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L-β-(5-Hydroxy-2-pyridyl)-alanine and L-β-(3-Hydroxyureido)-alanine from Streptomyces*

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In the course of the chemical screening study on the metabolites from actinomycetes, two unusual amino acids were isolated: L-β-(5-Hydroxy-2-pyridyl)-alanine (**1**) and L-β-(3-hydroxyureido)-alanine (**9**). The structures of **1** and **9** were elucidated from the physico-chemical properties of these amino acids and derivatives, and supported by synthesis. Antibacterial activities of **1** and its methylester (**2**) were antagonized by L-tyrosine, and those of **9** and its derivative (**17**) by L-glutamine.

Structural analogs of amino acids have been isolated from a variety of microorganisms and plants.[2] Most of them showed the antibiotic activity in synthetic media, and this has been utilized to detect analogs in the fermentation broths of actinomycetes and bacteria.[3]

In the course of our chemical screening program on metabolites from actinomycetes, we have searched for compounds which showed color reactions by both ninhydrin and ferric chloride reagents, with the ultimate aim to find analogs of physiologically active phenolic amines. As a result, we have found two unusual amino acids, *i.e.*, L-β-(5-hydroxy-2-pyridyl)-alanine (**1**) which is a L-tyrosine analog, and L-β-(3-hydroxyureido)-alanine (**9**) which is a L-glutamine antagonist. The isolation and structure determination as well as some of the biological properties of these amino acids are subjects of this article.

L-β-(5-Hydroxy-2-pyridyl)-alanine (1)

This amino acid, originally designated as SF-1346 substance, has been obtained from a fermentation broth of *Streptomyces chibaensis* SF-1346 which was isolated from soil in Okayama-city in Japan. The culture broth was filtered from mycelia, and passed through a column of Dowex 50W×2 (hydrogen form) resin. The 1 N ammonium hydroxide eluate was concentrated, and applied on a column of active carbon. The compound **1** showing brown color reactions to ninhydrin and ferric chloride was eluted with aqueous acetone, and crystallized from water. Treatment of **1** with methanolic hydrogen chloride gave crystalline methylester (**2**) dihydrochloride.

The gross structure of **1** was first implied by mass spectroscopy of **1**, **2**, and their trimethylsilyl (TMS) derivatives (Chart 1). In addition to molecular ions, fragment ions arising from typical α-cleavage of α-amino acids appeared. The positive charge was retained more favorably in the hydroxy-containing moiety, with or without hydrogen shift. The proton magnetic resonance (PMR) spectrum illustrated in Fig. 1 showed a doublet at δ 3.67 and a triplet at 4.57, assignable to the alanine moiety, in consistent with three ^{13}C signals of $-CH_2-\underset{|}{C}H-COOH$ at 33.5, 52.8, and 170.2 (Table I).

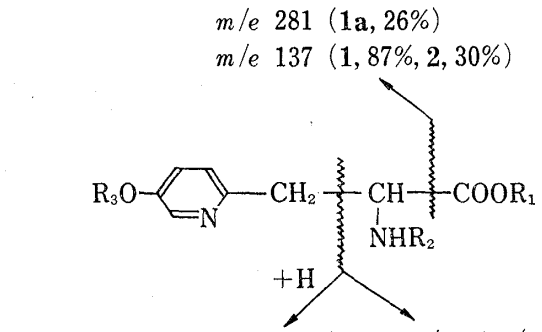
The presence of 5-hydroxypyridine nucleus was suggested by carbon-thirteen magnetic resonance (CMR) and ultraviolet (UV) spectroscopy. As shown in Table I, the chemical

* Dedicated to the memory of Prof. Eiji Ochiai.

1) Location: *Morooka, Kohoku-ku, Yokohama, 222, Japan.*

2) G. Nass, K. Poralia, and H. Zähner, *Naturwiss.*, **58**, 603 (1971).

3) D.L. Pruess, J.P. Scannell, M. Kellett, H.A. Ax, J. Janecek, T.H. Williams, A. Stempel, and J. Berger, *J. Antibiotics*, **27**, 229 (1974).



M^+ : *m/e* 398 (**1a**, 11%), 340 (**2a**, 1%), 196 (**2**, 3%), 182 (**1**, 9%)

Chart 1. Electron-impact Fragmentation of **1** ($R_1=R_2=R_3=H$), **2** ($R_1=CH_3$, $R_2=R_3=H$), **1a** ($R_1=R_2=R_3=$TMS) and **2a** ($R_1=CH_3$, $R_2=R_3=$TMS)

The TMS derivatives were prepared by heating with bis-trimethylsilylacetamide at 80° for 1 hr.

TABLE I. ^{13}C Chemical Shifts[a] of L-β-(5-Hydroxy-2-pyridyl)-alanine (**1**) and 5-Hydroxypyridine (**5-HP**) in Deuterium Oxide

		Alanine			Pyridine			
		CH_2	CH	COOH	C-2	C-3 or C-4	C-5	C-6
1	pH>1	33.5	52.8	170.2	141.3	130.6, 130.1	155.9	134.9
1	pH 10.0	39.8	56.9	178.7	142.9	127.4, 125.8	160.4	139.6
5-HP	pH 10.2				135.3	127.3, 125.8	162.0	139.7

a) Internal standard, dioxane (67.4 ppm relative to TMS). The assignment was supported by the partially decoupled spectra.

shifts of five ^{13}C signals were very close to those of 5-hydroxypyridine, and UV spectra of two compounds were quite similar, both showing maxima around 220, (255), 280 and 320 nm at neutral pH, 230 and 290 nm at acidic, and 240 and 305 nm at alkaline.

The remaining problem was the substituted position of the alanine residue on the 5-hydroxypyridine nucleus. CMR data presented in Table I favoured C-2 or C-6 substitution. Furthermore, the aromatic proton signals of **1** that appeared at 8.02 (two protons) and 8.37 (one proton) (Fig. 1), resembled to those of 5-hydroxy-2-acetoxymethylpyridine that appeared at 7.15 (H-3 and H-4) and 8.07 (H-6) in a (1:1) mixture of chloroform-d and dimethylsulfoxide-d_6.[4]

Compound **1** is optically active ($[\alpha]_D^{25}$ −33°), and the positive rotation change (+88°) observed by passing from the neutral to the protonated form indicated the L-configuration.[5]

Data presented so far suggested the structure **1** as the most probable one. In order to confirm the structure, in particular the location of the alanine residue, the racemic form of **1** was synthesized. Before its discovery in nature, Norton and coworkers[6] have already synthesized the DL form in 1961 *via* 7 steps from kojic acid.[6a]

4) S. Inouye, T. Tsuruoka, T. Ito, and T. Niida, *Tetrahedron*, **24**, 2125 (1968).
5) J.P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. 1, John Wiley & Sons, Inc., New York, 1961, p. 76.
6) *a*) S.J. Norton, C.G. Skinner, and W. Shive, *J. Org. Chem.*, **26**, 1495 (1961); *b*) S.J. Norton and P.T. Sullivan, *J. Heterocyclic Chem.*, **7**, 699 (1970).

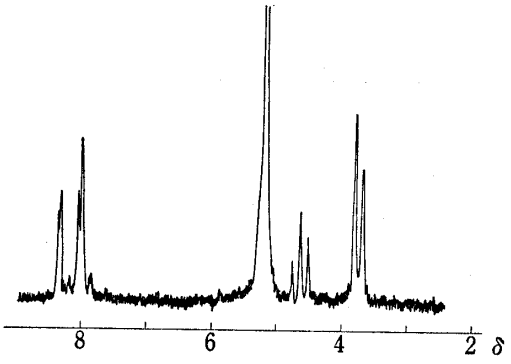


Fig. 1. 60 MHz PMR Spectrum of **1** in Deuterium Oxide at pH <1

The starting material in this synthesis was 3-hydroxy-6-hydroxymethyl-2(1H)-pyridone (**3**), readily obtained from an antibiotic nojirimycin by oxydation and subsequent dehydration.[7] Compound **3** was converted to the 3-O-benzyl derivative(**4**), which, on treatment with phosphorous oxychloride at 75—80° for 2 hr, gave the 2,6-dichloro derivative (**5**). Condensation of **5** with diethyl sodio-acetamidomalonate in ethanol, followed by decarboxylation and hydrogenation afforded DL-β-(5-hydroxy-2-pyridyl)-alanine (**1**), in addition to novel DL-β-(2-chloro-3-hydroxy-6-pyridyl)-alanine (**8**). The synthetic compound of **1** showed the same UV and PMR spectra and *Rf* values as those of the natural one, and a half of the antibacterial activity as described later.

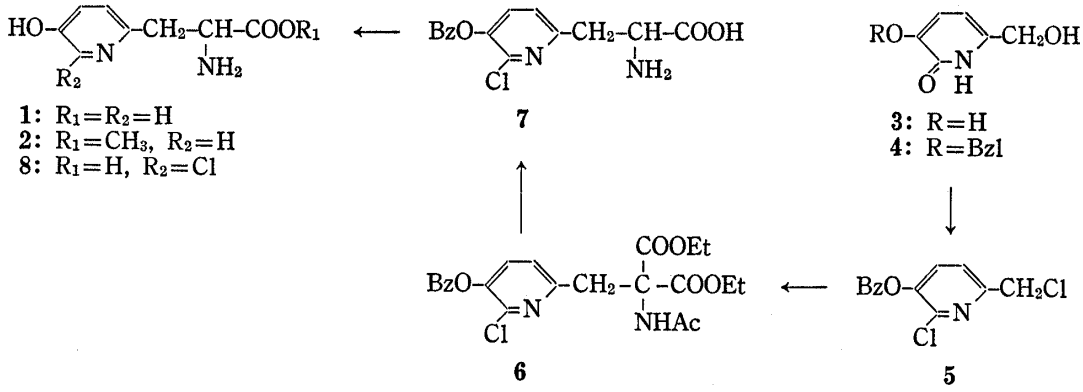


Chart 2

L-β-(3-Hydroxyureido)-alanine (9)

Streptomyces hygroscopicus SF-1293, isolated from soil in Fukui Prefecture in Japan, was found to produce two unusual amino acids, one of which is antifungal SF-1293 substance,[8a] identical with phosphinothricyl-alanyl-alanine.[8b] The other amino acid (**9**), originally named as SF-1293B substance, was isolated from a fermentation broth first by decolorizing with active carbon and then by adsorbing to Dowex 50WX 2 (hydrogen form) resin. The 1 N ammonium hydroxide eluate was concentrated, whereupon crystals of **9** were deposited. It showed purple color by ninhydrin and ferric chloride tests, and behaved as a neutral amino acid in paper electrophoresis at pH 6.4.

The CMR spectrum summarized in Table II revealed four carbon atoms, of which three could be assigned to the alanine moiety, as supported by the PMR spectrum showing $-C\underline{H}_2-C\underline{H}-$ signals at 3.88 and 4.32 in acidic deuterium oxide. The remaining one carbonyl carbon at 164.1, together with two oxygen and one nitrogen atoms could be accounted for by the ureido structure, which was suggested by the infrared (IR) spectrum. The multiple bands around 1660—1520 cm^{-1} were quite similar to the corresponding IR bands in L-citrulline used as a model amino acid.

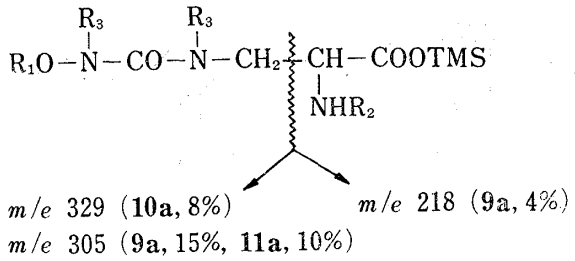
7) T. Tsuruoka, S. Inouye, and T. Niida, in preparation.
8) *a*) Y. Kondo, T. Shomura, Y. Ogawa, T. Tsuruoka, H. Watanabe, K. Totsugawa, T. Suzuki, C. Moriyama, J. Yoshida, S. Inouye, and T. Niida, *Sci. Reports, Meiji Seika Kaisha*, **13**, 34 (1973); Y. Ogawa, T. Tsuruoka, S. Inouye, and T. Niida, *ibid.*, **13**, 42 (1973); Y. Ogawa, H. Yoshida, S. Inouye, and T. Niida, *ibid.*, **13**, 54 (1973); *b*) E. Beyer, K.H. Gugel, H. Hägele, H. Hagenmaier, S. Jessipow, W.A. König, and H. Zähner, *Helv. Chim. Acta*, **55**, 224 (1972).

Table II. ^{13}C Chemical Shifts[a] of L-β-(3-Hydroxyureido)-alanine (**9**) and L-Albizziin (**13**) in Deuterium Oxide

		CH_2	CH	CO-NH	COOH
9	pH 12	44.5	57.0	163.4	181.6
13	pH 12	45.2	56.9	162.3	181.5
9	pH <1	39.9	54.4	164.1	170.7
13	pH <1	40.5	54.7	162.3	171.2

a) Internal standard, dioxane (67.4 ppm relative to TMS). The assignment was supported by the partially decoupled spectra.

Treatment of **9** with trifluoroacetic anhydride in trifluoroacetic acid gave crystalline O,N-di-trifluoroacetyl derivative (**10**), which showed two IR bands at 1825 and 1745 cm^{-1}. Attempted recrystallization of **10** from ethyl acetate resulted in the hydrolysis of an ester bond to give the N-trifluoroacetyl derivative (**11**). Mass spectra of the totally trimethylsilylated compounds of **9** and its trifluoroacetyl derivatives (**10** and **11**) gave the molecular ions at *m/e* 523 (penta-trimethylsilylate), 571 (tri-trimethylsilylate) and 547 (tetra-trimethylsilylate), respectively. Therefore, the presence of five active hydrogen atoms was demonstrated, of which two were replaceable with acyl groups. Principal fragment ions arised from the rupture of C-2 and C-3 bond, and the positive charge was preferably retained by the hydroxyureido moiety, as shown in Chart 3.



M^+ : *m/e* 571 (**10a**, 8%), 547 (**11a**, 8%), 523 (**9a**, 5%)

Chart 3. Electron-impact Fragmentation of **9a** ($R_1=R_2=R_3=$ TMS), **10a** ($R_1=R_2=CF_3CO$, $R_3=$ TMS), and **11a** ($R_1=R_3=$TMS, $R_2=CF_3CO$).

Trimethylsilylation was carried out with bis-trimethylsilyltrifluoroacetamide at 120° for 1 hr (**9a**), or with bis-trimethylsilylacetamide at 80° for 1 hr (**10a** and **11a**)

Fig. 2. 60 MHz PMR Spectrum of **10** in Trifluoroacetic Acid

The physico-chemical properties described above suggested the hydroxyureidoalanine as the most probable structure of **9**, which was proven by the following reactions.

Acid hydrolysis of **9** with refluxing 6N hydrochloric acid gave quantitatively L-2,3-diaminopropionic acid (**12**), identical with the authentic sample. Catalytic reduction of **9** over platinic oxide gave a deoxy compound (**13**), whose physical properties were indistinguishable from those of L-albizziin, first obtained from *Mimosaceae*.[9] For identification purpose, L-albizziin was synthesized from **12** by treating the latter with potassium cyanate. The synthetic compound and the deoxygenated product (**13**) were identical with respects to mp, IR, $[\alpha]_D$ and *Rf* values in paper partition chromatography(PPC) and thin-layer chromatography (TLC).

The remaining problem was the location of a hydroxy group, which may be at N^1 or N^3 of the ureido moiety. L-β-(1-Hydroxy-ureido)-alanine (**14**) was recently isolated from the

9) a) R. Gmelin, G. Straus, and G. Hasenmaier, *Hoppe-Seyler's Z.*, **314**, 28 (1959); b) A. Kjaer, P.O. Larsen, and R. Gmelin, *Experientia*, **15**, 253 (1959).

alkaline hydrolyzate of an anthelmintic quisqualic acid.[10a] Furthermore, L-N^5-hydroxyarginine (**15**) was discovered as a metabolite of bacteria[11a,c] and a fungus.[11b] The CMR of **15** indicated that the δ-carbon adjacent to a hydroxyimino group showed remarkable downfield shift (10 ppm) compared to that of L-arginine.[11c] A similar deshielding would be expected for the β-carbon of **14**. However, when the ^{13}C chemical shifts of **9** were compared to those of L-albizziin (**13**), the methylene carbon showed little change (Table II). This result favoured N^3 substitution of a hydroxy group, which was supported by the PMR of **10** in trifluoroacetic acid. As illustrated in Fig. 2, a doublet and a triplet appeared in the lower field were assigned to NH groups, since they disappeared in the spectrum in deuterium oxide. Decoupling experiment indicated the following partial structure, $-NH-CH_2-\underset{|}{CH}-NH-$. In compound **14**, a singlet NH signal in stead of a triplet should be observed.

Compound **9** was recently synthesized by Takemoto and co-workers,[10b] before it was discovered in nature. The compound synthesized from L-aspartic acid essentially *via* the same route as reported was identical with the natural product in mp, IR and *Rf* values in PPC and TLC.

Treatment of **9** with 2—3 moles of benzyloxycarbonyl chloride, followed by treatment with hydrogen bromide saturated in acetic acid, yielded an acidic amino acid (**17**). Elemental analysis and mass spectrum of the TMS derivative indicated the insertion of a carbonyl group with loss of two active hydrogen atoms. The IR spectrum of **17** showed bands at 1815 and 1740 cm^{-1}, characteristic for 3,5-dioxo-1,2,4-oxadiazolidine ring,[12] and was found to be identical with the IR spectrum of isoquisqualic acid,[10b] which is a position isomer of quisqualic acid, an active principle of a Chinese drug *Quisqualis Fructus*.[10a] Use of a large excess of benzyloxycarbonyl chloride afforded crystalline N,N′-di-benzyloxycarbonyl-isoquisqualic acid(**18**).

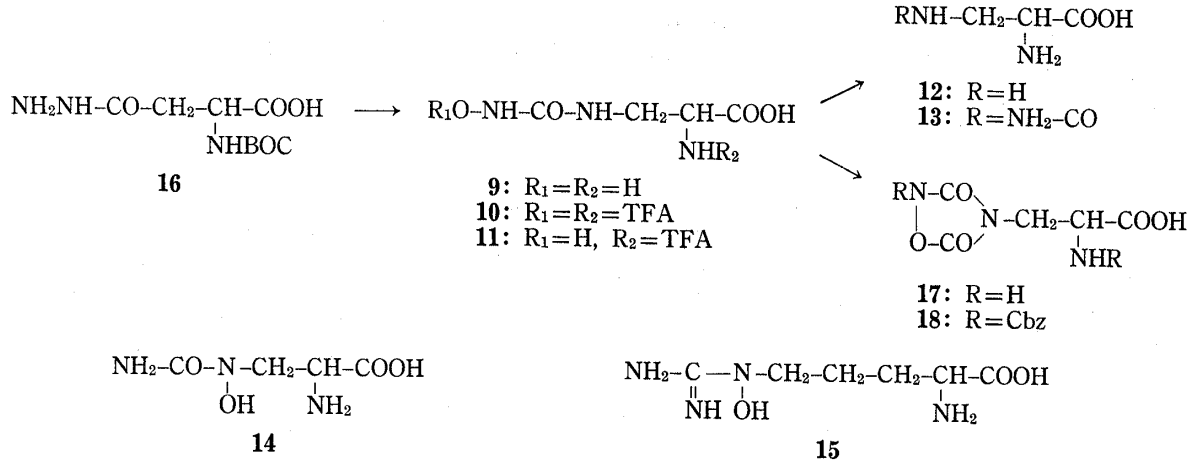


Chart 4

Antibacterial Property

Two natural amino acids (**1**, **9**) and their derivatives (**2**, **17**) showed antibacterial activity, in particular in synthetic media (Table III). As was expected, the activity of the pyridine analogs (**1**, **2**) of L-tyrosine was non-competitively[3] antagonized by the latter, and in lesser extent by L-tryptophan and L-phenylalanine. The synthetic DL form showed a half activity

10) *a*) T. Takemoto, N. Takagi, T. Nakajima, and K. Koike, *Yakugaku Zasshi*, **95**, 176 (1975); *b*) T. Takemoto, T. Nakajima, S. Arihara, and K. Koike, *ibid.*, **95**, 326 (1975).
11) *a*) H. Maehr, J.F. Blount, D.L. Pruess, L. Yarmchuk, and M. Kellett, *J. Antibiotics*, **26**, 284 (1973); *b*) B. Fischer, W. Keller-Schierlein, H. Kneifel, W.A. König, W. Loeffler, A. Müller, R. Muntwyler, and H. Zähner, *Arch. Mikrobiol.*, **91**, 203 (1973); *c*) D. Perlman, A.J. Vlietnick, H.W. Matthews, and F.F. Lo, *J. Antibiotics*, **27**, 826 (1974).
12) G. Zinner, and R.O. Weber, *Arch. Pharmazie*, **298**, 580 (1965).

of L-form against *Bacillus subtilis*, whereas **8** showed no activity so far examined. Although microbial products structurally related to **1** such as amiclenomycin,[13a] L-2,5-dihydrophenylalanine[13b] and anticapsin[13c] were reported, to our best knowledge, **1** is the first L-tyrosine analog of microbial origin.

TABLE III. Antibacterial Activities of **1**, **2**, **9** and **17** at a Concentration of 5 mg/ml

	Inhibition Zone (mm) Assayed by Paper Disc Method			
	1	**2**	**9**	**17**
Bacillus subtilis	17	14	0	10
Bacillus subtilis No. 8193[a]	29	28	15[b]	22[b]
Escherichia coli[c]	35	25	14	22
Pseudomonas aeruginosa[c]	28[b]	28	20[b]	14[b]

a) A mutant requiring L-tryptophan and L-methionine for its growth.
b) Faint zone
c) Synthetic medium

The activity of **9** and **17** was weakly inhibited by L-aspartic acid, L-glutamic acid and L-asparagine, and most strongly by L-glutamine. Comparison of these structures of **9**, **17**, and L-glutamine indicated that the former two are imino isosters of the γ-methylene of the latter, with an extra hydroxy (and carbonyl) group. It has been reported that N^5-hydroxyarginine (**15**) and L-2-amino-3-dimethylaminopropionic acid[14] where the γ-carbon of L-leucine is replaced by a nitrogen atom, are antimetabolites of the respective parent amino acids. There-Finally, fore, **9** and **17** may be regarded as new structural analogs of L-glutamine. it may be of interest to add that compound **1** and **17** showed antibacterial synergism against *Escherichia coli*.

Experimental[15]

General Procedure for Detection of Ninhydrin and Ferric Chloride-positive Compounds in Fermentation Broth——A filtered broth was spotted on silica gel thin-layer plates (E. Merck, Darmstadt), and the plates were developed with *n*-butanol–acetic acid–water (4: 1: 5 v/v). The plates were dried, and sprayed with a (1: 1) mixture of 2% ferric chloride and 1% potassium ferricyanate for detection of a phenol and/or with a (1: 1) mixture of 3.6% ninhydrin in acetone and 0.2% cadmium acetate in acetone–acetic acid–water (20: 1: 5) for detection of a primary amine. By this procedure, an antibiotic cycloserine could be found in addition to **1** and **9**.

Fermentation and Isolation of L-β-(5-Hydroxy-2-pyridyl)-alanine (1)——A fermentation medium (20 liters) consisting of malto syrup (4.0%), soybean meal (2.0%), soluble vegetable protein (0.5%), Pharmamedia (1.0%), soybean oil (0.3%), ferrous sulfate (0.001%), nickel chloride (0.0001%) and cobaltous chloride (0.0001%), was adjusted to pH 7.0 before sterilization, and inoculated with a seed culture of *Streptomyces chibaensis* SF-1346. The culture was incubated at 28° for 72 hr in a jar fermentor with aeration. The cultured broth was adjusted to pH 3 with concentrated hydrochloric acid, and the filtrate (17 liters) was passed through a column of Dowex 50WX 2 (H^+) resin (2.5 liters). The column was washed with water, and eluted with 1 N ammonium hydroxide. The first effluent (2 liters) was discarded, and the second one (4 liters) was collected, concentrated to 200 ml, and then passed through a column of active carbon. The column was washed with water, and eluted with 10% aqueous acetone. The eluate (1.6 liters) was concentrated to 50 ml, from which crystals of **1** (3.1 g) were deposited upon standing at 5° overnight. From

13) *a*) Y. Okami, T. Kitahara, M. Hamada, H. Naganawa, S. Kondo, K. Maeda, T. Takeuchi, and H. Umezawa, *J. Antibiotics*, **27**, 656 (1974); *b*) T. Yamashita, N. Miyairi, K. Kunugita, K. Shimizu, and H. Sakai; *J. Antibiotics*, **23**, 537 (1970); J.P. Scannell, D.L. Pruess, T.C. Demny, T.H. Williams, and A. Stempel, *J. Antibiotics*, **23**, 618 (1970); *c*) R. Shah, N. Neuss, M. Gorman, and L.D. Boeck, *J. Antibiotics*, **23**, 613 (1970).
14) A.D. Argoudelis, R.R. Herr, D.J. Mason, T.R. Pyke, and J.F. Zieserl, *Biochemistry*, **6**, 165 (1967).
15) Melting points were uncorrected.

the mother liquor were recovered further 1.8 g: Total yield, 4.9 g. Recrystallization from water gave a pure sample. mp 262—263° (decomp.). $[\alpha]_D^{25}$ −33° (c=1.0, H_2O), +55° (c=1.1, 1N HCl), −6.3° (c=1.4, 1 N NaOH). UV $\lambda_{max}^{H_2O}$ nm ($E_{1cm}^{1\%}$): 221 (396), 255 (sh), 281 (175), 320 (15). $\lambda_{max}^{0.1N\ HCl}$ 230 (245), 290 (318); $\lambda_{max}^{0.1N\ NaOH}$ 242 (565), 303 (210). IR ν_{max}^{KBr} cm^{-1}: 3370, 2950, 2650, 1620, 1595, 1570, 1480, 1410, 1345, 1295, 1255, 845. PMR (in D_2O containing CF_3COOH) δ: 3.67 (2H, doublet), 4.57 (1H, triplet), 8.02 (2H, multiplet), 8.35 (1H, multiplet). *Rf* Values of **1** and L-tyrosine on cellulose TLC (solvent system, *n*-BuOH–AcOH–H_2O=4: 1: 5 v/v), 0.28, 0.42; (*n*-BuOH–acetone–diethylamine–H_2O=30: 30: 6: 15 v/v) 0.21, 0.48. Compound **1** migrated to a cathod as an acid in paper electrophoresis at pH 9.4 (0.1 M Veronal buffer). Relative migration rate to L-aspartic acid, 0.63. *Anal.* Calcd. for $C_8H_{10}O_3N_2$: C, 52.74; H, 5.53; N, 15.38. Found: C, 52.24; H, 5.92; N, 14.99.

L-β-(5-Hydroxy-2-pyridyl)-alanine Methylester (2) Dihydrochloride——A solution of **1** (2.0 g) in 2 N methanolic hydrogen chloride (50 ml) was allowed to stand at room temperature overnight, and then heated at 80° for 3 hr. The reaction mixture was concentrated to dryness. The residue was crystallized from ethanol, and recrystallized from methanol–ethanol. Yield, 1.55 g. mp 224—225° (decomp.). $[\alpha]_D^{20}$ +38° (c=1.0, H_2O). IR ν_{max}^{KBr} cm^{-1}: 1755 (CO), 1630, 1570. PMR (in D_2O) δ: 3.69 (2H, doublet), 3.81 (3H, singlet), 4.72 (1H, triplet), 8.04 (2H, multiplet), 8.40 (1H, multiplet). *Anal.* Calcd. for $C_9H_{12}O_3N_2 \cdot 2HCl$: C, 40.16; H, 5.25; N, 10.41. Found: C, 40.19; H, 5.13; N, 10.50.

3-Benzyloxy-6-hydroxymethyl-2(1H)-pyridone (4)——To a suspension of 3-hydroxy-6-hydroxymethyl-2(1H)-pyridone (3) (28 g) in ethanol (350 ml) was added potassium hydroxide (24 g) in water (10 ml). The resulting clear solution was kept at 65—70°, and added dropwise benzyl chloride (60 ml) in ethanol (100 ml) during 2 hr. The reaction mixture was heated for further 3 hr, cooled, and filtered off to remove sodium chloride. Concentration of the filtrate gave crystals of **4** (35 g). mp 157—158°. UV $\lambda_{max}^{methanol}$ nm ($E_{1cm}^{1\%}$): 238 (260), 300 (310). *Anal.* Calcd. for $C_{13}H_{13}O_3N$: C, 67.52; H, 5.66; N, 6.06. Found: C, 67.26; H, 5.82; N, 5.93.

3-Benzyloxy-2-chloro-6-chloromethyl-2(1H)-pyridone (5)——A mixture of **4** (5 g) and phosphorous oxychloride (25 ml) was kept at 0° for 20 min to obtain clear solution, and then heated at 75—80° for 2 hr. The dark-brown solution was evaporated to dryness, and the residue was dissolved in chloroform (200 ml). After washing with aqueous sodium hydrogen carbonate and water, the chloroform layer was evaporated to dryness, and the residue was crystallized from ethanol. Yield, 1.5 g. Recrystallization from chloroform–ethanol gave an analytical sample. mp 113—115°. UV $\lambda_{max}^{methanol}$ nm ($E_{1cm}^{1\%}$): 233 (500), 284 (250). IR ν_{max}^{Nujol} cm^{-1}: 1560 (CO–NH). Mass Spectrum *m/e*: 269 (M^+), 91 ($C_7H_7^+$). *Rf* values of **4** and **5** on silica gel TLC (solvent system, $CHCl_3$–MeOH=10: 1 v/v), 0.28, 0.85. *Anal.* Calcd. for $C_{13}H_{11}ONCl_2$: C, 58.23; H, 4.14; N, 5.22. Found: C, 58.26; H, 4.12; N, 5.31.

DL-β-(5-Hydroxy-2-pyridyl)-alanine (1)——To a solution of diethyl acetamidomalonate (904 mg) in ethanol (15 ml) containing sodium (100 mg) was added **5** (1.07 g), and the mixture was stirred at 60—65° for 18 hr. The precipitated sodium chloride was removed by filtration, and the filtrate was evaporated to crystalline mass (**6**). *Rf* Values of **5** and **6** on silica gel TLC (solvent system, $CHCl_3$–MeOH=10: 1 v/v), 0.85, 0.76.

The above crystals, without further purification, were dissolved in 47% hydrobromic acid (10 ml), and refluxed for 4 hr. The reaction mixture was evaporated to dryness to give crystals of **7** hydrobromide. *Rf* Values of **6** and **7** on silica gel TLC (solvent system, *n*-BuOH–AcOH–H_2O=3: 1: 1 v/v), 0.81, 0.58.

The hydrobromide of **7** was dissolved in a (1: 1) mixture of water and methanol (40 ml). The solution was adjusted to pH 2.0 by aqueous sodium hydroxide, and hydrogenated over 5% Pd-carbon (300 mg) under 2.8 atm of hydrogen for 2 hr. The filtrate (pH, 1.5) was readjusted to pH 4.5 by aqueous sodium hydroxide, and evaporated to dryness. The residue was dissolved in water (3 ml), and the insoluble portion of **8** was separated by filtration. The filtrate was evaporated to a residue (350 mg), which was dissolved in water (10 ml), and applied on a column of active carbon (10 ml). After washing with water, the column was eluted with 30% aqueous acetone, and the effluents were collected in 3 ml fractions. Fractions 4—7 were combined, and concentrated to deposit racemic crystals (110 mg) of **1**. Recrystallization from water gave an analytical sample mp 247—248° (decomp.). UV $\lambda_{max}^{H_2O}$ nm ($E_{1cm}^{1\%}$): 221 (400), 255 (sh), 281 (185), 320 (17). $\lambda_{max}^{0.1N\ HCl}$ 230 (255), 290 (315); $\lambda_{max}^{0.1N\ NaOH}$ 242 (573), 304 (217).[16] IR ν_{max}^{Nujol} cm^{-1}: 3450 (H_2O), 1600. *Anal.* Calcd. for $C_8H_{10}O_3N_2 \cdot H_2O$: C, 47.99; H, 6.04; N, 13.99. Found: C, 47.96; H, 5.93; N, 13.97.

DL-β-(6-Chloro-5-hydroxy-2-pyridyl)-alanine (8)——The water insoluble portion (380 mg) described in "DL-β-(5-Hydroxy-2-pyridyl)-alanine (**1**)" was dissolved in water (20 ml) at 60°, and the clear solution was cooled to 3° to deposit crystals of **8** (190 mg). Further crystals (100 mg) were recovered from the mother liquor that was concentrated. Recrystallization from water gave an analytical sample. mp 265—267° (decomp.). UV $\lambda_{max}^{H_2O}$ nm ($E_{1cm}^{1\%}$): 224 (360), 286 (273), 320 (sh). $\lambda_{max}^{0.1N\ HCl}$: 224 (360), 285 (280); $\lambda_{max}^{0.1N\ NaOH}$ 245 (545), 310 (315). IR ν_{max}^{Nujol} cm^{-1}: 3100, 2720, 2680, 1650, 1620. Mass Spectrum of the tri-TMS derivative *m/e*: 432 (M^+), 417 (M^+-15), 315 (M^+—COOTMS), 243 (M^+—COOTMS—72), 218 ((CHNHTMS-COOTMS)$^+$), 215 (M^+—CHNHTMS—COOTMS+H). Relative *Rf* values and ninhydrin color of **1**, **7** and **8** on descending

16) Norton and coworkers[6b] reported mp 240—241° (decomp.), and UV λ_{max}^{pH3} 285 nm and λ_{max}^{pHI2} 306 nm.

PPC (solvent system, *n*-BuOH–AcOH–H_2O=3:1:1 v/v), **1**=1.00 (brown), **7**=1.62 (yellow), **8**=1.23 (brown). *Anal.* Calcd. for $C_8H_9O_3N_2Cl$: C, 44.36; H, 4.19; N, 12.93. Found: C, 44.66; H, 4.07; N, 13.11.

Fermentation and Isolation of L-β-(3-Hydroxyureido)-alanine (9)——A jar fermentor was inoculated with a seed culture of *Streptomyces hygroscopicus* SF-1293 in a medium (200 liters) consisting of D-glucose (3.0%), malto syrup (1.0%), wheat embryo (2.5%), soluble vegetable protein (0.5%), soybean oil (0.1%), yeast extract (0.1%), ferrous sulfate (0.001%), nickel chloride (0.0001%), and cobaltous chloride (0.0001%).

The fermentation was continued at 28° for 96 hr with aeration, and the cultured broth was adjusted to pH 3 and filtered. The filtrate (150 liters) was passed through a column of active carbon (7.5 liters), and washed with water (30 liters). The effluent and washings were combined, passed through a column of Dowex 50WX 2 (H^+) resin (9 liters). After washing with water, the column was eluted with 0.05 N ammonium hydroxide. Upon concentrating and cooling the eluate overnight, crystals (40 g) of **9** were deposited. Recrystallization from water gave a pure sample. mp 211—212° (decomp.). $[\alpha]_D^{25}$ −12° (*c*=1.0, 1 N HCl), $[\alpha]_D^{20}$ −2.0° (*c*=1.0, 1 N NaOH), pK_a', 1.6, 8.8, *ca.* 11.0 (titration equivalent, 174). No UV maximum above 200 nm. IR ν_{max}^{KBr} cm^{-1}: 3270, 3000, 2820, 1660, 1630, 1600, 1565, 1520, 1440, 1400, 1370, 1150, 1090, 840, 730, 680. PMR (in D_2O containing CF_3COOH) δ: 3.88 (2H, doublet,) 4.32 (1H, triplet), (in CF_3COOH) δ: 4.16 (2H, broad singlet), 4.68 (1H, broad singlet), 7.10 (1H, broad singlet), 7.82 (3H, broad singlet). It showed positive biuret, red-tetrazolium and Lemieux reactions, but negative Molish and Folin reactions. *Anal.* Calcd. for $C_4H_9O_4N_3$: C, 29.45; H, 5.56; N, 25.76. Found: C, 29.45; H, 5.53; N, 25.80.

O,N-Ditrifluoroacetyl-L-β-(3-hydroxyureido)-alanine (10)——A mixture of **9** (6 g), trifluoroacetic anhydride (12 ml) and trifluoroacetic acid (18 ml) was kept at room temperature overnight. After addition of ether (50 ml), crystals (6.75 g) that were deposited were separated by filtration. Concentration of the mother liquor and addition of ether yielded further crystals (1.30 g). Total yield, 8.05 g. mp 162—163°. $[\alpha]_D^{24}$ 0° (*c*=2.8, CF_3COOH). IR ν_{max}^{Nujol} cm^{-1}: 3300, 1825, 1745, 1700, 1670. PMR (in CF_3COOH) δ: 4.07 (2H, triplet, J=5.5 Hz), 5.07 (1H, quartet, J=5.5, 7.5), 6.79 (1H, triplet, J=5.5), 8.28 (1H, doublet, J=7.5). *Anal.* Calcd. for $C_8H_7O_6N_3F_6$: C, 27.05; H, 1,99; N, 11.83. Found: C, 26.84; H, 2.34; N, 11.45.

N-Trifluoroacetyl-L-β-(3-hydroxyureido)-alanine (11)——Compound **10** (2 g) was dissolved in ethyl acetate (50 ml) and the solution was filtered. The filtrate was concentrated to deposit crystals of **11**, 1.4 g. mp 160.5—161°. $[\alpha]_D^{24}$ −7.4° (*c*=1.2, H_2O). IR ν_{max}^{Nujol} cm^{-1}: 3450, 3350, 3280, 1710. *Anal.* Calcd. for $C_6H_8O_5N_3F_3$: C, 27.81; H, 3.11; N, 16.21. Found: C, 28.24; H, 3.17; N, 15.94.

L-2,3-Diaminopropionic Acid (12) Hydrochloride——A solution of **9** (10 g) in 4 N hydrochloric acid (100 ml) was refluxed for 6 hr, and evaporated to dryness. To the residual syrup was added water (30 ml), and the solution was evaporated again. The residue was dissolved in water (20 ml) and addition of ethanol (200 ml) caused crystallization of **12** monohydrochloride (4.6 g). Further crops of 2.5 g were recovered from the mother liquor. Total yield, 7.1 g. It was recrystallized from water. mp 236—237° (decomp.). $[\alpha]_D^{23}$ +25° (*c*=1.0, 5 N HCl). *Anal.* Calcd. for $C_3H_8O_2N_2 \cdot HCl$: C, 25.62; H, 6.45; N, 19.92. Found: C, 25.37; H, 6.54; N, 19.69. The IR spectrum was superimposable on that of the authentic sample of **12** hydrochloride.

L-2-Amino-3-ureidopropionic Acid (L-Albizziin) (13)——a) A solution of **9** (300 mg) in water (30 ml) was hydrogenated over platinic oxide (50 mg) at room temperature for 2 hr, and filtered to remove catalyst. The filtrate was evaporated to dryness, and the residue (300 mg) was crystallized from water–ethanol. mp 216—217° (decomp.). $[\alpha]_D^{23}$ −66.2° (*c*=1.0, H_2O), −23.6° (*c*=1.0, 1 N HCl), −3.2° (*c*=0.5, 1 N NaOH.[17]) IR ν_{max}^{KBr} cm^{-1}: 1675, 1610, 1560. Mass Spectrum of the tetra-TMS derivative, *m/e*: 435 (M^+), 420 (M^+-15), 217 ($(CH_2NTMS-CO-NHTMS)^+$). It showed negative ferric chloride test. *Anal.* Calcd. for $C_4H_9O_3N_3$: C, 32.65; H, 6.17; N, 28.56. Found: C, 32.44; H, 6.20; N, 28.13.

b) To a suspension of **12** hydrochloride (2.8 g) in water (30 ml) was added solid potassium cyanate (1.5 g) in small portions, and the mixture was stirred at room temperature until complete solution was effected. The reaction mixture was concentrated and allowed to stand at room temperature overnight. The precipitate (480 mg) was separated, and the filtrate was passed through a column of Dowex 50WX 2 (H^+) resin (2.8×20 cm). After washing with water, the column was eluted with 0.2 N ammonium hydroxide, the eluate was concentrated, and ethanol was added. The resulting precipitate was recrystallized from water and ethanol to give **13** (690 mg), mp 218—219° (decomp.). $[\alpha]_D^{25}$ −63.4° (*c*=1.0, H_2O), −21.9° (*c*=1.0, 1 N HCl), −4.0° (*c*=1.0, 1 N NaOH).[17] The synthetic compound was indistinguishable from **13** obtained in a) with respects to IR, optical rotations,[18] elemental analysis and *Rf* values on paper and thin-layer chro-

17) The negative sign in 1 N NaOH, which is opposite to the positive rotation reported,[9a] was confirmed by ORD (*c*=1.0, 1 N NaOH), $[\alpha]^{20}$ (nm): −3.5° (589), −7° (350), 0° (287), +38° (250). Very recently, Professor R. Gmelin sent us a sample of natural L-albizziin, which showed $[\alpha]_D^{24}$ −3.3° (*c*=3.3, 1 N NaOH), in consistent with ours.

18) For natural L-albizziin, mp 217° (decomp.), $[\alpha]_D$ −66.2° (H_2O), −22.2° (1 N HCl), + 3.2° (1 N NaOH) were reported.[9a] In this synthesis, no formation of L-3-amino-2-ureidopropionic acid was observed, for which Gmelin and coworkers[9b] reported mp 204—210° (decomp.), $[\alpha]_D$ +3.2° (H_2O), −43.0° (1 N HCl), +24.5° (1 N NaOH).

matographies. *Rf* Values on cellulose TLC of **9**, **12** and **13**: (solvent system, *n*-BuOH–AcOH–H_2O=2: 1: 1 v/v), 0.27, 0.23, 0.34.

The first precipitate (480 mg), which was far less soluble in water than L-albizziin, was recrystallized from hot water. mp 220—221° (decomp.). It could not be differentiated from **13** by TLC, and mass, NMR and elemental analysis, but lacked in optical activity. Its IR spectrum was slightly different from that of **13**. Based on the above data, the first precipitate was determined to be DL form of **13**, probably being formed by the partial racemization under alkaline condition.

N-*t*-Butyloxycarbonyl-L-aspartic Acid γ-Hydrazide (16) Hydrazine Salt——A solution of L-aspartic acid γ-ethylester hydrochloride (20 g), triethylamine (10 ml) and *t*-butyl azidoformate (15 ml) in a (1: 1) mixture of water and dioxane (120 ml) was stirred at room temperature for 2 days. The reaction mixture was concentrated, and washed with ethyl acetate. The aqueous layer was adjusted to pH 3.0 by citric acid, and extracted with ethyl acetate. Evaporation of solvent from the extract left a syrup (13.4 g), which was dissolved in ethanol containing hydrazine hydrate (10 ml). The solution was allowed to stand at room temperature overnight, during which time crystals (14.5 g) were deposited. Recrystallization from ethanol gave **16** as hydrazine salt, mp 143—144°. $[\alpha]_D^{24}$ −4.7° (c=1.5, H_2O). IR ν_{max}^{KBr} cm^{-1}: 1680, 1640, 1610. *Anal.* Calcd. for $C_9H_{21}O_5N_5$: C, 38.70; H, 7.58; N, 25.08. Found: C, 38.93; H, 7.58; N, 25.27.

L-β-(3-Hydroxyureido)-alanine (9)——To a solution of **16** hydrazine salt (2.6 g) in methanol (50 ml) was added ethereal solution of diazomethane, and the mixture was evaporated to a syrupy α-methyl ester (2.5 g). This was suspended in cold 30% aqueous acetic acid (40 ml), and aqueous solution of sodium nitrite (500 mg) was added during 30 min. The reaction mixture was extracted with ether, washed repeatedly with saturated aqueous solution of sodium hydrogen carbonate to remove acetic acid, and dried over sodium sulfate. Evaporation of solvent gave the acid azide (600 mg) (IR ν_{max}^{Nujol} cm^{-1}: 2120 (N_3)), which, upon heating at 80° for 15 min under reduced pressure, was converted to the isocyanate (IR ν_{max}^{Nujol} cm^{-1}: 2220 (NCO)). Treatment of the latter with hydroxylamine (400 mg) in dioxane, followed by hydrolysis with 1 N sodium hydroxide and then with 90% trifluoroacetic acid, gave a crude product, which was purified by preparative PPC using a solvent system of *n*-butanol–pyridine–water (6: 4: 3 v/v). Aqueous extract from the band corresponding to **9** was passed through a column of Dowex 50WX4 (H^+) resin, and 0.05 N ammonium hydroxide eluate was concentrated to deposit crystals of **9** (8 mg), mp 208—210° (decomp.). The IR spectrum and *Rf* values were indistinguishable from those of **9** of natural origin. Lit.,[10b], mp 214—215° (decomp.).

L-β-(3,5-Dioxo-1,2,4-oxadiazolidin-4-yl)-alanine (Isoquisqualic Acid) (17)——To a solution of **9** (2.3 g) in water (100 ml) were added sodium hydrogen carbonate (3.6 g) and then dropwise benzyloxycarbonyl chloride (6.0 g). The reaction mixture was stirred at room temperature for 4 hr, and washed with ether. The aqueous layer was acidified with 5 N hydrochloric acid, and extracted with ether and subsequently with ethyl acetate. The extracts were combined, and, after drying over sodium sulfate, evaporated to a clear syrup. This was dissolved in acetic acid (15 ml) saturated with hydrogen bromide, and allowed to react at room temperature for 1 hr. An excess of dry ether was added, and the resulting precipitate was taken up by filtration. This was dissolved in water, neutralized with solid sodium hydrogen carbonate, and passed through a column of DEAE-Sephadex A-25 (1.9×15 cm). After washing with water, the column was eluted with 1 N acetic acid. The eluate was evaporated to dryness, and the residue was crystallized from iso-propanol. Yield, 250 mg. mp 193—194° (decomp.), $[\alpha]_D^{20}$ +1.7° (c=0.7, H_2O). It showed reddish brown color with ferric chloride, and purple color with ninhydrin. In high-voltage paper electrophoresis at pH 6.4, it behaved as an acid, with the same migration rate as L-glutamic acid. Mass Spectrum of the tri-TMS-derivative, *m/e*: 405 (M^+), 390 (M^+-15), 288 ($M^+-COOTMS$), 218 ($(CHNHTMS-COOTMS)^+$), 188 ($M^+-CHNHTMS-COOTMS+H$). IR ν_{max}^{Nujol} cm^{-1}: 1825, 1730. The IR spectrum of this crystals was considerably different from that of the authentic isoquisqualic acid.

Recrystallization from water gave another form of crystals, mp 213—214° (decomp.). The IR spectrum showed the characteristic bands at 1815, 1740, 1710, and 1605 cm^{-1}, and identical with that of isoquisqualic acid, but different from that of natural quisqualic acid. Lit.,[10b] mp for **13**, 204—206° (decomp.). *Anal.* Calcd. for $C_5H_7O_5N_3$: C, 31.75; H, 3.73; N, 22.22. Found: C, 31.46; H, 3.80; N, 21.87.

N,N′-Di-benzyloxycarbonyl-L-β-(3,5-dioxo-1,2,4-oxadiazolidin-4-yl)-alanine (18)——A mixture of **9** (800 mg), sodium hydrogen carbonate (2.5 g) and benzyloxycarbonyl chloride (4.5 g) in water (50 ml) was stirred at room temperature for 2 hr, and extracted with ether (70 ml). The ether layer was reextracted with 1 N sodium hydroxide (20 ml), and the aqueous layer was acidified with 5 N hydrochloric acid to precipitate **18** (950 mg). Repeated recrystallization from methanol gave an analytical sample, mp 183—185° (decomp.). $[\alpha]_D^{20}$ −1.9° (c=1.0, 1N NaOH). IR ν_{max}^{Nujol} cm^{-1}: 1800, 1770, 1705. PMR (in pyridine-d_5) δ: 3.83 (2H, doublet), 5.06 (1H, triplet), 5.31 (4H, singlet), 7.35 (multiplet), 10.77 (broad singlet), 12.00 (broad singlet). *Anal.* Calcd. for $C_{21}H_{19}O_9N_3$: C, 55.14; H, 4.19; N, 9.20. Found: C, 56.54; H, 4.53; N, 9.75.

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