STUDIES ON A NEW HERBICIDAL ANTIBIOTIC, HOMOALANOSINE†

SUSUMU FUSHIMI, SHIGERU NISHIKAWA, NOBUAKI MITO, MASAHIKO IKEMOTO††, MITSURU SASAKI and HARUO SETO†††

Agricultural Science Laboratory, Takarazuka Research Center,
Sumitomo Chemical Co., Ltd.,
Takatsukasa, Takarazuka, Hyogo 665, Japan
††Takatsuki Research Laboratory, Sumitomo Chemical Co., Ltd.,
Tsukahara, Takatsuki, Osaka 666, Japan
†††Institute of Applied Microbiology, The University of Tokyo,
Bunkyo-ku, Tokyo 113, Japan

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Homoalanosine having a herbicidal activity was isolated from the culture filtrate of a soil isolate SC-1688 which was classified as *Streptomyces galilaeus*. The chemical structure of homoalanosine was determined to be L-2-amino-4-nitrosohydroxyaminobutyric acid by analyses of spectral and biological data. The antibiotic has high herbicidal activity at low concentrations against especially common cocklebur and ladysthumb among the tested weeds and crops. Foliar application of this antibiotic inhibited the growth of roots and buds. This result indicated that homoalanosine had a systemic herbicidal activity.

In the course of our screening for new herbicidal antibiotics, we found that a soil isolate SC-1688 collected at Gonohe, Aomori Prefecture, produced an active material, homoalanosine. Physicochemical, NMR and biological studies of homoalanosine revealed that its chemical structure was L-2-amino-4-nitrosohydroxyaminobutyric acid. Although the racemate of this antibiotic had already been chemically synthesized[1] as a homolog of alanosine[2] and its insecticidal activity had been also reported[3], its isolation from natural sources and herbicidal activity have not been reported. This paper deals with the taxonomy of the producing strain and fermentation, purification procedures, structure determination, herbicidal activity and mechanism of action of homoalanosine.

Materials and Methods

Instruments

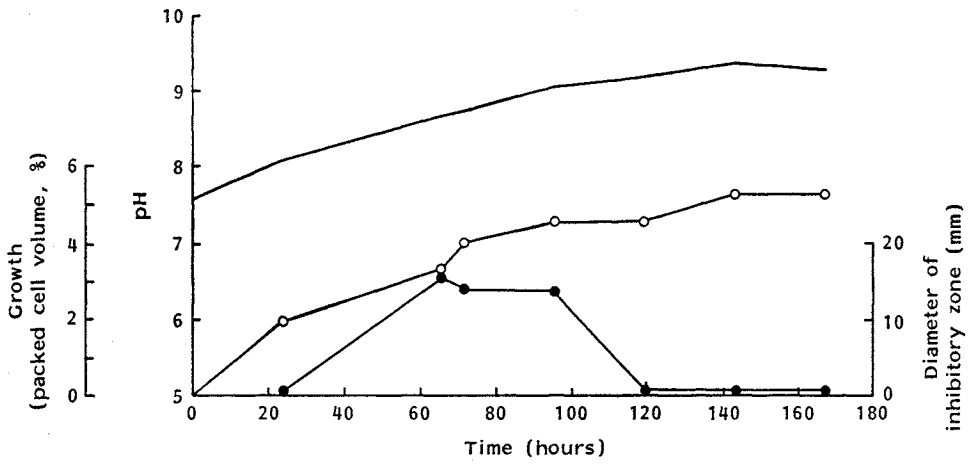
IR, NMR and secondary ion (SI)-MS spectra were recorded on a Hitachi IR spectrophotometer Model 270-30, a Varian NMR spectrometer XL-200 and a Hitachi M-80B mass spectrometer, respectively. Heteronuclear multiple bond correlation spectroscopy (HMBC) spectrum was recorded on a Jeol JNM-GX-500 spectrometer. A scanning electron micrograph was obtained with an electron microscope Hitachi S-430.

Screening Procedures

Actinomycetes isolated from soil samples were inoculated in Erlenmeyer flasks (200 ml) containing 60 ml of the screening medium consisting of soluble starch 3.0%, yeast extract 1.0%, NaCl 0.3% and $CaCO_3$ 0.3% (pH 7.2). After cultivating for 4 days at 28°C on a rotary shaker (150 rpm), broth filtrates were sprayed onto leaves of 1-week old radishes. The treated radishes were kept at 20~28°C for 2 weeks. Herbicidal activity was examined through a visual observation of the damaged area.

† Homoalanosine has been reported in Jpn. Kokai 259593 ('87), Nov. 11, 1987.

Fig. 1. Time course of homoalanosine production.
○ Growth, ● diameter of inhibitory zone, — pH.



Taxonomy

Taxonomy of the producing organism was performed by the method of SHIRLING and GOTTLIEB[4]. For experiments on cultural characteristics, all cultures were incubated at 28°C and were observed for 14~21 days. Utilization of carbon sources was examined by the method of PRIDHAM and GOTTLIEB[5]. The results were obtained after 14 days at 28°C. The type of diaminopimelic acid in the cell wall was determined by the method of BECKER *et al.*[6]

Culture Conditions

A loopful of a slant culture of SC-1688 was inoculated in a baffled Erlenmeyer flask (200 ml) containing 50 ml of the seed medium and incubated on a rotary shaker (150 rpm) at 28°C for 48 hours. The culture broth was transferred into baffled Erlenmeyer flasks containing 50 ml of the production medium and incubated again for 96 hours. The seed and production media were the same as the screening medium. Typical time course of homoalanosine production is shown in Fig. 1. Production of the antibiotic was monitored by measuring the inhibitory zone against *Bacillus subtilis* on synthetic medium (Davis medium).

Herbicidal Activity in Plow Field

Herbicidal activity of homoalanosine was examined by the following two experiments.

As the Expt 1, a mixture of the seeds of test plants were sowed to a vat which was filled with plow field soil and cultivated for 18 days at 20~28°C. The cultivated plants were treated with homoalanosine by foliar application and cultivated again. Visual assessment was made after 20 days by the observation of damaged area on a scale value 0 (no effect)~10 (dead).

Expt 2 was carried out in the same way as for Expt 1 except for that test plants were cultivated under condition of 15~19°C for 29 days followed by treatment with homoalanosine. Twenty five days after treatment, herbicidal activity was examined.

Herbicidal Activity under Flooded Condition in Paddy Field

Paddy field soil was filled in a Wagner pot, and then a mixture of the seeds of test plants were incorporated into the soil at the depth of 1 to 2 cm. After establishment of the condition of paddy field by flooding, rice plants at the 3rd leaf stage were transplanted and cultivated in a greenhouse at 20~28°C for 11 days. Homoalanosine diluted with water at appropriate concentrations was applied to the water surface, and the depth of water was maintained at 4 cm. During 2 days after treatment, water drainage was carried out at the rate of 3 cm/day. Twenty days after treatment, the herbicidal activity was examined in the same way as described above.

Antimicrobial Activity and Mechanism of Action

Antimicrobial activity of homoalanosine was examined by using paper-disc method. Each paper disc contained 20 μg of homoalanosine. Davis and nutrient agar media were used as synthetic and non-synthetic media, respectively. Davis medium consisted of glucose 0.5%, $Na_2HPO_4 \cdot 12H_2O$ 0.8%, KH_2PO_4 0.2%, trisodium citrate$\cdot 3H_2O$ 0.05%, $MgSO_4 \cdot 7H_2O$ 0.01%, $(NH_4)_2SO_4$ 0.1% and agar 1.5%.

Reversant was determined by using the counterdiffusion method[7].

Results

Taxonomy

Microscopic studies showed that aerial mycelia were formed from the branched substrate mycelia grown in various agar media. Mature spore-chains were flexuous or open spiral, and consisted of more than 10 spores per chain. Most of the spores were cylindrical ($0.36 \sim 0.56 \times 1.0 \sim 1.2$ μm) and possessed a smooth surface (Fig. 2). Typical verticillate aerial mycelium and the other special morphology were not observed. The cultural characteristics of strain SC-1688 on various media are presented in Table 1. Physiological properties of strain SC-1688 were as follows; gelatin liquefaction, starch hydrolysis, milk coagulation and melanin formation were all positive and temperature range for growth was 12~38°C. Strain SC-1688 utilized L-arabinose, D-xylose, D-glucose, D-fructose, sucrose,

Fig. 2. Scanning electron micrograph of the spores of strain SC-1688 (on yeast extract - malt extract agar).

Bar represents 0.5 μm.

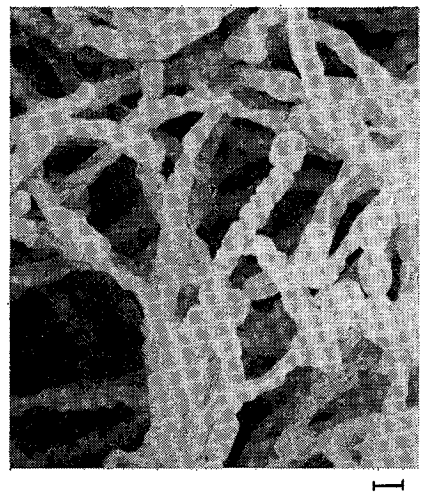


Table 1. Cultural characteristics of strain SC-1688.

Medium	Growth	Aerial mycelium	Reverse side color	Soluble pigment
Sucrose - nitrate agar	Good	Moderate, white (2)	Yellowish gray (20)	None
Glucose - asparagine agar	Good	Poor, white (1)	Pale yellow (46)	None
Glycerol - asparagine agar (ISP-5)	Good	Good, white (1)	White (2)	None
Inorganic salts - starch agar (ISP-4)	Good	Poor, white (1)	Grayish brown (78)	None
Tyrosine agar (ISP-7)	Good	Good, yellowish white (14)	Dark grayish brown (104)	Dark reddish brown (212)
Nutrient agar	Moderate	Very poor, medium gray (6)	Yellowish gray (20)	None
Yeast extract - malt extract agar (ISP-2)	Good	Good, brownish gray (25)	Dark brown (228)	None
Oatmeal agar (ISP-3)	Good	Good, medium gray (7)	Dark grayish brown (104)	None

Incubated at 28°C for 14 days.

Color and number in parenthesis followed "Color Tone Manual", Nihon Shikisai Kenkyujo, Japan.

inositol, L-rhamnose and raffinose, but did not utilize D-mannitol. Since whole cell hydrolysate contained LL-diaminopimelic acid, cell wall type of strain SC-1688 was classified as Type I. The microbial characteristics of strain SC-1688 were in good agreement with those of *Streptomyces galilaeus*. Therefore, the strain SC-1688 was identified as *S. galilaeus*. This organism has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with the accession No. FERM P-8715.

Purification of Homoalanosine

Purification procedures for homoalanosine are shown in Scheme 1. Broth filtrate was passed through an activated carbon column, and then adsorbed on a column of Amberlite CG-400 (OH^-). After the column was washed with water, the antibiotic was eluted with 0.2 N AcOH. The eluate was adsorbed on a column of Amberlite CG-120 (Na^+). After washing the column with water, homoalanosine was eluted with 0.9% aqueous ammonia. The eluate was concentrated *in vacuo* to afford small amount of solution and was chromatographed on a column of Amberlite CG-400 (equilibrated with 86 mM phosphate buffer pH 5.8) using the same buffer as an eluting solution. Active fractions were combined and applied to a column of Amberlite CG-120 (H^+). The column was washed with water and eluted with 0.9% aqueous ammonia. The eluate containing homoalanosine was concentrated *in vacuo* to afford a crude syrup. It was dissolved in water and applied to a column of Sepha-

Scheme 1. Purification procedures for homoalanosine.

Broth filtrate (2 liters)
↓
Activated carbon (850 g)
↓
Filtrate
↓
Amberlite CG-400 (OH^-) (640 ml)
  washed with water
  eluted with 0.2 N AcOH
Amberlite CG-120 (Na^+) (650 ml)
  washed with water
  eluted with 0.9% aq ammonia
  concd *in vacuo*
Amberlite CG-400 (125 ml)
  (equilibrated with 86 mM phosphate buffer pH 5.8)
  eluted with 86 mM phosphate buffer pH 5.8
Amberlite CG-120 (H^+) (80 ml)
  washed with water
  eluted with 0.9% aq ammonia
  concd *in vacuo*
Sephadex G-10 (3.5 × 131 cm)
  eluted with water
  concd *in vacuo*
Silica gel TLC, Kieselgel 60 F_{254}
  developed with
  BuOH - EtOH - $CHCl_3$ - 28% aq ammonia (4 : 5 : 2 : 8)
Amberlite CG-120 (H^+) (12 ml)
  washed with water
  eluted with 0.9% aq ammonia
  concd *in vacuo*
Colorless powder 10 mg

dex G-10 developed with water. Active fractions were combined and concentrated *in vacuo* to afford a pale yellowish powder. Further purification was carried out using preparative TLC. The pale yellowish powder was dissolved in water and chromatographed on silica gel TLC (Kieselgel 60 F_{254}) using solvent system consisting of BuOH - EtOH - $CHCl_3$ - 28% aqueous ammonia (4:5:2:8). Homoalanosine was collected under irradiation with UV lamp at 254 nm. The silica gel powder containing homoalanosine was extracted with water, and then adsorbed on a column of Amberlite CG-120 (H^+). After the column was washed with water, the antibiotic was eluted with 0.9% aqueous ammonia. The eluate was concentrated *in vacuo* to afford homoalanosine as a colorless powder. From 2 liters of culture broth, 10 mg of homoalanosine were obtained.

Structure Determination

Physico-chemical properties of homoalanosine are summarized in Table 2. The IR spectrum (Fig. 3) showed strong absorption at 1620 and 1407 cm^{-1} signifying the presence of nitrosamine and carboxylic acid groups, respectively.

The molecular formula of homoalanosine was established to be $C_4H_9O_4N_3$ by elemental analysis (Calcd for $C_4H_9O_4N_3 \cdot 1\frac{1}{2}H_2O$: C 25.26, H 6.32, N 22.11. Found: C 24.78, H 5.95, N 23.24) and analyses of NMR and MS (SI-MS 164 $(M+H)^+$) spectra. The ^{13}C NMR spectra revealed the presence of 4 carbons, which were

Table 2. Physico-chemical properties of homoalanosine.

Appearance	Colorless powder
Solubility	
Soluble:	H_2O
Insoluble:	MeOH, EtOH, acetone
MP	181~183°C
Molecular formula	$C_4H_9O_4N_3$
Elemental analysis	
Calcd for $C_4H_9O_4N_3 \cdot 1\frac{1}{2}H_2O$:	C 25.26, H 6.32, N 22.11
Found:	C 24.78, H 5.95, N 23.24
MW (SI-MS) *m/z*	164 $(M+H)^+$
$[\alpha]_D^{25}$	−11° (*c* 1.0, H_2O)
UV	
$\lambda_{max}^{0.1N\ HCl}$ nm (ε)	227 (5,470)
$\lambda_{max}^{0.1N\ NaOH}$ nm (ε)	248 (5,860)
Color reaction	Ninhydrin, positive (purple)
Rf values	0.48[a]
	0.10[b]

[a] Silica gel TLC, Kieselgel 60 F_{254}, BuOH - EtOH - $CHCl_3$ - 28% aq ammonia (4 : 5 : 2 : 8).
[b] Cellulose TLC, Cellulose F_{254}, BuOH - AcOH - H_2O (6 : 2 : 2).

Fig. 3. IR spectrum of homoalanosine (KBr).

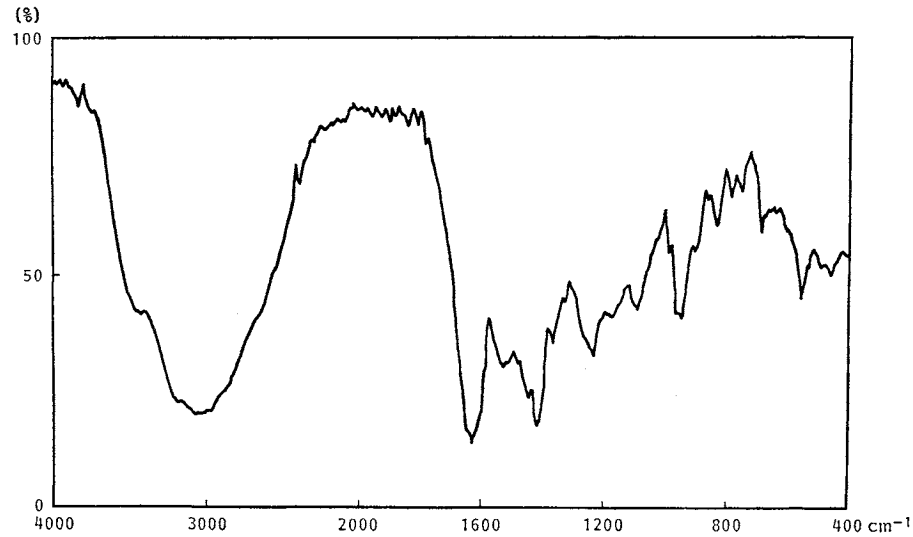
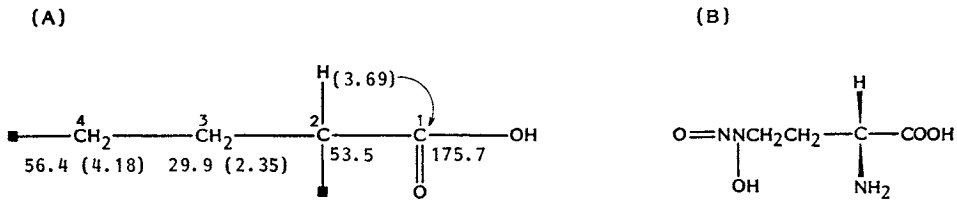


Fig. 4. Partial structure (A) and whole structure (B) of homoalanosine.



$\delta_C(\delta_H)$ in D_2O, pD 8.7. →: Long range coupling in HMBC.

attributed to two methylene carbons (29.9 and 56.4 ppm), one methine carbon (53.5 ppm) and one carbonyl (175.7 ppm). The ^1H NMR spectra showed the presence of 5 non-exchangeable protons which were ascribed to two methylene protons and one methine proton. Since total protons in homoalanosine are 9, 4 protons are exchangeable. The spin decoupling experiments clearly showed the presence of the spin system $-CH_2CH_2CH-$ in the molecule of this compound (Fig. 4A). The remaining carbon was carbonyl. The binding position of the carbonyl was determined to be at C-2 by the long range coupling between 2-H and carbonyl in the HMBC spectrum[8]. The C-1 carbonyl proved to exist as a carboxylic acid by the IR absorption at 1620 cm^{-1} and fragment ion peak at m/z 45 $(COOH)^+$.

The presence of an amino group was established by the color reaction (purple) to ninhydrin. The binding position of the amino group was determined by comparison of the NMR chemical shifts between homoalanosine and 2,4-diaminobutyric acid. The ^1H and ^{13}C NMR spectral data of 2,4-diaminobutyric acid are as follows: pD 8.7 (D_2O), δ_{C-1} 177.0 (s), δ_{C-2} 54.0 (d), δ_{C-3} 30.6 (t), δ_{C-4} 37.8 (t), δ_{2-H} 3.70 (t), δ_{3-H} 2.15 (m), δ_{4-H} 3.20 (t). The NMR chemical shifts at C-2 position (3.69 and 53.5 ppm) of homoalanosine were almost identical with those at C-2 (3.70 and 54.0 ppm) of the model compound. Therefore the binding position of the amino group was determined to be at C-2 carbon. Downfield shifts at C-4 position in NMR (4.18 and 56.4 ppm) suggested that a strong electron-withdrawing group was bound to C-4. The established partial structure at this point was confirmed by the corresponding fragment ion peak at m/z 102 $(C_4H_8O_2N)^+$.

Since there remained two oxygen, two nitrogen and one exchangeable proton, a nitramine or nitrosohydroxyamine group was bound to C-4. The characteristic UV absorption of the antibiotic indicated the presence of nitrosohydroxyamine. In acidic water solution, it showed a UV absorption maximum at 227 nm (ε 5,470), which shifted to 248 nm (ε 5,860) in alkaline water solution. This UV spectral characteristic was identical with those of alanosine[2] and propanosine[9] which had a nitrosohydroxyamine group in the structure. Since UV absorption of 2-amino-4-nitraminobutyric acid is 230 nm at pH 6.2 and 12.5[10], it was reasonable to conclude that homoalanosine had a nitrosohydroxyamine group at C-4 position. The IR absorption at 1407 cm^{-1} signifying the presence of the nitrosamine group supported this structure.

Thus, the whole planar structure of homoalanosine was determined as shown in Fig. 4B. The absolute configuration of C-2 proved to be L by the study on the action mechanism of homoalanosine described below. Although the racemate of this antibiotic had already been chemically synthesized as a homolog of alanosine[1], so far as we are aware, there was no report on isolation of homoalanosine from natural sources.

Herbicidal Activity of Homoalanosine

Although this antibiotic has been known to have insecticidal activity[3], its strong herbicidal ac-

Table 3. Herbicidal activities of homoalanosine.

Expt 1: Activity in plow field

Scientific designation	Common name	Leaf stage	Rate (g/a)[a]		
			40	10	2.5
Gossypium hirsutum	Cotton	1	7[b]	0	0
Pharbitis purpurea	Tall morning glory	4	6	1	0
Xanthium strumarium	Common cocklebur	3	10	9	8
Abutilon theophrasti	Velvetleaf	2	10	9	1
Solanum nigrum	Black nightshade	1	10	6	1
Zea mays	Corn	3	10	6	0
Echinochloa crus-galli	Barnyardgrass	2	7	5	0
Setaria viridis	Green foxtail	3	8	5	0

Expt 2: Activity in plow field

Scientific designation	Common name	Leaf stage	Rate (g/a)		
			40	10	2.5
Beta vulgaris	Sugar beat	0.5	5	6	3
Polygonum persicaria	Ladysthumb	2	10	10	10
Galium aparine	Cleavers	2	3	3	3
Stellaria media	Common chickweed	3	6	4	4
Veronica persica	Persian speedwell	1	6	6	7
Avena fatua	Wild oats	2	9	6	3
Alopecurus misouroibes	Black grass	3	2	2	2

Expt 3: Activity under flooded condition in paddy field

Scientific designation	Common name	Leaf stage	Rate (g/a)		
			40	10	2.5
Oryza sativa	Rice	4	0	0	0
Echinochloa oryzicola	Barnyardgrass	2	10	3	0
Eleocharis acicularis	Needle spickerush	4	10	4	0
Scirpus juncoides	Hardstem bulrush	2	7	0	0
Sagittaria pygmaea	Arrow head	3	10	0	0

Visual assessment was conducted on 20th day (Expt 1 and Expt 3) or on 25th day (Expt 2).

[a] g/are.

[b] Control rating (0: no effect, 10: all plants dead).

tivity has not been reported. Herbicidal activities of homoalanosine are summarized in Table 3. In Expt 1, homoalanosine showed herbicidal activity against various plants, especially, against common cocklebur and velvetleaf. Appearance of herbicidal activity was very slow. About 13 days after treatment, herbicidal effect began to appear. Since homoalanosine gave damage to buds and roots rather than treated leaves, the antibiotic was considered to be translocated in the plant symplastically. In Expt 2, this antibiotic showed herbicidal activity against various plants especially against ladysthumb. Appearance of herbicidal effect was very slow as in Expt 1. About 18 days after treatment, inhibition of the growth of roots and buds began to appear. In a paddy field experiment (Expt 3), homoalanosine gave damage not to rice plant but to various weeds.

Antimicrobial Activity and Mechanism of Action

Homoalanosine has not only herbicidal activity but also antimicrobial activity. Homoalanosine

exhibited antimicrobial activity against some Gram-positive bacteria only on synthetic media (Table 4). This characteristic in antimicrobial activity indicated that homoalanosine acts as an antimetabolite. The chemical structure of homoalanosine suggested that the antibiotic acted as an amino acid antimetabolite. Twenty kinds of standard L-amino acids were examined by using the counterdiffusion method. The results indicated L-aspartic acid and L-glutamic acid to be reversant (data are not shown). Although D-aspartic acid and D-glutamic acid were also examined, these D-form amino acids were not reversant. These biological activities of L and D isomers indicated that the absolute configuration of homoalanosine was L.

Table 4. Antimicrobial activity of homoalanosine.

Microorganisms	Diameter of inhibitory zone (mm)	
	Synthetic medium	Non-synthetic medium
Bacillus subtilis	41	0
Streptococcus faecalis	32	0
Pseudomonas fluorescens	0	0
P. glumae	0	0
Erwinia carotovora	0	0

Synthetic and non-synthetic media used were Davis and nutrient agar media, respectively. Each paper disc contained 20 μg of homoalanosine.

Discussion

The mechanism of action proved that homoalanosine is an L-aspartic acid and L-glutamic acid antimetabolite. In general, amino acid antimetabolites are translocated in the plant symplastically. Homoalanosine was also translocated in the plant symplastically because, when leaves were treated with this antibiotic the damaged parts of the plant were buds and roots rather than leaves. Since symplastic movement is required for herbicides to control perennial weeds, amino acid antimetabolites are suitable herbicides for controlling perennial plants. Among known amino acid antimetabolites, rhizobitoxin[11,12], L-2-amino-4-(2-aminoethoxy)-*trans*-3-butenoic acid[13], wildfire toxin[14,15], bialaphos[16~18], methionine sulfoximine[19] and oxetin[20] are known to have herbicidal activity. Bialaphos is in practical use. Modification of these compounds may lead to the appearance of novel herbicides to control perennial weeds.

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