STRUCTURE OF ALAHOPCIN (NOURSEIMYCIN), A NEW DIPEPTIDE ANTIBIOTIC

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The structure of alahopcin (nourseimycin) (**1**), a new dipeptide antibiotic isolated from *Streptomyces*, has been established to be (2*S*,3*R*)-2-[(L-alanyl)amino]-4-formyl-3-(hydroxyaminocarbonyl)butyric acid. **1** exists in two cyclic hemiacetal type tautomers formed by intramolecular ring closure between the hydroxyamino group and the formyl group in aqueous solution. The structure of the new weakly acidic amino acid (**2**), a constituent of **1**, is revealed to be (2*S*,3*R*)-2-amino-4-formyl-3-(hydroxyaminocarbonyl)butyric acid, and **2** also exists in two cyclic hemiacetal type tautomers in aqueous solution.

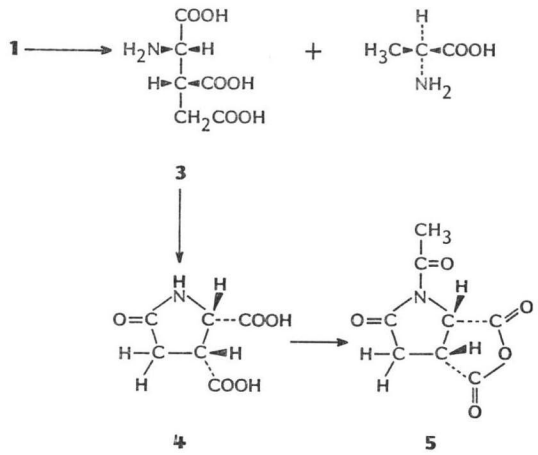
Alahopcin (B-52653) was isolated from the fermentation broth of *Streptomyces albulus* subsp. *ochragerus* in a screening program for new antibiotics selectively active against the *in vitro* antibiotic resistant mutant of *Staphylococcus aureus*[1,2], and nourseimycin (T-804A) was isolated from the fermentation broth of *Streptomyces noursei* in a project to find new antimetabolites produced by microorganisms[3]. In the course of structural elucidation studies of alahopcin and nourseimycin, it was found that these two biologically active substances are identical by comparison of ^{1}H NMR, ^{13}C NMR, IR and mass spectra, optical rotation, and Rf values of TLC. In this report, the structural elucidation of alahopcin (nourseimycin) (**1**) is described, and the structure of the amino acid (**2**), obtained by the enzymatic hydrolysis of **1** using α-amino acid ester hydrolase[4], is also discussed.

The antibiotic **1** is a water-soluble white powder having a weakly acidic and amphoteric character (*pKa'* 8.8, 7.6 and 3.1) and no characteristic UV absorption. The molecular formula of **1** was established to be $C_9H_{15}N_3O_6$ from elemental analysis and MS (SIMS) *m/z* 262 $(M+H)^+$ [2,3].

Hydrolysis of **1** with 6 N HCl (at 120°C in a sealed tube for 16 hours) gave L-alanine, ammonia and an amino acid (**3**) which showed shorter retention time than aspartic acid by standard automatic amino acid analysis [yield (mol/mol): the acidic amino acid **3**, 0.82; Ala 0.86; NH_3 0.92] and was identified as 1-amino-1,2,3-propanetricarboxylic acid (α-aminotricarballylic acid)[5] by spectroscopic data ($C_6H_9NO_6$; MS (SIMS) *m/z* 192 $(M+H)^+$; ^{1}H NMR (D_2O) δ 2.90 (2H, d, J=6.8), 3.57 (1H, td, J=6.8, 6.8, 4.8), 4.21 (1H, d, J=4.8); ^{13}C NMR (D_2O) δ 34.0 (t), 42.5 (d), 55.6 (d), 172.3 (s), 175.4 (s), 176.2 (s)).

The stereochemistry (1*S*,2*R*) of **3** was established by the following experiments. Refluxing of an aqueous solution (at pH 2.9) of **3** gave the corresponding 5-oxo-2,3-pyrrolidinedicarboxylic acid (**4**) of which the optical rotation ($[\alpha]_D^{25}$ +46.7° (*c* 1, H_2O)) and ^{1}H NMR spectrum ((D_2O) δ 2.88 (2H, d, J=8),

Fig. 1.



3.92 (1H, q, J=8), 4.77 (1H, d, J=8)) are identical with those of the synthetic 2*S*,3*R* isomer prepared according to the procedure of GREENSTEIN *et al.*[5]. The *cis*-configuration of the vicinal carboxyl group of **4** was also confirmed by the comparison of ^{1}H NMR spectra of mono sodium salt of the 2,3-*cis* and 2,3-*trans* isomer of 5-oxo-2,3-pyrrolidinedicarboxylic acid[5] (the free acid of *trans* isomer is sparingly soluble in D_2O) (the mono sodium salts of **4** and the synthetic 2,3-*cis* isomer; (D_2O) δ 2.81 (2H, d, J=8), 3.77 (1H, q, J=8), 4.59 (1H, d, J=8), the mono sodium salt of the synthetic 2,3-*trans* isomer; (D_2O) δ 2.81 (2H, d, J=8), 3.61 (1H, td, J=6, 8, 8), 4.57 (1H, d, J=6)) and by the conversion of **4** into *N*-acetyl-5-oxo-2,3-pyrrolidinedicarboxylic anhydride (**5**)[6].

Treatment of **1** with 2,4-dinitrofluorobenzene gave the 2,4-dinitrophenyl (DNP) derivative (**6**), and then **6** was hydrolyzed with 6 N HCl (at 120°C in a sealed tube for 16 hours) to give DNP-L-alanine as an only DNP-amino acid.

Enzymatic hydrolysis of **1**, using α-amino acid ester hydrolase produced by *Acetobacter turbidans* ATCC 9325[7] and *Xanthomonas citri* IFO 3835[8], gave a new amino acid (**2**)[4] and L-alanine. The amino acid **2** is a water soluble crystalline compound having a weakly acidic and amphoteric character (*pKa'* 8.9, 7.4 and 2.6) and no characteristic UV absorption. The molecular formula of **2** was established to be $C_6H_{10}N_2O_5$ from elemental analysis and MS (SIMS) m/z 191 $(M+H)^+$. The IR spectrum of **2** shows the presence of –CONH–, $-COO^-$, $-NH_3^+$ (IR ν_{max} (KBr, cm^{-1}) 1690, 1628, 1405, 1365, 1130, 1023), but no absorption at 1800~1700 cm^{-1} region[4].

The above data show that the amino group of the L-alanine moiety of **1** is free, the carboxyl group of L-alanine forms a peptide bond with the amino group of the amino acid **2**, and the acidic amino acid **3** is produced as an artifact from the amino acid **2** moiety by acid hydrolysis of **1**.

Treatment of **1** with *p*-nitrophenylhydrazine in 50% acetic acid gave the *p*-nitrophenylhydrazone (**7**). In the ^{1}H NMR spectra (400 MHz, D_2O) of **1** and **2**, no proton signal was observed over the formyl proton region of δ 9.0 to 11.0, but proton signals were observed in hemiacetal methine region (**1**: δ 5.27 (dd, J=2.1, 6.9) and 5.23 (dd, J=4.6, 7.1), total 1H, and **2**: δ 5.34 (dd, J=2.0, 6.3) and 5.32 (dd, J=4.9, 7.0), total 1H) (the presence of hemiacetal like structure).

Esterification of the per-*N*-acetyl derivative of **7** with diphenyldiazomethane gave the mono (diphenylmethyl) ester (^{1}H NMR ($CDCl_3$) δ 1.29 (3H, d, J=7.5, Ala CH_3), 1.89 and 2.20 (each 3H, s, acetyl), 6.77 and 7.97 (each 2H, d, J=9.3, *p*-NO_2-C_6H_4), 6.86 (1H, s, CH-Ph_2), 7.26 (10H, s, $CH(C_6H_5)_2$)) (the presence of one free carboxylic group in **1**).

Reduction of the aldehydic functional group of **1** with sodium borohydride gave the dihydro derivative (**8**) ($C_9H_{17}N_3O_6$; MS (SIMS) m/z 264 $(M+H)^+$; ^{1}H NMR (D_2O) δ 1.77 (3H, d, J=7, Ala CH_3), 1.9~2.25 (2H, m, CH_2), 3.05 (1H, q, J=7, CH), 3.8~4.0 (2H, m, CH_2O), 4.40 (1H, q, J=7, Ala CH), 4.66 (1H, d, J=7, CH)) which gave a positive (cherry-purple) color test with ferric chloride. Hydrolysis of **8** with 1 N HCl (at room temperature for 18 hours) gave the lactone (**9**) ($C_9H_{14}N_2O_5$; MS (SIMS) m/z

Fig. 2.

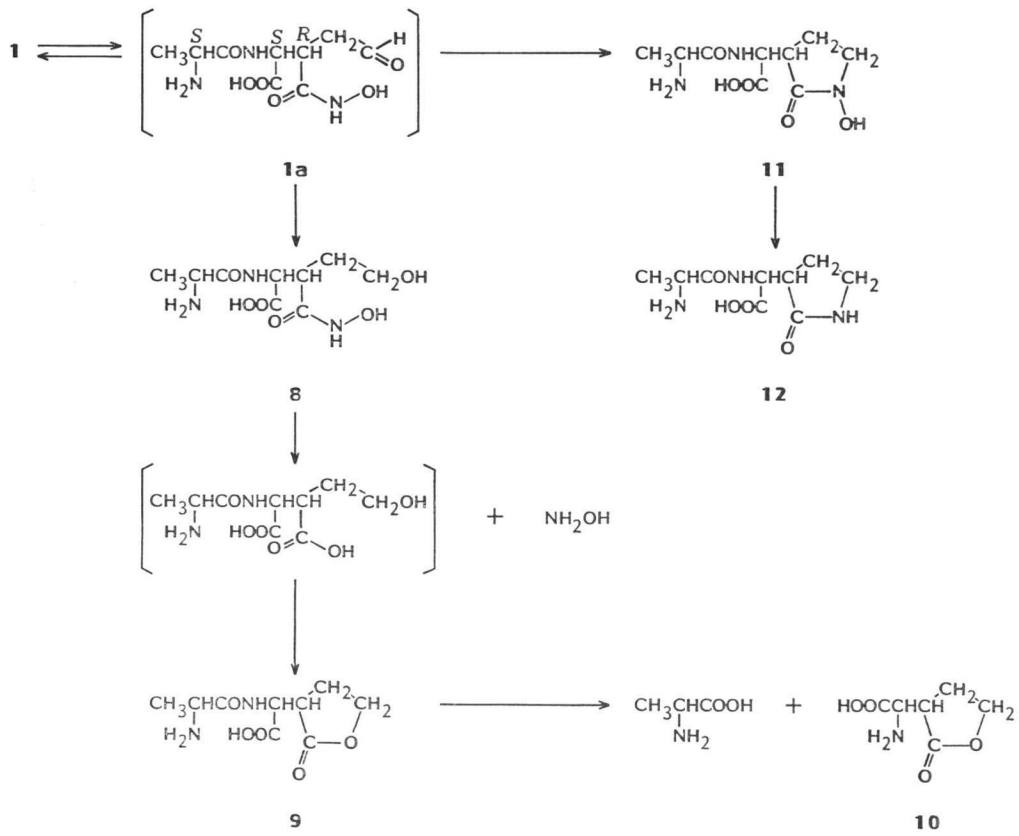
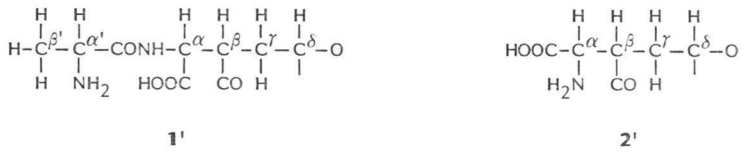


Fig. 3.



231 $(M+H)^+$; IR ν_{max} (KBr, cm^{-1}) 1756 (lactone); ^1H NMR (D_2O) δ 1.79 (3H, d, J=7, Ala CH_3), 2.05~2.9 (2H, m, CH_2), 3.47 (1H, dt, J=4.5, 10, 10, CH), 4.35 (1H, q, J=7, Ala CH), 4.45~4.7 (2H, m, CH_2O), 4.86 (1H, d, J=4.5, CH)) and hydroxylamine which was isolated as *m*-nitrobenzaldehyde oxime (the location of formyl group and the presence of masked *N*-hydroxyamide group in **1**). Hydrolysis of **9** with 6 N HCl (at 120°C in a sealed tube for 16 hours) gave L-alanine and 2-(2-oxotetrahydrofuran-3-yl)glycine (**10**). As described above, **1** gave ammonia almost quantitatively by acid hydrolysis with 6 N HCl, and TLC showed that there was no detectable amount of hydroxylamine in the hydrolysate of **1**. This unusual reaction mechanism will be discussed below. Incidentally, these experimental results led to the erroneous molecular formula, in which one oxygen was missing, at an early stage of structure elucidation studies of alahopcin[1].

^1H and ^{13}C NMR spectra of **1** and **2** (Tables 1 and 2, and also see Refs 2 and 3 for the ^1H NMR spectral data of **1**) show rather complicated spectra, which are explicable as **1** and **2** exist in a mixture of

Table 1. ^{1}H NMR data (at 400 MHz) of the amino acid **2**[a].

Assignment of protons		The major isomer[b]	The minor isomer[b]
γ	$-CH_2-$	2.23 (ddd, J=2.0, 9.0, 14.3)	1.67 (ddd, J=4.9, 8.0, 13.2)
		2.28 (ddd, J=6.3, 8.3, 14.3)	2.77 (ddd, J=7.0, 9.8, 13.2)
β	-CH-	3.38 (ddd, J=5.1, 8.3, 9.0)	3.21 (ddd, J=5.1, 8.0, 9.8)
α	-CH-	4.43 (d, J=5.1)	4.46 (d, J=5.1)
δ	-CH-	5.34 (dd, J=2.0, 6.3)	5.32 (dd, J=4.9, 7.0)

[a] In D_2O+DCl, chemical shift δ in ppm from internal DSS (J in Hz), with a Jeol JNM-GX400 FT NMR Spectrometer.
[b] Approximately in the ratio of the integration 1.7~1.9: 1.

Table 2. ^{13}C NMR data of **1** and **2**[a].

Carbon atom	Alahopcin (**1**)[b]	Amino acid **2**[c]
Cγ	29.0 (t)	28.8 (t) and 28.6 (t)[d]
Cβ	40.5 (d) and 41.1 (d)[d]	38.2 (d) and 38.9 (d)[d]
Cα	55.8 (d) and 56.0 (d)[d]	53.8 (d)
Cδ	82.3 (d) and 82.0 (d)[d]	81.6 (d) and 82.0 (d)[d]
Cβ'	17.3 (q)	
Cα'	50.0 (d)	
C=O	170.9 (s)	168.4 (s) and 169.4 (s)
	171.5 (s)	169.6 (s)
	175.1 (s) and 175.4 (s)	

[a] At 25.2 MHz, chemical shift δ in ppm from Me_4Si, calculated from internal 1,4-dioxane.
[b] In D_2O.
[c] In D_2O+DCl.
[d] The chemical shift of the minor component of tautomers.

Table 3. Comparison of ^{1}H NMR data of alahopcin (**1**)[a] in D_2O and D_2O plus DCl solution.

Assignment of protons		The major isomer Chemical shift (δ, ppm)		The minor isomer Chemical shifts (δ, ppm)	
		in D_2O	in D_2O+DCl	in D_2O	in D_2O+DCl
β'	CH_3-	1.53	1.53 (0.00)	1.53	1.53 (0.00)
γ	$-CH_2-$	2.04	2.16 (+0.12)	1.58	1.60 (+0.02)
		2.16	2.19 (+0.03)	2.62	2.69 (+0.07)
β	-CH-	3.17	3.41 (+0.24)	3.04	3.27 (+0.23)
α'	-CH-	4.07	4.12 (+0.05)	4.08	4.12 (+0.04)
α	-CH-	4.54	4.91 (+0.37)	4.56	4.91 (+0.35)
δ	-CH-	5.25	5.27 (+0.02)	5.20	5.27 (+0.07)

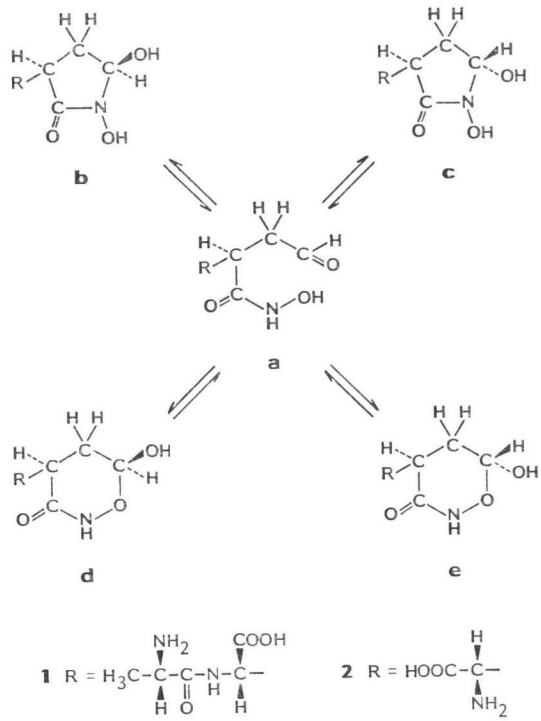
[a] Chemical shift δ in ppm from internal DSS with a Jeol JNM-FX400 FT NMR Spectrometer.

two tautomeric forms in aqueous solution and have the partial structure **1'** and **2'** (Fig. 3).

Furthermore, the ^{1}H NMR spectra (400 MHz) of **1** were measured in D_2O plus DCl to confirm the location of the free carboxyl group in **1**. As shown in Table 3, the α methine proton signals in the amino acid **2** moiety were shifted downfield by 0.35~0.37 ppm relative to those (δ 4.54 (major component) and 4.56 (minor component)) measured in D_2O. Additionally, coupling between the protons (α methine proton) corresponding to the signals at δ 4.41 (major component) and δ 4.38 (minor component) and the proton (amide proton) corresponding to the signal at δ 8.25 was revealed in the ^{1}H NMR spectral decoupling experiment of **1** in DMSO-d_6.

Reduction of **1** with sodium cyanoborohydride in aqueous acetic acid gave the deoxy derivative (**11**) ($C_9H_{15}N_3O_5$, MS (SIMS) m/z 246 $(M+H)^+$) which shows a positive (cherry-purple) color test with ferric chloride, and the structure of **11** was determined to be L-alanyl-2-(1-hydroxy-2-oxopyrrolidin-3-yl)glycine from its spectral data (^1H NMR (D_2O) δ 1.78 (3H, d, J=7, Ala CH_3), 1.8~2.8 (2H, m, CH_2), 3.22 (1H, ddd, J=5, 8, 9.5, CH), 3.79 (2H, dd, J=7, 8, CH_2), 4.35 (1H, q, J=7, Ala CH), 4.74 (1H, d, J=5, CH); IR ν_{max} (KBr, cm^{-1}) 1682, 1609). Catalytic hydrogenolysis of **11** with Raney Ni gave the dideoxy derivative (**12**) ($C_9H_{15}N_3O_4$, MS (SIMS) m/z 230 $(M+H)^+$) which shows a negative color test with ferric chloride, and the structure of **12** was determined to be L-alanyl-2-(2-oxopyrrolidin-3-yl)glycine from its spectral data (^1H NMR (D_2O) δ 1.79 (3H, d, J=7, Ala CH_3), 1.9~2.8 (2H, m, CH_2), 3.18 (1H, dt, J=5, 9, 9, CH), 3.58 (2H, dd, J=6, 8, CH_2), 4.35 (1H, q, J=7, Ala CH), 4.74 (1H, d, J=5, CH); IR ν_{max} (KBr, cm^{-1}) 1679, 1608).

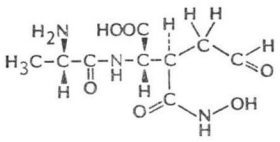
Fig. 4. Structures and probable tautomeric forms of alahopcin (**1**) and the amino acid **2**.



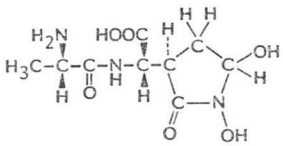
From the above results, the structure of **1** was determined to be (2*S*,3*R*)-2-[(L-alanyl)amino]-4-formyl-3-(hydroxyaminocarbonyl)butyric acid (**1a**, the open chain form of **1**), and it was revealed that **1** exists in the mixture of two forms of four probable cyclic hemiacetal type tautomeric forms (**1b**, **1c**, **1d** and **1e**) formed by the intramolecular ring closure between hydroxyamino group and formyl group of the open chain structure **1a** in aqueous solution, but the cyclic structures of two tautomers which are observed in the ^1H and ^{13}C NMR spectra have not yet been specified. The NMR spectra data of **1** are satisfactorily explicable as **1** exists in the mixture of two five-membered ring tautomers, L-alanyl-L-[(3*R*,5*S*)-1,5-dihydroxy-2-oxopyrrolidin-3-yl]glycine (**1b**) and L-alanyl-L-[(3*R*,5*R*)-1,5-dihydroxy-2-oxopyrrolidin-3-yl]glycine (**1c**) in aqueous solution, and it is also difficult to rule out the possibility of the six-membered ring tautomeric structures, L-alanyl-L-[(4*R*,6*S*)-6-hydroxy-3-oxoperhydro-1,2-oxazin-4-yl]glycine (**1d**) and L-alanyl-L-[(4*R*,6*R*)-6-hydroxy-3-oxoperhydro-1,2-oxazin-4-yl]glycine (**1e**). Additionally, the formation of **3** and ammonia by the hydrolysis of **1** with 6 N HCl can be understood by the fission of N–O bond in the 6-hydroxy-3-oxoperhydro-1,2-oxazin-4-yl moiety of the tautomeric form **1d** and **1e**.

On the basis of the results of the structural elucidation study of **1** and the spectral data of **2** described above, the structure of the open-chain tautomer (**2a**) of **2** was determined to be (2*S*,3*R*)-2-amino-4-formyl-3-(hydroxyaminocarbonyl)butyric acid and it is considered that **2** also exists in the mixture of two forms of four probable cyclic hemiacetal type tautomers (**2b**, **2c**, **2d** and **2e**) in aqueous solution. However, a significant difference was observed in the acid hydrolysis of **2** with 6 N HCl (at 120°C in a sealed tube for 16 hours) as compared with that of **1**. The acid hydrolysis of **2** resulted in extremely

Fig. 5. Structures of open chain form (**1a**) and probable cyclic form (**1b,c**) of alahopcin (**1**).

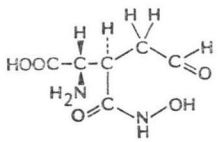


1a

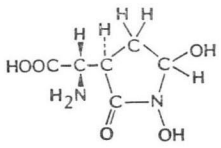


1b, c

Fig. 6. Structures of open chain form (**2a**) and probable cyclic form (**2b,c**) of the amino acid **2**.



2a



2b, c

low yield of **3** (0.17 mol/mol) and ammonia (0.23 mol/mol) with browning, and hydroxylamine was detected in the hydrolysate by TLC. These experimental results complicate the problem of the tautomerism of **2**, and the interaction between the α-amino group and the formyl group as well as the interaction between the hydroxyamino group and the formyl group must be taken into consideration in this case. Therefore, the structures of two ring tautomers of **2** which are observed in the ^{1}H and ^{13}C NMR spectra remain to be elucidated, although the five membered ring structures (**2b** and **2c**) are most likely from the ^{1}H and ^{13}C NMR spectral data of **2**.

Experimental

General

Melting points were determined with a Yamato MP-21 apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer model 141 or a polarimeter Jasco DIP-181. 90 MHz and 100 MHz ^{1}H NMR spectra were recorded with TMS as an external standard in D_2O and as an internal standard in $CDCl_3$ and DMSO-d_6 with a Varian EM-390 spectrometer (90 MHz) or a Varian XL-100A spectrometer (100 MHz). 400 MHz ^{1}H NMR spectra were recorded with sodium 2,2-dimethyl-2-silapentane-5-sulfate (DSS) as an internal standard with a Jeol JNM-GX400 or a Jeol JNM-FX400. ^{13}C NMR spectra were recorded with 1,4-dioxane as an internal standard with a Varian XL-100A spectrometer at 25.2 MHz, and chemical shifts are reported in ppm downfield from TMS. Mass spectra [SIMS (Secondary Ion Mass Spectra)] were recorded with a Hitachi M-80A mass spectrometer. IR spectra were recorded with a Hitachi 270-30 infrared spectrometer or a Jasco IRA-1 spectrometer. Amino acid analyses were performed on a Hitachi HPLC Model 835 amino acid analyzer (column: Hitachi Ion-exchange Resin #2619). Silica gel TLC was performed on pre-coated TLC plates Silica gel 60 F_{254} (Merck). Cellulose TLC was performed on pre-coated TLC plates Cellulose F (Merck) with BuOH - AcOH - H_2O (3: 1: 1) as a developing solvent. Ratios for a mixture of solvents are expressed by volume (v/v). Organic solvents were dried over anhydrous sodium sulfate before concentration. Solutions were concentrated under diminished pressure using a rotary evaporator.

Acid Hydrolysis of Alahopcin (**1**): Isolation of L-Alanine and α-Aminotricarballylic Acid (**3**) from the Acid Hydrolysate of **1**

A solution of **1** (1.0 g) in 6 N HCl (100 ml) was heated at 120°C in a sealed tube for 16 hours. The hydrolysate was concentrated to dryness. The residue was applied to a column of Dowex 1X2 (AcO^-, 100 ml). The column was developed with water to give L-alanine. The fractions containing L-alanine were concentrated to dryness. The residue was applied to a column of Dowex 50WX8 (400 ml, buffered with 0.1 M pyridine-formic acid buffer) and eluted with 0.1 M pyridine-formic acid buffer (pH 3.1). The eluate was concentrated to dryness and the residue was recrystallized from water - EtOH to give L-alanine (0.28 g) as colorless crystals; $[\alpha]_D^{23}$ +11.6° (*c* 0.5, 5 N HCl).

Anal Calcd for $C_3H_7NO_2$: C 40.44, H 7.92, N 15.73.
Found: C 40.22, H 8.25, N 15.43.

After elution of L-alanine with water, the Dowex 1X2 (AcO^-) column was washed with 1 M AcOH and then eluted with 3 M AcOH to give α-aminotricarballylic acid (**3**). Ninhydrin-positive fractions were

concentrated to dryness. The residue (369 mg) was chromatographed on a column of activated carbon (20 ml) with water and the eluate was concentrated to dryness. The residue (293 mg) was applied to a column of Sephadex LH-20 (500 ml, swollen with 70% MeOH) and the column was developed with 70% MeOH. The eluate was concentrated and the resulting aqueous solution was lyophilized to give **3** (208 mg) as a white solid; MS (SIMS) *m/z* 192 $(M+H)^+$; ^1H NMR (100 MHz, D_2O) δ 2.90 (2H, d, J=6.8 Hz, $-CH_2-$), 3.57 (1H, td, J=6.8 Hz, 6.8 Hz, 4.8 Hz, –CHCO–), 4.21 (1H, d, J=4.8 Hz, $-COCHNH_2$); ^{13}C NMR (D_2O) δ 34.0 (t), 42.5 (d), 55.6 (d), 172.3 (s), 175.4 (s), 176.2 (s).

Anal Calcd for $C_6H_9NO_6$: C 37.70, H 4.75, N 7.33.
Found: C 37.59, H 4.95, N 7.43.

(2*S*,3*R*)-5-Oxo-2,3-pyrrolidinedicarboxylic Acid (**4**)

A solution of **3** (600 mg) in water (60 ml) was adjusted to pH 2.9 and refluxed for 7 hours. After cooling to room temperature, the mixture was stirred with activated carbon. The filtrate was concentrated and lyophilized to give a white solid (550 mg), which was applied to a column of Sephadex LH-20 (500 ml, swollen with 70% MeOH). The column was developed with 70% MeOH, and the eluate was concentrated to dryness to give **4** (220 mg); $[\alpha]_D^{25}$ +46.7° (*c* 1, H_2O); ^1H NMR (90 MHz, D_2O) δ 2.88 (2H, d, J=8 Hz, 4-CH_2), 3.92 (1H, q, J=8 Hz, 3-CH), 4.77 (1H, d, J=8 Hz, 2-CH). The mono sodium salt of **4**: ^1H NMR (90 MHz, D_2O) δ 2.81 (2H, d, J=8 Hz, 4-CH_2), 3.77 (1H, q, J=8 Hz, 3-CH), 4.59 (1H, d, J=8 Hz, 2-CH).

N-Acetyl-5-oxo-2,3-pyrrolidinedicarboxylic Anhydride (**5**)

A solution of **4** (100 mg) in acetic anhydride (18 ml) was heated at 100°C for 30 minutes and at 140°C for 15 minutes. The mixture was cooled to room temperature to give **5** (33 mg) as colorless crystals. An additional crop (10 mg) was obtained by the concentration of the mother liquid; mp 224~225°C (dec); IR ν_{max} (KBr, cm^{-1}) 1870, 1808, 1750, 1717.

Anal Calcd for $C_5H_7NO_5$: C 48.74, H 3.58, N 7.11.
Found: C 48.47, H 3.60, N 7.18.

N-(2,4-Dinitrophenyl) Derivative of **1** (**6**)

A 5% (v/v) solution of 2,4-dinitrofluorobenzene in EtOH (10 ml) was added to a solution of **1** (480 mg) and $NaHCO_3$ (480 mg) in water (5 ml). The mixture was stirred for 5 hours at room temperature and concentrated to remove EtOH. The concentrate was washed with ethyl ether and acidified with 1 N HCl to give a yellow precipitate. The precipitate was collected by filtration and washed with ethyl ether to give the 2,4-dinitrophenyl derivative **6** (750 mg) as yellow powder, which was used for the subsequent hydrolysis without further purification.

Isolation of *N*-(2,4-Dinitrophenyl)alanine from the Acid Hydrolysate of **6**

A solution of **6** (500 mg) in 6 N HCl (50 ml) was heated in a sealed tube at 120°C for 16 hours. The mixture was extracted with ethyl ether and the organic layer was concentrated. The concentrate was applied to a preparative paper partition chromatography (Toyo filter paper No. 526; developing solvent, 1.5 M phosphate buffer (pH 6, 1 M NaH_2PO_4+0.5 M Na_2PO_4)). The appropriate band was cut out from the paper and extracted with water. The aqueous extract was acidified with 1 N HCl and extracted with ethyl ether. The etheral extract was concentrated to dryness and the residue was crystallized with ethyl ether - hexane to give *N*-(2,4-dinitrophenyl)alanine (50 mg); ^1H NMR (90 MHz, DMSO-d_6) δ 1.54 (3H, d, J=7.5 Hz, CH_3CH-), 4.62 (1H, quintet, J=7.5 Hz, CH_3CHNH-), 7.18 (1H, d, J=9.5 Hz, aromatic H at 6), 8.28 (1H, dd, J=9.5 Hz, 3 Hz, aromatic H at 5), 8.83 (1H, d, J=3 Hz, aromatic H at 3), 8.87 (1H, d, J=7.5 Hz, –NH–).

p-Nitrophenylhydrazone of **1**

1 (1.1 g) was dissolved in a 50% AcOH solution (40 ml) of *p*-nitrophenylhydrazine (1.2 g). The solution was stirred for 5 hours at room temperature. The mixture was filtered and the filtrate was concentrated to dryness. The residue was partitioned between ethyl ether (100 ml) and water (100 ml). The aqueous layer was washed with ethyl ether and concentrated to dryness. The residue (1.7 g) was dissolved in 1% AcOH (30 ml). The solution was chromatographed on Amberlite XAD-2 (120 ml) and eluted with 1% AcOH. The eluate was concentrated to dryness and triturated with EtOH to give

an orange powder (**7**, 0.85 g), which was used without further purification for the subsequent steps.

Acetylation of **7**

Acetic anhydride (1.0 ml) was added to a solution of **7** (0.66 g) in AcOH (6.5 ml). The resulting solution was stirred for 1 hour at room temperature and then MeOH (2.5 ml) was added to the solution to work up. The solution was concentrated to dryness and ethyl ether was added to the residue to give a yellow powder (0.78 g). The yellow powder was dissolved in MeOH (10 ml) and applied to a column of Sephadex LH-20 (370 ml, swollen with MeOH). The column was eluted with MeOH to give the *p*-nitrophenylhydrazone diacetate (530 mg), which was used without further purification for the subsequent steps.

Esterification of the *p*-Nitrophenylhydrazone Diacetate with Diphenyldiazomethane

The diacetate (0.34 g) was dissolved in MeOH (1 ml), and diphenyldiazomethane (200 mg) in MeOH (10 ml) was added to the solution. The solution was stirred for 1 hour at room temperature. Additional diphenyldiazomethane (40 mg) in MeOH (2 ml) was added and the solution was stirred for 1 hour at room temperature. The mixture was concentrated to dryness. The residue was triturated with ethyl ether to give a yellow powder. The yellow powder was extracted with chloroform and the chloroform solution was concentrated to dryness. The residue was dissolved in MeOH and chromatographed on a column of Sephadex LH-20 (380 ml, swollen with MeOH) with MeOH. The eluate was concentrated to dryness to give a yellow powder; ^{1}H NMR (90 MHz, $CDCl_3$) δ 1.29 (3H, d, J=7.5 Hz, CH_3CH–), 1.89 (3H, s, CH_3CO), 2.20 (3H, s, CH_3CO), 6.77 and 7.97 (each 2H, d, J=9.3 Hz, *p*-NO_2–C_6H_4–), 6.86 (1H, s, –CH–Ph_2), 7.26 (10H, s, –$CH(C_6H_5)_2$).

Sodium Borohydride Reduction of **1**: Preparation of L-Alanyl-5-hydroxy-3-(hydroxyaminocarbonyl)norvaline (**8**, Dihydroalahopcin)

Sodium borohydride (2.0 g) was added to a solution of **1** (1.0 g) in water (40 ml) under cooling (ice-water bath) and stirred for 3 hours at room temperature. The mixture was adjusted to pH 6.4 with 2 N HCl under cooling (ice-water bath) and concentrated. The residue was chromatographed on a column of activated carbon (600 ml) with water. The eluate was concentrated and the residue was re-chromatographed on a column of activated carbon (400 ml) with water. The eluate was concentrated and then lyophilized to give dihydroalahopcin **8** (400 mg) as a white solid; $[\alpha]_D^{21}$ −4.0° (*c* 1, H_2O); IR ν_{max} (KBr, cm^{-1}) 1674, 1613, 1539; MS (SIMS) *m/z* 264 $(M+H)^+$; ^{1}H NMR (100 MHz, D_2O) δ 1.77 (3H, d, J= 7 Hz, –CHCH_3), 1.9~2.25 (2H, m, 4-CH_2), 3.05 (1H, q, J=7 Hz, 3-CH), 3.8~4.0 (2H, m, 5-CH_2), 4.40 (1H, q, J=7 Hz, CH_3CH–), 4.66 (1H, d, J=7 Hz, 2-CH); TLC (cellulose) Rf 0.25 (*cf.* **1** Rf 0.23, **2** Rf 0.16).

Anal Calcd for $C_9H_{17}N_3O_5 \cdot 3/2H_2O$: C 37.24, H 6.94, N 14.48.
Found: C 37.08, H 7.09, N 14.14.

Acid Hydrolysis of Dihydroalahopcin (**8**) with 1 N HCl: Preparation of L-Alanyl-2-(2-oxotetrahydrofuran-3-yl)glycine (**9**)

A solution of **8** (275 mg) in 1 N HCl (20 ml) was allowed to stand for 18 hours at room temperature. The mixture was adjusted to pH 6.0 and concentrated. The residue was chromatographed on a column of MCI GEL CHP20P (400 ml; a synthetic porous polymeric absorbent, 150~300 μm, Mitsubishi Chem. Ind., Ltd.) and the column was eluted with water. The eluate was concentrated and the residue was re-chromatographed on a column of MCI GEL CHP20P (700 ml) with water. The eluate was concentrated to about 10 ml. EtOH (60 ml) was added to the concentrate and the mixture was allowed to stand in a refrigerator to give **9** as colorless crystals (168 mg); $[\alpha]_D^{21}$ +42.2° (*c* 1, H_2O); MS (SIMS) *m/z* 231 $(M+H)^+$; IR ν_{max} (KBr, cm^{-1}) 1756, 1677, 1638, 1607, 1524; ^{1}H NMR (90 MHz, D_2O) δ 1.79 (3H, d, J=7 Hz, –CHCH_3), 2.05~2.9 (2H, m, 4-CH_2), 3.47 (1H, dt, J=4.5 Hz, 10 Hz, 10 Hz, 3-CH), 4.35 (1H, q, J=7 Hz, –C*H*CH_3), 4.45~4.7 (2H, m, 5-CH_2), 4.86 (1H, d, J=4.5 Hz, α-CH); TLC (cellulose) Rf 0.42.

Anal Calcd for $C_9H_{14}N_2O_5$: C 46.95, H 6.13, N 12.17.
Found: C 46.56, H 6.11, N 12.21.

Proof of the Presence of Hydroxylamine in the Hydrolysate of **8** with 1 N HCl (by the Conversion of Hydroxylamine to *m*-Nitrobenzaldehyde Oxime)

A solution of **8** (50 mg) in 1 N HCl (5 ml) was allowed to stand for 18 hours at room temperature. The mixture was concentrated to dryness and dried *in vacuo*. A solution of *m*-nitrobenzaldehyde (100 mg) in MeOH (10 ml) was added to the solution of the residue in water (0.2 ml) and stirred for 3 hours at room temperature. The mixture was concentrated and the residue was partitioned between water and ethyl ether. The organic layer was separated, washed with water and concentrated. The residue was chromatographed on a column of silica gel (50 ml, Kieselgel 60, 70~230 mesh; Merck) and the column was washed with toluene and eluted with toluene - EtOAc (19: 1). The eluate was concentrated and petroleum ether was added to the residue to give *m*-nitrobenzaldehyde oxime as crystals (5 mg). IR spectrum (KBr) of the crystals was identical with that of the authentic samples; TLC (silica gel; toluene - EtOAc, 19: 1) Rf 0.17.

Acid Hydrolysis of L-Alanyl-2-(2-oxotetrahydrofuran-3-yl)glycine (**9**): Isolation of 2-(2-Oxotetrahydrofuran-3-yl)glycine (**10**)

The dipeptide **9** (20 mg) was dissolved in 6 N HCl (4 ml) and heated in a sealed tube for 16 hours at 120°C. The mixture was concentrated to dryness and dried *in vacuo*. The residue was chromatographed on a column of activated carbon (50 ml). The column was eluted with water and the eluate (70~220 ml) was concentrated to dryness. MeOH (60 ml) was added to the residue to give **10** (5.8 mg) as colorless needles; IR ν_{max} (KBr, cm^{-1}) 1760 (lactone); ^1H NMR (400 MHz, D_2O) δ 2.18 (1H, dq*, J= 9.3 Hz, 9.5 Hz, 9.8 Hz, 12.7 Hz, 4-CH), 2.52 (1H, dddd, J=2.8 Hz, 7.3 Hz, 9.8 Hz, 12.7 Hz, 4-CH), 3.28 (1H, dt, J=4.9 Hz, 9.8 Hz, 9.8 Hz, 3-CH), 4.16 (1H, d, J=4.9 Hz, α-CH), 4.38 (1H, dt*, J=7.3 Hz, 9.0 Hz, 9.5 Hz, 5-CH), 4.49 (1H, dt*, J=2.8 Hz, 9.0 Hz, 9.3 Hz, 5-CH) (*apparent multiplicity); TLC (cellulose) Rf 0.33.

Anal Calcd for $C_6H_9NO_4$: C 45.28, H 5.70, N 8.80.
Found: C 45.35, H 5.63, N 8.79.

Results of automatic amino acid analysis of the hydrolysate of **8** and **9** (each sample (5 mg) for hydrolysis was dissolved in 6 N HCl and heated in a sealed tube at 120°C for 16 hours) are set out as right Table.

Amino acid	Ala	**10**
Sample (retention time)	(43 minutes)	(19 minutes)
Hydrolysate of **8**	1.03 mol/mol	1.02 mol/mol
Hydrolysate of **9**	1.00 mol/mol	1.00 mol/mol

Sodium Cyanoborohydride Reduction of **1**: Preparation of L-Alanyl-2-(1-hydroxy-2-oxopyrrolidin-3-yl)glycine (**11**)

Sodium cyanoborohydride (2.0 g) was added to a solution of **1** (1.0 g) in AcOH - water (1: 2, 24 ml) and stirred overnight at room temperature. Water (200 ml) and Dowex 50WX8 (H^+, 100 ml) were added to the mixture and stirred for 1 hour at room temperature. The mixture was poured onto a column of Dowex 50WX8 (H^+, 60 ml) and the column was washed with water and eluted with 0.5 N ammonium hydroxide. The eluate was concentrated to dryness *in vacuo*. The residue was chromatographed on a column of Amberlite IRA-68 (AcO^-, 150 ml) and the column was eluted with water. The eluate was concentrated and the residue was chromatographed on a column of MCI GEL CHP20P (750 ml). The column was eluted with water and the eluate was concentrated and then lyophilized to give **11** (720 mg) as a white solid; $[\alpha]_D^{21}$ +49.3° (*c* 1, H_2O); IR ν_{max} (KBr, cm^{-1}) 1682, 1609; MS (SIMS) *m/z* 246 $(M+H)^+$; ^1H NMR (100 MHz, D_2O) δ 1.78 (3H, d, J=7 Hz, $-CHCH_3$), 1.8~2.8 (2H, m, 4-CH_2), 3.22 (1H, ddd, J=5 Hz, 8 Hz, 9.5 Hz, 3-CH), 3.79 (2H, dd, J=7 Hz, 8 Hz, 5-CH_2), 4.35 (1H, q, J=7 Hz, $-CHCH_3$), 4.74 (1H, d, J=5 Hz, α-CH); TLC (cellulose) Rf 0.34.

Anal Calcd for $C_8H_{15}N_3O_5 \cdot H_2O$: C 41.06, H 6.51, N 15.96.
Found: C 41.21, H 6.42, N 15.92.

Catalytic Reduction of L-Alanyl-2-(1-hydroxy-2-oxopyrrolidin-3-yl)glycine (**11**): Preparation of L-Alanyl-2-(2-oxopyrrolidin-3-yl)glycine (**12**)

A solution of **11** (200 mg) in water (20 ml) was adjusted to pH 7.2 with 1 N sodium hydroxide. The solution was hydrogenated with Raney Ni catalyst (200 mg) under atmospheric pressure at room tem-

perature for 4 hours (16 ml of hydrogen was absorbed). After the catalyst was removed, the filtrate was concentrated and the residue was chromatographed on a column of activated carbon (250 ml). The column was washed with water and eluted with water to MeOH gradient. The eluate was concentrated and the residue was chromatographed on a column of MCI GEL CHP20P (200 ml). The column was eluted with water, and the eluate was concentrated and then lyophilized to give **12** (86 mg) as a white solid; $[\alpha]_D^{21}$ +27.1° (*c* 1, H_2O); IR ν_{max} (KBr, cm^{-1}) 1679, 1608; MS (SIMS) *m/z* 230 $(M+H)^+$; ^1H NMR (90 MHz, D_2O) δ 1.79 (3H, d, *J*=7 Hz, $-CHCH_3$), 1.9~2.8 (2H, m, 4-CH_2), 3.18 (1H, dt, *J*=5 Hz, 9 Hz, 9 Hz, 3-CH), 3.58 (2H, dd, *J*=6 Hz, 8 Hz, 5-CH_2), 4.35 (1H, q, *J*=7 Hz, $-CHCH_3$), 4.74 (1H, d, *J*=5 Hz, α-CH); TLC (cellulose) Rf 0.38.

Anal Calcd for $C_9H_{15}N_3O_4 \cdot 3/2H_2O$: C 42.18, H 7.08, N 16.40.
Found: C 42.16, H 7.37, N 16.51.

Acid Hydrolysis of the Amino Acid **2** with 6 N HCl

The amino acid **2** (5.0 mg) was dissolved in 6 N HCl (1 ml) and heated in a sealed tube at 120°C for 16 hours. The hydrolysate was concentrated to dryness *in vacuo*, followed by amino acid analyses which gave **3** (0.17 mol/mol) and ammonia (0.23 mol/mol). The rest of the sample solution for amino acid analysis was concentrated to dryness. The residue was dissolved in water (0.1 ml), followed by adding a solution of *m*-nitrobenzaldehyde (10 mg) in MeOH (10 ml). The presence of *m*-nitrobenzaldehyde oxim in the reaction mixture was detected by TLC (silica gel; toluene - AcOH, 19: 1; Rf 0.17).

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