the incubation of the chloroacetyl acid with aminoacylase for several days, the (+)-amino acid, $[\alpha]_D^{25}$ +16.0° (1 N HCl), was isolated by column chromatography over Dowex 50×8 (H^+ form). The (−)-antipode, $[\alpha]_D^{25}$ −4.5° (1 N HCl), was obtained partially resolved after hydrolysis of the unreacted acyl acid.

The lactam (III B) of the 5-pyronyl isomer gave the (−)-antipode, $[\alpha]_D^{25}$ −30.0° (H_2O), as the less soluble fraction in the repeated recrystallization (aqueous methanol) of the brucine-lactam salt. The (+)-antipode, $[\alpha]_D^{25}$ +28.5° (H_2O), was obtained from the mother liquors.

Infrared spectra of each synthetic and natural amino acids are shown in Fig. 2. The IR spectra of active form of stizolobic and stizolobinic acid differ slightly from that of the racemic form but are identical with that of the natural amino acids.

The absolute configuration of (−)-stizolobinic and (+)-stizolobic acid is expected to be L, because the enzymes employed in this study are known to react predominantly with the L-isomers of racemic amino acids.

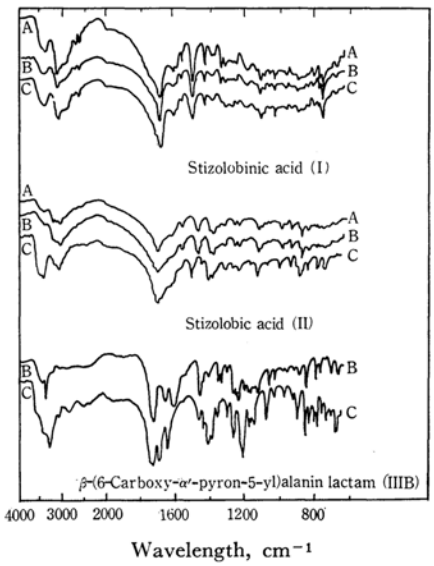

Fig. 2. Infrared spectrum of the natural and synthetic amino acids (KBr disk).
A: Natural amino acid
B: Optical active amino acid
C: Racemic amino acid

According to the rule of Lutz and Jirgensons[7] the optical rotatory power of the L-form of an α-amino acid in aqueous solution becomes more positive on protonation. We have found the ORD. spectra at two different dissociation stages of the amino acid in aqueous solution to be simple dispersion curves in the 350—700 mμ region. Figure 3 shows the ORD curves of (+)-stizolobic acid, (−)-stizolobinic acid and the lactam of (−)-β-(6-carboxy-α'-pyron-5-yl)alanine assigned L-form.

Since the content of the natural amino acids in

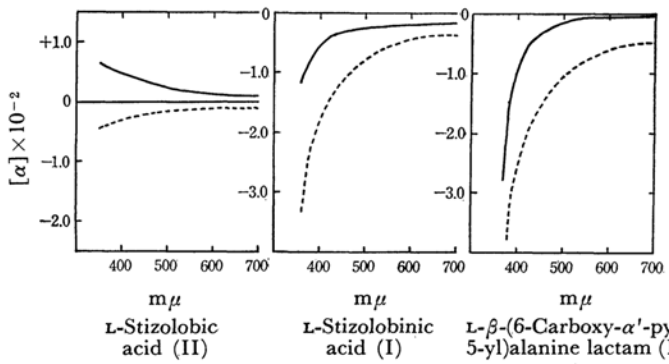

Fig. 3. ORD curves of the optical resolved amino acids.
Solid line: 1N aqueous hydrochloric acid solution at 25°C
Broken line: 0.5 M acetate buffer (pH 5.0) at 25°C

5) M. Bergmann and H. Fraenkel-Conrat, *J. Biol. Chem.*, **119**, 707 (1937).
6) K. Okawa, This Bulletin, **29**, 486 (1956).
7) O. Lutz and B. Jirgensons, *Ber.*, **63**, 448 (1930); *cf.* M. Winitz and J. P. Greenstein, *J. Am. Chem. Soc.*, **77**, 716 (1955).

the plant was very small, it was difficult to study the configuration directly by ORD or chemical methods.

The configuration of aspartic acid can be determined on a micro scale by a sensitive enzymatic method. Therefore, the amino acids were ozonized in order to obtain aspartic acid. Table 2 summarises the results of ozonization of the natural and synthetic amino acids. The amount of L-form of aspartic acid obtained in Table 2 was determined by the use of pig heart glutamic-oxaloacetic transaminase and pig heart malate dehydrogenase (Table 3).

A control experiment with authentic samples of aspartic acid gave unequivocal results as expected: approximately 50% of L-form was detected in racemic aspartic acid and none in the D-acid. After ozonolysis of the samples of stizolobinic acid (I), we obtained about 50% of L-aspartic acid from the racemic acid, over 96% from the (−)-antipode and natural acid, and 4% from the (+)-antipode. Thus the configuration of the alanine side chain in each antipode is L for the levorotatory and D for the dextrorotatory form in agreement with the ORD curves. The natural amino acid is identical with the (−)-isomer and is termed L-β-(6-carboxy-α'-pyron-3-yl)alanine.

The analytical results of the samples of stizolobic acid (II) showed over 89% of L-aspartic acid from the (+)-isomer, 39.5% from the −4.5° isomer and 84% from the natural acid. The (+)-isomer and the natural acid are assigned as the L-form, the (−)-isomer the D-form. Therefore, the configuration of the natural acid is L-β-(6-carboxy-α'-pyron-4-yl)alanine.

Similarly, the lactam (IIIB) of β-(6-carboxy-α'-pyron-5-yl)alanine must be L in the (−)-isomer and D in the (+)-isomer.

Experimental*1

Optical Resolution of DL-Stizolobinic Acid (I). Method A: *(+)- and (−)-Stizolobinic Acid.* Free L-lysine was freshly prepared from the hydrochloride by Dowex 50W×2 (H^+ form) column chromatography. A mixture of 2.27 g of DL-stizolobinic acid and 1.5 g of L-lysine was dissolved in 10 m*l* of water and filtered. After the addition of the same volume of ethanol, the solution was allowed to stand overnight in a refrigerator. The precipitate was collected, recrystallized three times from 50% aqueous ethanol, and then three times from 55% aqueous methanol. Yield of the less soluble lysine salt was 0.33 g; $[\alpha]_D^{20}$ +16.5° (*c* 1, water).

The aqueous solution of this salt was acidified with hydrochloric acid, the crystalline precipitate formed was collected and recrystallized from 3 N hydrochloric acid to give 106 mg of (+)-stizolobinic acid hydrochloride. The hydrochloride was converted easily to the free acid by treatment with water; $[\alpha]_D^{25}$ +19.5° (*c* 1, 3 N HCl).

Found: C, 47.73; H, 4.07; N, 6.31%. Calcd for $C_9H_9NO_6$: C, 47.58; H, 3.99; N, 6.17%.

All the filtrates from the less soluble lysine salt were combined, and evaporated to dryness *in vacuo*. The residual material was dissolved in 55% aqueous methanol, seeded with (+)-stizolobinic acid - L-lysine salt, kept in a refrigerator overnight and filtered. The filtrate was evaporated to dryness, dissolved in water and acidified with hydrochloric acid until crystalline precipitate had formed. The precipitate was collected and recrystallized four times from 3 N hydrochloric acid to give 89 mg of (−)-stizolobinic acid hydrochloride. Treatment with water produced the free acid; $[\alpha]_D^{25}$ −20.5° (*c* 1, 3 N HCl).

Found: C, 47.80; H, 4.01; N, 6.20%. Calcd for $C_9H_9NO_6$: C, 47.58; H, 3.99; N, 6.17%.

(+)- and (−)-Stizolobinic acid are identical with natural stizolobinic acid with regard to infrared and ultraviolet spectra.

Method B: *N-Acetyl-DL-stizolobinic Acid.* To a mixture of 1 g of DL-stizolobinic acid and 3 g of sodium bicarbonate in 25 m*l* of water was added dropwise 2.5 m*l* of acetic anhydride under vigorous agitation in an ice bath. Stirring was continued for 1 hr at 0°C and allowed to stand overnight at room temperature. The reaction mixture was acidified with 3 m*l* of concentrated hydrochloric acid and chilled. The precipitate was collected and recrystallized from hot water, yielding 0.84 g of colorless crystals, mp 251—252°C (decomp.).

Found: C, 49.32; H, 4.11; N, 5.21%. Calcd for $C_{11}H_{11}NO_7$: C, 49.07; H, 4.12; N, 5.20%.

(+)-Acetylstizolobinic Acid. A mixture of 1.12 g of strychnine and 0.9 g of *N*-acetyl-DL-stizolobinic acid was dissolved in 50 m*l* of hot ethanol and filtered. The filtrate was chilled and the bulky precipitate formed was collected. Three recrystallization from ethanol gave 370 mg of colorless crystals, mp 255—257°C (decomp.); $[\alpha]_D^{20}$ +11.6° (*c* 1, water).

Found: C, 63.41; H, 5.78; N, 6.98%. Calcd for $C_{32}H_{33}N_3O_9$: C, 63.67; H, 5.51; N, 6.96%.

The strychnine salt (340 mg) was decomposed by dissolving in 20 m*l* of aqueous sodium bicarbonate solution (100 mg). The aqueous solution was extracted with chloroform and acidified with hydrochloric acid to yield the crystalline precipitate. The precipitate was collected and recrystallized from hot water to give (+)-acetylstizolobinic acid, mp 230—232°C; $[\alpha]_D^{20}$ +98.5° (*c* 1, methanol).

Found: C, 49.24; H, 4.20; N, 5.18%. Calcd for $C_{11}H_{11}NO_7$: C, 49.07; H, 4.12; N, 5.20%.

(+)-Stizolobinic Acid. (+)-Acetylstizolobinic acid (100 mg) in 5 m*l* of concentrated hydrochloric acid was refluxed for 3 hr. Then the solution was cooled and the precipitate was collected. Recrystallization from hot water gave 40 mg of free (+)-stizolobinic acid; $[\alpha]_D^{25}$ +18.5° (*c* 1, 3 N HCl).

Found: C, 47.71; H, 3.96; N, 5.87%. Calcd for $C_9H_9NO_6$: C, 47.58; H, 3.99; N, 6.17%.

Method C. *N-Carbobenzyloxy-DL-stizolobinic Acid.* To a mixture of 1 g of DL-stizolobinic acid and 3 g of sodium bicarbonate in 20 m*l* of water and 15 m*l* of ether was added dropwise 13.6 g of 30% carbobenzyloxychloride in toluene solution under vigorous agitation in an ice bath. Stirring was continued for 3 hr at 0°C and for 3 hr at room temperature. After separation from the organic layer, the aqueous layer was

*1 All melting points are uncorrected.

extracted with ether and acidified with 3 ml of concentrated hydrochloric acid. The oily product was extracted three times with ethyl acetate, the combined extracts were washed with water and evaporated to dryness under reduced pressure. The residual oil solidified gradually and was recrystallized twice from ethyl acetate - chloroform - petroleum ether, yielding 1.34 g of colorless crystals, mp 165.5—167°C.

Found: C, 56.47; H, 4.19; N, 3.98%. Calcd for $C_{17}H_{15}NO_8$: C, 56.51; H, 4.18; N, 3.88%.

Activation of Papain. Papain was activated by the method of Doherty and Popenoe as follows.[8] A mixture of 2 g of papain (commercial grade, E. Merk, 1 : 350 digestive powder) and 0.1 g of potassium cyanide was suspended in 10 ml of 0.04 M citrate buffer (pH 4.9) and adjusted the pH to 5.0 with glacial acetic acid. The suspension was stirred for an hour at room temperature, filtered through a mat of Dicalite on a glass filter, and the filtrate was reserved for use.

Carbobenzyloxystizolobinic Acid Anilide and (+)-Stizolobinic Acid. To 9.12 g of *N*-carbobenzyloxy-DL-stizolobinic acid, dissolved in a mixture of 15 ml of 0.1 M citrate buffer (pH 4.9) and 37 ml of 2 N sodium acetate, was added 4.1 ml of freshly distilled aniline and 50 ml of the activated papain solution described above. The mixture was incubated at 37°C for 24 hr under nitrogen. The anilide formed was collected, suspended in a large volume of hot ethanol and filtered. The filtrate was evaporated to dryness and the residue was recrystallized twice from aqueous ethanol to give 2.09 g of crystals, mp 239—243°C (decomp.); $[\alpha]_D^{24}$ −18.0° (*c* 1, glacial acetic acid).

Found: C, 63.31; H, 4.55; N, 6.10%. Calcd for $C_{23}H_{20}N_2O_7$: C, 63.30; H, 4.62; N, 6.42%.

The mother liquor of the first crop was further incubated with the addition of 10 ml of enzyme solution for 3 days. The anilide was filtered and recrystallized from aqueous ethanol to give 0.61 g of the anilide; $[\alpha]_D^{24}$ +11.7° (*c* 1, glacial acetic acid).

The mother liquor of the second crop was acidified with concentrated hydrochloric acid and extracted many times with ethyl acetate. The combined extracts were evaporated to dryness to give 6 g of the unreacted acid. The mixture of 1 g of the unreacted acid in 25 ml of concentrated hydrochloric acid and 25 ml of glacial acetic acid was refluxed for 7 hr under nitrogen, and evaporated to dryness. The residue was recrystallized twice from 3 N hydrochloric acid and treated with water to give 0.33 g of (+)-stizolobinic acid; $[\alpha]_D^{25}$ +20.0° (*c* 1, 3 N HCl).

(−)-Stizolobinic Acid. One gram of the (−)-anilide was dissolved in glacial acetic acid saturated with hydrogen chloride, hydrolyzed in a sealed tube at 100°C for 40 hr and chilled. The crystalline material was collected and recrystallized from 3 N hydrochloric acid, yielding 0.44 g of (−)-stizolobinic acid hydrochloride; $[\alpha]_D^{22}$ −16.8° (*c* 1, 3 N HCl).

The Optical Resolution of DL-Stizolobic Acid (II). *N-Chloroacetyl-DL-stizolobic Acid.* *N*-Chloroacetyl-derivative was prepared from 1.5 g of DL-stizolobic acid, 4.1 g of chloroacetyl chloride and 4.5 g of sodium bicarbonate by the same procedure for the preparation of *N*-carbobenzyloxy-DL-stizolobinic acid. The product was recrystallized twice from acetone-benzene-petroleum ether and once from acetone, mp 175°C.

Found: C, 43.35; H, 3.78; N, 4.57%. Calcd for $C_{11}H_{10}NO_7Cl$: C, 43.51; H, 3.32; N, 4.61%.

Preparation of Aminoacylase.[6] Three grams of "Takadiastase" was suspended in 30 ml of water, extracted one hour at 0°C under stirring, and filtered through a mat of Dicalite. The filtrate was used for the resolution.

Isolation of (+)-Stizolobic Acid and (−)-Chloroacetylstizolobic Acid. A reaction mixture consisting of 1 g of *N*-chloroacetyl-DL-stizolobic acid, 30 ml of aminoacylase solution, 0.3 ml of 37% formaldehyde solution, 100 ml of 0.1 M phosphate buffer (pH 6.8) and water to make a final volume of 170 ml, was incubated at 37°C for 3 weeks with the occassional addition of the enzyme solution. The progress of the reaction was examined by the spot test with ninhydrin.

After filtration, the reaction mixture was applied to a column of Dowex 50W×8 (H^+ form, 2×30 cm) and eluted with water. The eluate could be divided into two fractions by the measurement of the optical density at 303 mμ. The first fraction (A) was acidic and contained phosphate and the second (B) was neutral and free from phosphate. The fraction B was concentrated to dryness under reduced pressure, and the residue (0.24 g) was recrystallized from hot water. The crystalline material (0.16 g) was dissolved in 20 ml of water and applied to Amberlite IR 45 column (OH^- form, 2×15 cm). After washing with 200 ml of water, eluted with 0.01 N hydrochloric acid. The eluate was evaporated to dryness, and the residue was recrystallized from hot water to give 65 mg of (+)-stizolobic acid; $[\alpha]_D^{25}$ +16.0° (*c* 0.5, 1 N HCl).

Found: C, 47.50; H, 4.13; N, 6.07%. Calcd for $C_9H_9NO_6$: C, 47.58; H, 3.99; N, 6.17%.

(+)-Stizolobic acid was identical with respect to infrared spectra and mixed melting point with the natural acid.

The fraction A was extracted with ethyl acetate, and the extract was evaporated to dryness. The residual oil solidified partially by storage at room temperature and was separated by filtration. The solid product was dissolved in hot ethanol, filtered, and evaporated to dryness. This procedure was repeated several times. Weighed 0.3 g; $[\alpha]_D^{25}$ −10° (*c* 1, ethanol).

(−)-Stizolobic Acid. A solution of (−)-chloroacetylstizolobic acid (0.3 g) obtained above in glacial acetic acid saturated with dried hydrogen chloride was saponified on a steam bath for 48 hr. The precipitate was collected, washed with glacial acetic acid and recrystallized twice from hot water to yield 0.15 g of colorless crystals; $[\alpha]_D^{25}$ −4.5° (*c* 1, 1 N HCl).

Optical Resolution of DL-β-(6-Carboxy-α'-pyron-5-yl)-alanine Lactam (III B). *(−)- and (+)-β-(6-Carboxy-α'-pyron-5-yl)alanine Lactam.* A mixture of 9.32 g of brucine and 4.82 g of DL-β-(6-carboxy-α'-pyron-5-yl)alanine lactam monomethanolate was recrystallized three times from 70% methanol and once from 50% aqueous methanol. The crystals weighed 3.7 g; $[\alpha]_D^{20}$ −40° (*c* 1, 50% aqueous methanol).

The less soluble brucine salt obtained above was dissolved in water and decomposed by passing through a Dowex 50W×2 (H^+ form) column. The effluent was concentrated to dryness. Recrystallization from water gave 1.2 g of the (−)-lactam; $[\alpha]_D^{25}$ −30.0° (*c* 1, water).

8) D. G. Doherty and E. A. Popenoe, *J. Biol. Chem.*, **189**, 447 (1951).

Found: C, 51.27; H, 3.47; N, 6.72%. Calcd for $C_9H_7NO_5$: C, 51.68; H, 3.37; N, 6.70%.

The mother liquor of the brucine salt of (−)-lactam was evaporated to dryness and the residue was recrystallized three times from 90% aqueous methanol to yield 1.9 g of the (+)-brucine salt; $[\alpha]_D^{20}$ +12° (*c* 1, 50% aqueous methanol).

The decomposition of the (+)-salt and recrystallization of the product was carried out by the same procedure mentioned above, and 0.5 g of the (+)-lactam, $[\alpha]_D^{25}$ +28.5° (*c* 1, water), was obtained.

Found: C, 51.36; H, 3.56; N, 6.81%. Calcd for $C_9H_7NO_5$: C, 51.68; H, 3.37; N, 6.70%.

Enzymatic Determination of the Configuration of Each Aspartic Acid Derived from the Ozonolysis of Natural Stizolobinic and Stizolobic Acids and Six Optical Active Amino Acids. *Materials.* Yields of the samples of each aspartic acid were analyzed by the ninhydrine method and summarized in Table 2.

Ozonolysis of Racemic, Optical Active and Natural Stizolobinic Acid (I). The ozonization of stizolobinic acid was carried out in ice-cold 0.5 N hydrochloric acid solution (1 mg/1 m*l*) for 2 hr. The ozonide was decomposed with the addition of 0.1 m*l* of 3% hydrogen peroxide on a steam bath for 10 min. The solution was concentrated to dryness in reduced pressure, the residue was dissolved in 1 m*l* of water and applied to a column of Dowex 50×2 (H^+ form, 0.5×5 cm). The column was washed with water. The first acidic eluate (approximately 20 m*l*) was discarded, the next 50 m*l* was collected and evaporated to dryness. The residue was dissolved in exact 2 m*l* of water, and the solution was reserved for the sample of enzymatic determination of the configuration of aspartic acid.

Ozonolysis of Racemic, Optical Active and Natural Stizolobic Acid (II). The ozonization was carried out in cold water solution (1 mg/1 m*l*). The following procedures are as described in the ozonolysis of stizolobinic acid.

Ozonolysis of Racemic and Optical Active β-(6-Carboxy-α'-pyron-5-yl)alanine Lactam (III B). The ozonization was carried out in cold 0.5 N hydrochloric acid solution (1 mg/1 m*l*). After ozonization, the reaction mixture was made up to 2 N hydrochloric acid solution, heated on a steam bath for 2 hr and evaporated to dryness. The residue was dissolved in 1 m*l* of water and then applied to Dowex 50 column as mentioned above.

Enzymes. Pig heart glutamic-oxaloacetic transaminase and pig heart malate dehydrogenase employed for the enzymatic assay of aspartic acid are both analytical reagent grade and purchased from C. F. Boehringer & Soehne GmbH.

Enzymatic Assay of Aspartic Acid. The results of enzymatic assay of the samples of each aspartic acid obtained above are summarized in Table 3. The mixture containing 0.1 to 0.4 μmol of aspartic acid, 25 μmol of sodium α-ketoglutarate, 10 γ of pig heart glutamic-oxaloacetic transaminase (approx. 180 units/mg), 750 μmol of phosphate buffer (0.5 M, pH 8.0), and water to make a final volume of 3.5 m*l* was incubated for 30 min at 37°C and chilled. To 3.0 m*l* of the incubation mixture was added 0.1 m*l* of aqueous NADH*2 (0.4 μmol) and 0.1 m*l* of malate dehydrogenase (50 γ of approx. 720 units/mg of the enzyme). The amount of oxaloacetate in the mixture was assayed spectrophotometrically by measuring the decrease in optical density at 340 mμ for the period of 15 min at room temperature.[9]

Control experiment without aspartic acid was taken as a blank.

We are indebted to Dr. Kaoru Kuriyama of Shionogi Research Laboratory, Shionogi & Co., Ltd. for the measurement of ORD spectra.

9) S. Ochoa, "Methods in Enzymology," Vol. I, Academic Press Inc., New York (1955), p. 735.

*2 Reduced nicotinamide-adenine dinucleotide.