γ-CHLORONORVALINE, A LEUCINE ANALOG FROM *STREPTOMYCES*

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γ-Chloronorvaline (AL-719) and γ-hydroxynorvalinelactone (AL-719Y) were isolated from the culture broth of *Streptomyces griseosporeus* AL-719. Physico-chemical studies led to the structure elucidation of AL-719 and 719Y, with their respective configurations of (2*S*, 4*S*) and (2*S*, 4*R*). AL-719 shows antibacterial activity on a synthetic agar, especially against *Pseudomonas aeruginosa*, which was reversed by L-leucine. The producing strain AL-719 was characterized.

A wide variety of amino acid analogs has been discovered from plants and microorganisms,[1] in particular in the culture broths of *Streptomyces* sp.[2]. In the course of our screening program for new antibiotics, a *Streptomyces* strain with a code number AL-719 was found to produce an antimicrobial agent that was active against *Pseudomonas aeruginosa* on a synthetic medium. Chemical study of this agent herein-after designated as substance AL-719 revealed that it was a chlorine-containing antimetabolite of L-leucine, with one of the two methyl groups replaced by a chlorine.

This paper concerns the taxonomy of the producing organism, isolation and characterization of substance AL-719, together with its conversion product named substance AL-719Y.

Materials and Methods

Microbiological Assay

A paper disc agar-diffusion bioassay was used to detect and quantitatively measure substance AL-719 in broth and crude preparation. Inoculum was prepared from *Pseudomonas aeruginosa* grown overnight at 35°C on a rotary shaker in a medium composed of 0.2% yeast extract, 0.5% peptone, 2% sucrose, 0.2% Na glutamate, 0.2% K_2HPO_4, 0.01% $MgCl_2 \cdot 6H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$ and 0.001% $MnSO_4 \cdot xH_2O$ (pH 6.8). The cells were washed with water and added to one liter of liquefied minimal-agar containing glucose (10 g), Na glutamate (5 g), K_2HPO_4 (1 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), KCl (0.1 g), Na citrate (5 g) and agar (15 g).

Minimal-agar of the following composition was used for activity determination against other bacteria: glucose (10 g), NaCl (5 g), K_2HPO_4 (3 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $(NH_4)H_2PO_4$ (1 g) and agar (15 g).

Physico-chemical Measurements

Melting points were taken with a Yamato capillary apparatus, and are uncorrected. Spectral data were obtained as follows: IR spectra in KBr discs with a Hitachi model 215 IR spectrometer, NMR spectra in D_2O using a Varian XL-100 spectrometer, mass spectra with a JMS-01SG mass spectrometer,

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ORD and CD curves with a JASCO J-20 spectrometer at 20°C, and optical rotations with a Perkin-Elmer 141 polarimeter. D_2O and dioxane were used as external standard of PMR and internal one of CMR spectra, respectively. E. Merck silica gel F_{254} plates developed with *n*-BuOH - AcOH - H_2O (2: 1: 1) were used for thin-layer chromatography.

Antiviral Test

Monolayers of L-929 cells were grown in stationary microplates for 3 days. A dosage of one hundred $TCID_{50}$ of vesicular stomatitis virus Indiana strain in 0.05 ml of maintenance medium was added to the plates. Following viral adsorption, AL-719 and 719Y were added to the plates dissolved in the maintenance medium (0.05 ml). After 5 days of incubation at 37°C, cultures were examined microscopically for the presence or absence of viral cytopathic effect.

Efficacy was expressed by the difference in 50% infective titers of the sample and the control (Δ log $TCID_{50}$/ml). A value above 1.0 was considered as slightly effective, and that above 1.5 as effective. The maximum dose tolerated was defined as the highest concentration of the compound that did not affect the growth of cells.

Results and Discussion

Characterization of the Strain

Strain AL-719 was isolated from a soil sample collected from Jalalabad, U. P., India. The culture was maintained on yeast-malt agar and preserved by lyophilization. Standard methods of the International Streptomyces Project[3] were used for characterization. Observation on morphological, cultural and physiological characteristics were made on cultures grown at 28°±1°C for 14~21 days. The colors were designated by reference to the Colour Harmony Manual[4].

Strain AL-719 has the following morphological characteristics: The culture belongs to the section *Spirales*. The aerial mycelium is characterized by simple branching. Spore chains are produced in

Table 1. Cultural characteristics of strain AL-719*.

Medium	Growth and reverse color	Aerial mycelium	Soluble pigment
Yeast extract-malt extract agar (ISP medium 2)	excellent growth, yellowish to reddish brown	abundant, light grey to dark grey (2 ge/3 fe)	none
Oatmeal agar (ISP medium 3)	excellent growth, yellow to brown	abundant, light brownish grey (3 fe)	none
Inorganic salts starch agar (ISP medium 4)	excellent growth, yellow to brown	abundant, grey (2 fe)	light brown
Glycerol asparagine agar (ISP medium 5)	good growth, light yellowish brown	light bluish grey (d)	none
Peptone yeast extract iron agar (ISP medium 6)	good growth, dark blackish brown	none	dark blackish brown
Tyrosine agar (ISP medium 7)	excellent growth, dark brownish black	abundant, dark grey (2 dc/2fe)	light brownish black
CZAPEKS sucrose nitrate agar	good growth, yellowish brown	scant, whitish grey	light brown
Glucose asparagine agar	excellent growth, yellow to brown	abundant, grey (2 dc)	light brown
Yeast starch agar	excellent growth, dark brown	abundant, grey (2 fe)	light brown
Nutrient agar	scant growth, light yellowish brown	none	light brown

* grown at 28°C for 2 weeks.

simple spirals which are long, open and loose. Spores are cylindrical to ovoid and smooth-walled. The cultural characteristics are described in Table 1. The culture belongs to the "Gray Series" of TRESNER and BACKUS[5]. Physiological characteristics are given in Table 2. It utilized most of carbohydrates tested except cellulose. Sodium chloride tolerance is less than 4%. Growth of the strain is inhibited by low level of streptomycin.

Table 2. Physiological properties of strain AL-719.

Criterion	Remarks
Melanin formation	positive
Hydrolysis of starch	positive
Liquefaction of gelatine	negative
Peptonization of skim milk	negative
Coagulation of skim milk	negative
Nitrate reduction	positive
H_2S formation	positive

On the basis of these characteristics the strain most resembled *Streptomyces griseosporeus* NIIDA and OGASAWARA[6]. The sole difference was in the form of spore chains. Strain AL-719 clearly formed spirals, while *Streptomyces griseosporeus* did not and belonged to *Retinaculi-aperti*. However, the difference was ambiguous, particularly in the shorter-chained forms. Therefore, the strain was named as *Streptomyces griseosporeus* AL-719. It has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under accession number FERM-P4993.

Fermentation and Isolation

Inocula for production were prepared in a seed medium consisting of 20 g soluble starch, 10 g Polypeptone, 3 g meat extract and 0.5 g K_2HPO_4 per liter of tap water and adjusted to a final pH of 7.0. Test tubes containing 10 ml of this medium were inoculated, each with a loop of spores from the stock culture and incubated at 28°C for 20 hours on a reciprocal tube shaker at 300 rpm. These seed cultures were inoculated, one per flask, into forty 500-ml Erlenmeyer flasks, each containing 80 ml of the production medium consisting of 1.0% sucrose, 0.5% glycerol, 1.0% soybean meal, 0.25% fish meal and 0.01% $CaCO_3$ (pH 7.0), and incubated at 28°C for 22 hours on a rotary shaker at 220 rpm. The maximum potency (100 μg/ml) was obtained 20~24 hours after inoculation. Thereafter, the potency dropped gradually.

The broth of 20 hours culture (pH 6.5) was adjusted to pH 3.0 with 6 N HCl, and was filtered with 4% w/v of filter aid. The filtrate (2.6 liters) was passed through a column of Dowex 50W×2 (H^+ form, 100 ml). After washing with water, the column was eluted with 0.5 N NH_4OH, and the alkaline eluate

Fig. 1. IR spectrum of substance AL-719 in KBr.

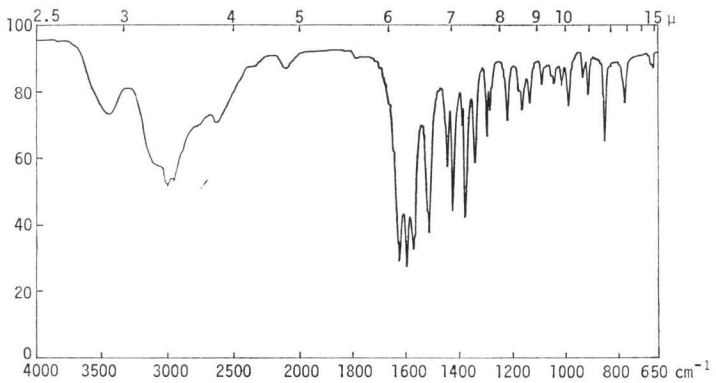
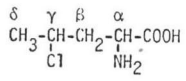
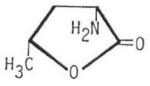


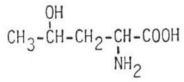
Chart 1.



I



II



III

Table 3. PMR data of substance AL-719 (I), AL-719Y (II) and γ-hydroxynorvaline (III) in D_2O.

Proton	Chemical shift and coupling constant		
	I	II	III*
α-CH	4.21	4.52	3.87
β-CH_2	2.35	2.04, 2.92	1.96
γ-CH	4.45	*ca* 4.8	3.96
δ-CH_3	1.60	1.45	1.20
$J_{\alpha\beta}$, $J_{\alpha\beta'}$	6, 8 Hz	8, 12 Hz	6 Hz
$J_{\beta\gamma}$, $J_{\beta'\gamma}$		4.5, 11	7.5
$J_{\gamma\delta}$	7	7	7

* Assignment was confirmed by double resonance.

was quickly evaporated to remove ammonia. The concentrated solution (250 ml) was passed through a column of DEAE-Sephadex A-25 (30 ml). The effluent was concentrated and chromatographed over Sephadex G-10 (500 ml), developing it with water. Active fractions were monitored by means of silica gel TLC. Rich fractions of Rf 0.48 (30 ml) were collected and lyophilized to give 97 mg of white powder. This was crystallized from water-methanol to afford colorless plates (50 mg) of AL-719. The isolation procedure described above was carried out in a cold room around 3°C due to unstability of the active ingredient.

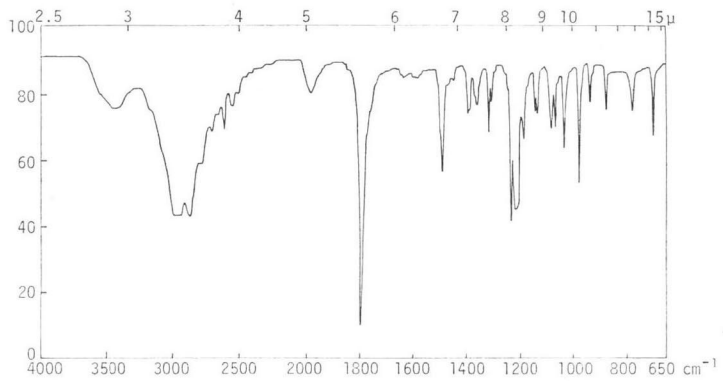
During the Sephadex G-10 chromatography, a ninhydrin-positive product of Rf 0.60 that was named substance AL-719Y was eluted subsequent to AL-719. Evaporation of solvent gave 100 mg of colorless crystals, which were recrystallized from water - methanol to give 60 mg of AL-719Y.

Physico-chemical Properties and Structures

Substance AL-719 occurred in colorless plates and melted at 162~165°C. It is soluble in water, and is hardly soluble in methanol, ethanol, chloroform, ethyl acetate and other organic solvents. It shows positive color reactions to ninhydrin and potassium permanganate. It is optically active, showing $[\alpha]_D^{25}$ +70° (*c* 0.2, 0.01 N HCl). No ultraviolet absorption maximum was observed. FD-mass spectrum showed $(M+1)^+$ at *m/z* 152, and an isotope-peak of a chlorine atom at *m/z* 154.

Elemental analysis: Found: C 39.49, H 6.57, N 9.12, O 22.15, Cl 22.67. Calcd. for $C_5H_{10}NO_2Cl$: C 39.60, H 6.60, N 9.24, O 21.12, Cl 23.43. IR spectrum of AL-719, shown in Fig. 1, indicated typical

Fig. 2. IR spectrum of substance AL-719Y in KBr.



bands of amino acid at 3150~2850 ($\nu_{NH_3^+}$), 1622 ($\delta_{NH_3^+}$), 1598 ($\nu_{C=O}$), 1518 ($\delta_{NH_3^+}$) and 1422 ($\nu_{C=O}$) cm^{-1}. PMR datum is summarized in Table 3, and its analysis led to the structure **I**, γ-chloronorvaline for AL-719. A quartet at 4.21 with AB_2 spin coupling characteristic for α-amino acid, could be assigned to α-methine. β-Methylene protons appeared at 2.35 as multiplet. A methyl doublet at 1.60 together with γ-methine sextet at 4.45, highly deshielded due to substitution of chlorine atom was in agreement with the partial structure $CH_3-\underset{|}{CH}-Cl$.

Substance AL-719Y was crystallized as hydrochloride, colorless plates melting at 198~200°C. It is soluble in water, less soluble in methanol, and showed yellow color by ninhydrin reagent. $[\alpha]_D^{25}+4.0°$ (*c* 1, H_2O). It showed end absorption in the ultraviolet spectrum. The molecular formula determined by CMR, mass and elemental analyses was $C_5H_{11}NO_2 \cdot HCl$; Calcd.: C 35.39, H 7.08, N 8.26, O 28.32, Cl 20.94. Found: C 35.73, H 7.10, N 8.36, O 27.66, Cl 21.15.

The off-resonance CMR analysis revealed, of the five carbons, one carbonyl at 174.4, two methines at 77.87 and 50.88, one methylene at 35.31, and one methyl at 20.39. IR spectrum, illustrated in Fig. 2, showed a strong band at 1800 cm^{-1} assignable to γ-lactone. EI-mass spectrum of AL-719Y hydrochloride showed ions at *m/z* 115 (M^+), 67 and 52, which were consistent with those reported for the γ-hydroxynorvalinelactone[7]. By combining these spectral data, the cyclic structure **II**, γ-hydroxynorvalinelactone was proposed for AL-719Y. This was supported by the PMR datum in Table 3. Ring opening of **II** by the addition of two molar equivalents of KOH followed by Sephadex G-10 chromatography yielded γ-hydroxynorvaline (**III**), whose structure was again supported by the PMR in Table 3.

Both AL-719 and 719Y have two asymmetric centers at C-2 and C-4, On measurement of ORD and CD curves in 0.1 N HCl - 90% MeOH, AL-719 showed a positive CD maximum at 213 nm with molecular ellipticity ($[\theta]+6340$), and a positive COTTON effect with the first extrema at 226 nm ($[\phi]+2660°$). This suggested L(*S*)-configuration at C-2, since L-amino acid shows a positive CD in absence of extra chromophore[8]. Analogously, 2 L(*S*) configuration was assigned to AL-719Y, since it exhibited CD curves similar to those of L-homoserinelactone[9], showing a positive maximum at 224 nm ($[\theta]+637$) at pH 2 and 220 nm ($[\theta]+6375$) at pH 7.5 in 90% MeOH.

4*R* configuration of AL-719Y was determined from the proton chemical shift of 4-methyl group. An epimeric mixture at C-4 was prepared by heating AL-719Y with 6 N HCl at 110°C for 48 hours[10], and showed two methyl doublets at 1.45 and 1.39. The methyl signal of natural origin was found to correspond to the more deshielded one at 1.45, indicating that CH_3 and NH_3^+ were in *cis*-orientation[10] in AL-719Y. The methyl signal at 1.39 coincided to that of (2*S*, 4*S*)-γ-hydroxynorvalinelactone, which was obtained by acid-catalized lactonization (6 N HCl at 110°C for 2 hours) of the γ-hydroxynorvaline from *Lathyrus odoratus*[11].

4*S* Configuration of AL-719 was deduced indirectly. AL-719 was very unstable in neutral and alkaline aqueous solution, and was quantitatively converted into AL-719Y, when kept at pH 6 at room temperature for 20 hours. Since there was no other product as examined by TLC of the reaction mixture, the formation of **II** from **I** could be rationalized by the intramolecular nu-

Table 4. Antibacterial spectrum of substance AL-719 on synthetic medium.

Test organism	Inhibitory diameter (mm)*			
	1,000 μg/ml	500 μg/ml	250 μg/ml	125 μg/ml
Pseudomonas aeruginosa	40.4	38.8	35.0	31.2
Serratia marcescens	17.0	13.0	10.0	t
Escherichia coli	0	0	0	0
Klebsiella pneumoniae	(18.2)	(16.0)	(14.0)	(11.0)
Bacillus subtilis	(16.0)	(13.0)	(t)	0

* t: trace; ( ): hazy zone

cleophilic replacement of carboxylate anion to the chlorine-containing γ-carbon, being accompanied with the inversion of C-4 configuration. Accordingly, (2*S*, 4*R*) and (2*S*, 4*S*) configurations were proposed to AL-719Y and AL-719, respectively. The above result suggested also that AL-719Y isolated from the fermentation broth might be an artifact, at least partly, formed from AL-719 during the isolation process.

Biological Properties

The antimicrobial spectrum of AL-719 was tested by the paper-disc diffusion method. It showed antibacterial activity only on a synthetic agar medium, as summarized in Table 4. AL-719 was active against *Pseudomonas aeruginosa*, and weakly so against *Serratia marcescens, Klebsiella pneumoniae* and *Bacillus subtilis*, but not against *Escherichia coli*. The antagonistic property of known amino acids was examined on the inoculated agar plates according to PRUESS and SCANNELL[2]. The anti-*Pseudomonas* activity of AL-719 was antagonized strongly by L-leucine, and weakly by L-isoleucine, L-valine and L-methionine. As judged from the semicircular distortion of the inhibition zone[2], competitive antagonism was suggested between them.

Substance AL-719Y was devoid of antibacterial activity, but both AL-719 and 719Y showed weak antiviral activity against vesicular stomatitis virus in the cell culture. They showed, respectively, Δlog $TCID_{50}$ values of 1.10 and 1.66 at the concentration of 500 μg/ml. The maximum tolerated dose in cell culture was 1,000 μg/ml in both the cases. No mice died by administering AL-719 at a dose of 100 mg/kg intraperitoneally.

Discussion

L-γ-Chloronorvaline (**I**) was once considered to be an antimetabolite of L-leucine[12], but no report appeared on the isolation and characterization of this compound. WIELAND *et al* prepared a diastereomeric mixture of **I** by the photochemical chlorination of L-norvaline, but, without isolation, converted it to γ-hydroxynorvalinelactone[13]. L-2-Amino-4,4-dichlorobutanoic acid (armentomycin), another leucine analog related to AL-719 was reported as a metabolite of *Streptomyces armentosus* var. *armentosus*[14].

γ-Hydroxynorvaline related to AL-719Y was found as a component of desmethylphalloin that was derived from phalloidins, phytotoxins of *Amanita phalloides*[10]. The free amino acid was discovered in the seed of *Lathyrus odoratus*[11].

Acknowledgement

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