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Detection, Characterization, and Phytotoxic Activity of the Nucleoside Antibiotics, Blasticidin S and 5-Hydroxylmethyl-Blasticidin S

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Abstract. Screening for herbicidal compounds carried out on culture broths of *Streptomyces* strains isolated from soil resulted in the detection of potent phytotoxic activity. The active principles were isolated, and identified as the nucleoside antibiotics Blasticidin S (Bl-S) and 5-Hydroxymethyl-Blasticidin S (H-M-Bl-S). Bl-S was more active than H-M-Bl-S on seedling germination in petri dishes and in postemergence greenhouse tests. Moreover both antibiotics were more phytotoxic to dicotyledonous than to monocotyledonous plants. The increased sensitivity of dicots was confirmed in carrot and rice cell cultures. Both compounds also inhibited [^{14}C]amino acid incorporation into proteins of rice and carrot cell cultures. Protein synthesis was more affected in carrot than rice.

Microorganisms influence plant growth in many different ways (Cutler 1988a). Increased sensitivity of environmental risks associated with the use of synthetic agrochemicals has stimulated greater interest in microorganisms as a potential source for safer pesticides (Cutler 1988b) and particularly for herbicides (Cutler 1988c, Duke and Lyndon 1987, Fisher and Bellus 1983). Studies of antibiotics to control plant diseases have been made since 1950, but the first "plant" antibiotic, Blasticidin S (Bl-S), was developed only in 1958, and about 6 years later its industrial production began.

During our screening program for new natural product herbicides, we found that a strain of Streptomyces sp. SD-702 (Donegani Collection) isolated from soil, produced a spent culture broth that was strongly active against dicotyledonous plants. Chemical analysis revealed that the active principles in the broth were Bl-S and 5-Hydroxymethyl-Blasticidin S (H-M-Bl-S) (Fig. 1).

Bl-S, a nucleoside antibiotic produced by *Streptomyces griseochromogens* (Otaki et al. 1966, Takeuki et al. 1958), is active against the phytopathogenic fungus *Piricularia oryzae* and it also possesses some antimicrobial activity (Yonehara 1984). It acts against fungi and bacteria by inhibiting protein synthesis with a mechanism similar to Puromycin (Yonehara 1984). H-M-Bl-S has recently been isolated together with Bl-S from the culture broth of *Streptomyces setonii* (Larsen et al. 1989).

A number of nucleoside antibiotics have been described as plant growth inhibitors, and, among them, gougerotin (Murao and Hayashi 1983) and rodaplutin (Dellweg et al. 1987) are structurally very similar to Bl-S. Further examples of phytotoxic nucleoside antibiotics are tubericidin (Fisher and Bellus 1983), 5'-O-sulfamoyl-tubericidin (Isono 1988), toyocamycin (Yamada et al. 1972), sangivamycin (Fisher and Bellus 1983), herbiplanin (Fisher and Bellus 1983), and the herbicidins (Arai et al. 1973).

Although some data concerning Bl-S phytotoxicity have already been reported (Agnistikova et al. 1970, Shogaki and Yoshida 1977), no systematic work on its herbicidal properties have been carried out and, concomitantly, nothing is known about its mode of action in plants. Moreover, no previous data on the phytotoxic activity of H-M-Bl-S have been reported.

This paper deals with the isolation, identification, phytotoxic properties, and mode of action of these metabolites.

Materials and Methods

Culture Conditions

An agar slant culture of *Streptomyces* sp. SD-702 (Donegani collection) was used to inoculate an Erlenmeyer flask (500 ml) con-

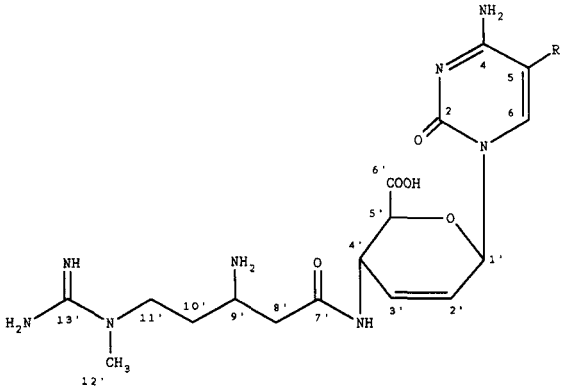

Fig. 1. Structure of Bl-S and H-M-Bl-S. R = H, Bl-S; R = CH_2OH, H-M-Bl-S.

taining 100 ml of a seed culture medium consisting of soluble starch (Carlo Erba, Italy; 20 g/L), glucose (10 g/L), $CaCO_3$ (3 g/L), casein hydrolysate (Oxoid, UK; 2 g/L), cottonseed flour (Traders Protein Div., USA; 2 g/L), yeast extract (Oxoid, UK; 2 g/L), and beef extract (Difco, USA; 2 g/L) adjusted to pH 7.0 with 1 N HCl.

The flask was incubated on a rotary shaker (180 rpm) at 28°C for 55 h, then the culture was transferred into 1 L of the same medium. After an incubation period of 40 h at 28°C (150 rpm), the culture broth was used to inoculate a 40-L fermentor containing 28 L of a medium consisting of glycerol (30 g/L), cottonseed flour (20 g/L), and $CaCO_3$ (3 g/L) adjusted to pH 6.5–7.0 with 1 N HCl.

The final fermentation was carried out at 28°C for 72 h with a minimal O_2 concentration of 20%.

Isolation of Antibiotics

After fermentation, 50 L of culture broth were separated from the mycelium cake by centrifugation. The mycelium cake was stirred overnight with a solution of water–ethanol ratio of 1:1 (vol/vol). The extract obtained was filtered to remove cell debris, and then concentrated under vacuum until the ethanol was completely removed. The remaining aqueous solution was added to the broth. The solution was ultrafiltered on a Desal G50 membrane (cut-off ≈15000) with a Hydro Air module (Hydro Air Research srl, Milan, Italy), and concentrated by reverse osmosis on a DDS-HC50 membrane (cut-off ≈500) with a DDS module (DK4900 Nakskow, Denmark).

The concentrate was adsorbed onto a macroreticular neutral resin, XAD-4 (Rohm and Haas Co., Philadelphia, PA USA), washed with two bed volumes of water, and eluted with a gradient of 20–80% acetonitrile in water.

The separation of H-M-Bl-S from Bl-S was achieved by chromatography on a reverse-phase silica C-18 (Amicon Europe, Lausanne, Switzerland) column by eluting with a solution of 10 mM KH_2PO_4 adjusted to pH 3 with H_3PO_4. After neutralization, the two groups of active fractions, containing each antibiotic, were pooled and concentrated. The antibiotics were desalted by adsorbing on macroreticular neutral resin XAD-4 and washing with water until no salts were detected in the effluent. The antibiotics were eluted with an acetonitrile-water solution, 1:1 (vol/vol), concentrated, and freeze-dried to obtain H-M-Bl-S (approximately 50 mg) and Bl-S (approximately 30 mg) as a white amorphous powder.

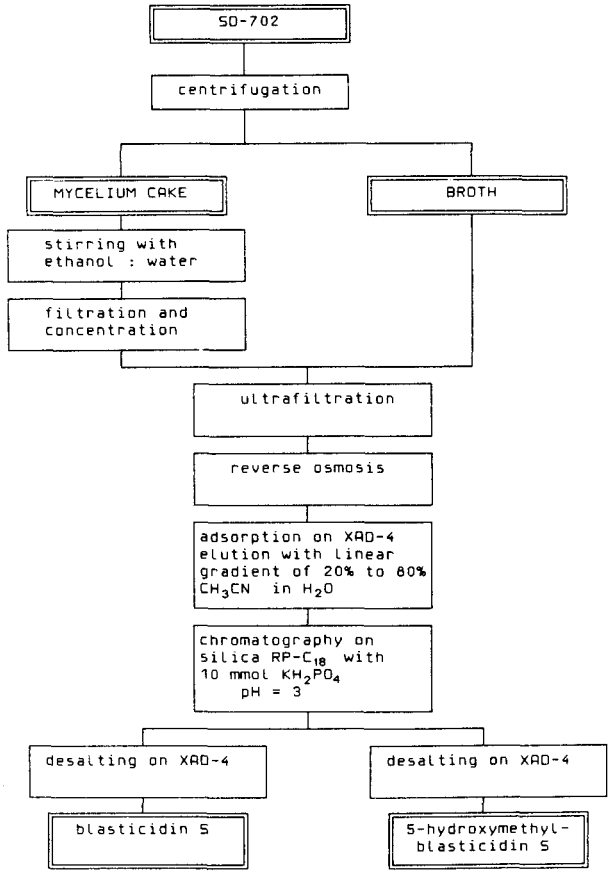

Fig. 2. Isolation procedure of the antibiotics.

High-performance liquid chromatographic (HPLC) assays were performed on a Lichrosorb RP-18 (Merck, Darmstadt, Germany) cartridge column (7 μm, 250 × 4 mm ID) equipped with a guard-cartridge, μBondapak RP-18 (Millipore/Waters, Milford, MA, USA), maintained at 40°C. A 10 mM KH_2PO_4 solution, adjusted to pH 3 with H_3PO_4, was used as the mobile phase at a flow rate of 1 ml/min. The chromatograms were analyzed with a photodiode array UV detector (Waters). The following instruments were used: two M510 pumps, a U6K injector, a 990 S photodiode array detector, a RCM-100 column chamber (all from Millipore/Waters) and an APC III computer (NEC Corporation, Tokyo, Japan). The retention time of H-M-Bl-S was 11.45 min, whereas the retention time of Bl-S was 10.27 min.

The steps leading to the isolation are outlined in Fig. 2.

Phytotoxicity Bioassays

Seedlings. Bl-S and H-M-Bl-S were tested in the laboratory on barley (*Hordeum vulgare* L. cv. Opale), rice (*Oryza sativa* L. cv. Balilla), and garden cress (*Lepidium sativum* L.) seedlings. The compounds were dissolved in distilled water to yield a 2.4 × 10^{-2} M stock solution. Ten seeds per sample were sown in Petri dishes (9 cm diameter) on two filter paper disks soaked with 5 ml of water with or without inhibitors and incubated in the dark at 26°C (relative humidity = 90%) for 3 days (cress), 4 days (bar-

ley), or 8 days (rice). The length of roots and stems or coleoptiles was then measured, and the dose inhibiting growth by 50% (ID_{50}) was derived from the dose–response curves. These bioassays were also carried out during fermentation of the producing microorganism and the purification steps, to monitor the biological activity.

Postemergence. Bl-S and H-M-Bl-S were tested in postemergence tests on the following monocot and dicot weeds: barnyard grass (*Echinochloa crusgalli* Beauv.), smooth broome (*Bromus inermis* Leyss.), Italian ryegrass (*Lolium multiflorum* Lam.), common cowpea (*Vigna sinensis* Endl.), common chickweed (*Stellaria media* Cyr.), bush morning glory (*Ipomea leptofilla* Torr.), field bindweed (*Convolvolus arvensis* L.), and Persian speedwell (*Veronica persica* Poir.). Seeds were sown in sterilized soil and grown in a greenhouse, with a temperature of 20°C at night and 25°C during the day. As soon as the first leaf was expanded (Graminaceous weeds), or the cotyledonary leaves were produced (dicot weeds), the plants were treated by foliar application. An area of 0.2 m^2 was sprayed with 20 ml of a solution containing the inhibitor at the concentration of 1 g/L (1000 ppm), that corresponded to a 1 kg/ha dose. Four weeks after treatment, herbicidal activity was examined. Visual assessment was made on a percentage scale: 0, no effect; 100, all dead plants (Table 2).

Cell Culture Bioassays

Culture Conditions. Carrot (*Daucus carota* L. cv. Lunga di Amsterdam) and rice (*Oryza sativa* L. cv. Roncarolo) were grown in a cell suspension culture at 26°C on a rotary shaker with a photoperiod of 16 h. Carrot cultures were maintained by transferring, every 10 days, 10 ml of cell suspension in 100 ml of Murashige and Skoog (MS) culture medium (Murashige and Skoog 1962) containing 2×10^{-6} M 2,4-D (2,4-dichlorophenoxyacetic acid) and 10^{-6} M kinetin. Rice culture maintenance was carried out by transferring, every 14 days, 10 ml of cell suspension in 100 ml of R2 medium (Ohira et al. 1973) with 2 mg/L 2,4-D.

Growth Inhibition. Cell samples were withdrawn from the stock cultures during the late exponential growth phase. Spent culture medium was discarded and cells were inoculated into 50-ml culture flasks containing 10 ml of medium (MS or R2) supplemented with different concentrations of Bl-S and H-M-Bl-S. The inoculum size was calculated on a dry weight basis and was approximately 10 or 20 mg for carrot and rice. Cells were incubated until controls reached the stationary growth phase, monitored by controlling the increase in sedimented cell volume in the graduated side-arm of the flasks. Fresh weight of cell samples was determined after vacuum filtration through a nylon cloth (pore size 25 μm). Dry weight was determined on the same samples after drying in an oven at 100°C for 24 h. The dose (based on net increase in cell dry weight) inhibiting growth by 50% (ID_{50}) was derived from the resulting dose–response curves.

Uptake and Incorporation of [^{14}C]Amino Acids. Experiments were carried out as described by Ciarrocchi et al. (1981) and Cella et al. (1982). Five milliliters of freshly diluted cultures of carrot and rice, corresponding to approximately 50 mg dry weight, were pretreated with or without the inhibitors for 1 h in a rotary shaker at 27°C before adding 0.5 μCi/ml (18.5 KBq/ml) of L-[U-^{14}C]amino acids mixture. The final amino acid concentration was 1 mM. After 30 min at 27°C with careful shaking, 0.5-ml aliquots were collected by vacuum filtration on Whatman GF/C filters, and the samples were washed with 20 ml of the same unlabeled ice-cold medium in which the amino acid concentration was 100 mM. Cells were then resuspended in 2 ml of distilled water, broken in a Teflon-in glass potter homogenizer, and 100 μl of 10% SDS (sodium dodecyl sulfate) were added. For the uptake experiments, 200-μl aliquots of the homogenate were withdrawn and placed directly into scintillation vials, together with 4 ml of Instagel (Packard). Proteins to be measured for [^{14}C]amino acid incorporation were precipitated from the remaining 1.8 ml cell homogenate with 3 ml of 10% TCA (trichloroacetic acid). The precipitate was filtered on a Whatman GF/C membrane, washed with 4 × 10 ml of 5% TCA, and finally with 95% ethanol. Filters were then dried under an infrared lamp and their radioactivity was counted after the addition of 4 ml of Instagel. Radioactivity was measured by a Packard scintillation counter. Incorporation values were calculated by the ratio between incorporated and taken up radioactivity, in order to correct for possible uptake inhibition.

Results

Identification of Antibiotics

Bl-S and H-M-Bl-S were identified by means of MS and NMR data.

The physicochemical data for Bl-S have already been reported in several papers (Otake et al. 1966, Takeuchi et al. 1958, Yonehara 1984); therefore, we report only those that supplement the reported data (Larsen et al. 1989) for H-M-Bl-S.

The physicochemical properties of H-M-Bl-S are very similar to those reported for Bl-S, the only structural difference being the presence of the hydroxymethyl group on the cytosine ring in H-M-Bl-S (Fig. 1). The absorptions of this group are lacking in the NMR spectra of Bl-S, while they are clearly displayed at 4.43 ppm in the ^{1}H spectrum and at 60.5 ppm in the ^{13}C spectrum of H-M-Bl-S. Both ^{1}H and ^{13}C NMR spectra (Fig. 3) were obtained in D_2O with a Bruker AM 300 spectrometer. Chemical shift assignments are listed in Table 1.

H-M-Bl-S Exhibited pH-Dependent UV Spectra

The absorption maximum was at 276.8 nm (ϵ = 9605) in 0.1 N HCl and at 270.0 nm (ϵ = 7765) in 0.1 N NaOH.

The molecular weight of H-M-Bl-S is 452 Da, corresponding to a molecular formula $C_{18}H_{28}N_8O_6$. The molecular weight was determined by FAB-MS experiments, which gave a $[M + H]^+$ ion at m/z 453 in the positive ion mode, using xenon as bombarding gas and glycerol as matrix.

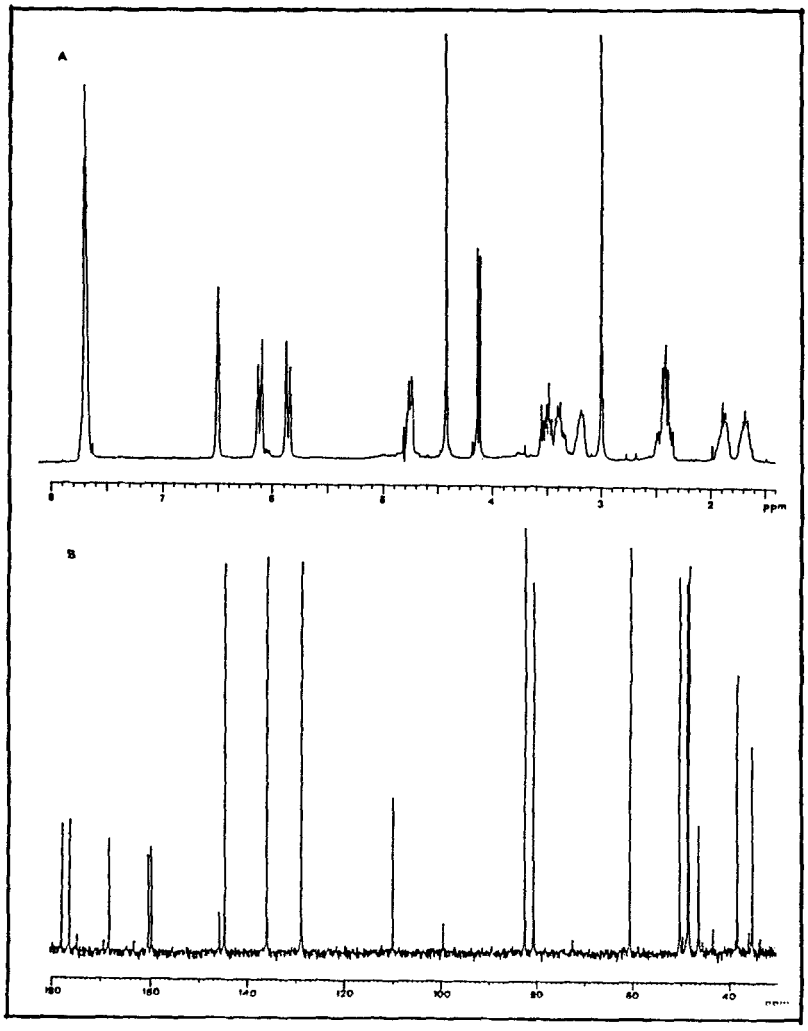

Fig. 3. NMR spectra of Bl-S and H-M-Bl-S in D_2O: **(A)** ^1H NMR; **(B)** ^{13}C NMR.

Phytotoxic Activity of Bl-S and H-M-Bl-S

Bl-S and H-M-Bl-S inhibited shoot and root elongation in barley, rice, and garden cress seedlings germinated in Petri dishes. The data in Fig. 4 show that garden cress was the most sensitive species to both Bl-S (ID_{50} 1.23 and 0.28 μM for shoots and roots, respectively) and H-M-Bl-S [ID_{50} 3.47 (shoot) and 1.4 (root) μM]. Barley and rice had considerably higher than those detected in garden cress. the ID_{50} for barley coleoptiles was greater than 71 μM for treatment with Bl-S and greater than 66 μM for treatment with H-M-Bl-S; for rice coleoptiles, the ID_{50} values were 18.8 and 42.8 μM for treatments with Bl-S and H-M-Bl-S, respectively. The ID_{50} for Bl-S in barley and rice roots was 8.5 and 9.9 μM, respectively, whereas the ID_{50} of H-M-Bl-S was 38.0 and 20.2 μM, respectively. Taken as a whole, these results indicate that (a) root growth is inhibited more than shoot growth; (b) Bl-S is a stronger inhibitor than H-M-Bl-S, the activity of Bl-S being approximately two to five times higher than that of H-M-Bl-S.

To confirm the differences among the ID_{50} values determined in garden cress (dicot), rice, and barley (monocots)—possibly indicating a good selectivity of the two inhibitors toward dicot plants—a few monocot and dicot weeds were treated postemergence at a 1 kg/ha dose of the inhibitors (Table 2). After a 4-week incubation period, monocots were almost unaffected (5–10% of growth reduction), whereas dicots exhibited strong growth inhibition which was higher for Bl-S (average inhibition value = 98%), but lower for H-M-Bl-S (average inhibition value = 64%). Only chickweed (*Stellaria media*) was insensitive to the latter compound.

The observed differential susceptibility of monocot and dicot plants could be caused by morphological or metabolic differences. As "in vitro" cultures are a particularly suitable system for investigating metabolic parameters, we studied the effects of Bl-S and H-M-Bl-S on the growth of rice and carrot

Table 1. Chemical shift assignments of ^{1}H and ^{13}C NMR in D_2O of H-M-Bl-S.

Index of atoms	^{1}H NMR (ppm)	^{13}C NMR (ppm)
2	/	159.7
4	/	160.3
5	/	110.0
6	7.64	144.5
1′	6.48	82.5
2′	6.10	135.9
3′	5.86	128.8
4′	4.75	48.3
5′	4.13	80.7
6′	/	177.7
7′	/	176.1
8′	2.46–2.40	46.3
9′	3.21	50.2
10′	1.90–1.69	35.1
11′	3.52–3.40	48.6
12′	3.00	38.3
13′	/	168.2
5-CH_2OH	4.43	60.5

cells. The results of these experiments (Fig. 5A and B) strongly confirmed: (a) the higher phytotoxicity of Bl-S, and (b) the higher sensitivity of dicot versus monocot, with either inhibitor. In fact, the ID_{50} value for Bl-S was more than three times lower than that for H-M-Bl-S, in either rice or carrot cells, and the ID_{50} of both antibiotics was 500 times lower in the dicot, carrot, than in the monocot, rice.

Effect of Bl-S and H-M-Bl-S on Protein Synthesis

Since Bl-S is known as a protein synthesis inhibitor in fungi (Yonehara 1984), we investigated the effects of Bl-S and H-M-Bl-S on the uptake and incorporation into proteins of [^{14}C]amino acids in carrot and rice cell cultures. Figure 6 shows that the [^{14}C]amino acid uptake was inhibited by increasing either Bl-S or H-M-Bl-S concentration in the medium. In the 1–470 μM concentration range, Bl-S inhibited amino acid uptake from 41–76% in carrot cells, and from 21–35% in rice cells (Fig. 6A). H-M-Bl-S showed a lower inhibitory activity, ranging from 2–70% in carrot cells, and from 0–22% in rice cells (Fig. 6B). The effects of Bl-S and H-M-Bl-S on amino acid incorporation into rice and carrot proteins are shown in Fig. 6C and D. In carrot, amino acid incorporation was inhibited by Bl-S, the values varying from 77–87% in a concentration range from 1–470 μM. For rice (Fig. 6C), the same increase in Bl-S concentration corresponded to a greater reduction in amino acid incorporation (from 19–81%).

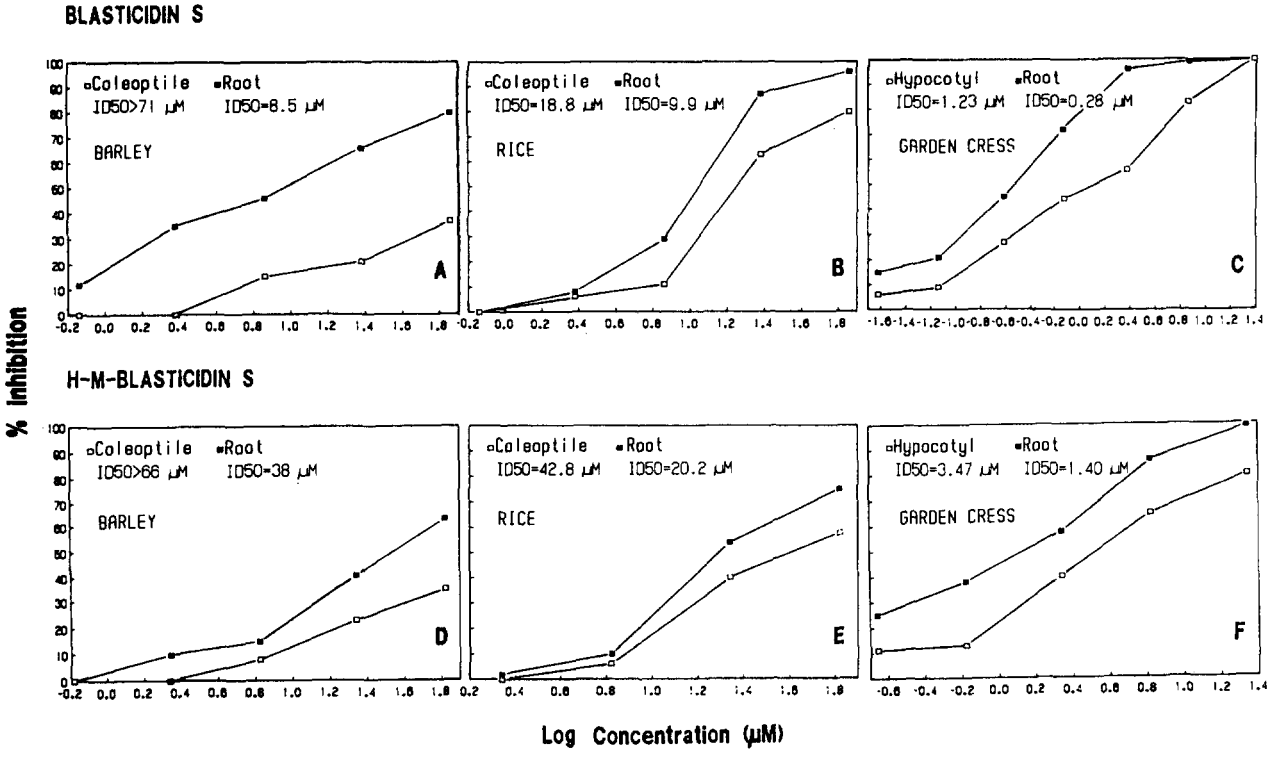

Fig. 4. Effect of Bl-S (A–C) and H-M-Bl-S (D–F) on the growth of rice, barley, and garden cress seedlings. Each value is the mean of six replicates. SE did not exceed ±5%.

Table 2. Effect of postemergence Bl-S and H-M-Bl-S application on shoot growth of several weeds.

Species tested	% of shoot growth inhibition	
	Bl-S	H-M-Bl-S
Monocot weeds		
Echinochloa crus-galli	10	10
Bromus inermis	10	10
Lolium multiflorum	5	5
Dicot weeds		
Vigna sinensis	100	70
Stellaria media	95	10
Ipomea purpurea	100	100
Convolvolus arvensis	95	70
Veronica persica	100	70

Inhibitors were added at 1 kg/ha rate. Treatments and control were repeated twice.

H-M-Bl-S markedly inhibited amino acid incorporation into carrot cells proteins in a dose-dependent manner, the inhibition values varying from 23% for the lower (1 μM) concentration, to 89% at 470 μM (Fig. 6D). When the antibiotic was similarly fed to rice cells, a considerable decrease in amino acid incorporation (approximately 70%) was observed only at the highest concentration.

Discussion

We have presented the isolation, purification, and some biological properties of Bl-S and H-M-Bl-S, two nucleoside antibiotics of particular interest because of their phytotoxic activity.

An interesting feature of both nucleoside compounds is their selectivity against dicot cells and plants. In Petri dish tests, garden cress seedlings were more sensitive than rice and barley, and, in greenhouse postemergence tests, dicot weeds were strongly damaged, whereas monocot weeds were mostly unaffected. In order to verify that such an apparent selectivity was not caused by morphological differences in whole plants, we employed cell cultures. They are homogeneous, cuticle-free systems that require little intercellular transport, and the environmental conditions can be rigorously controlled. Moreover, absorption and translocation to the active site are the most important limiting factors for the induction of herbicidal activity. Our results, from rice (monocot) and carrot (dicot) cells, indicate that the observed selectivity relies on some metabolic mechanism, rather than on morphological differences. A few possible hypotheses may be proposed to explain the different sensitivities of rice and carrot cells. First, a difference in the uptake rate at the plasmalemma level, second, a different inhibitory activity at the target level, and, third, a different potential for metabolizing the antibiotic molecule. As far as the first two aspects are concerned, two mechanisms of resistance to Bl-S in fungi have been reported (Misato 1967). In *Pellicularia sasakii* (Cooke), a naturally resistant species, the protein-synthesizing system is tolerant to the antibiotic both in whole cells and in cell-free extracts. On the other hand, a mutant of *Piricularia oryzae* (Cavara) exhibits tolerance based on impermeability to the antibiotic. We are not aware of any report describing the degradation of Blasticidin-like compounds by any organisms. However, we know that the only difference between Bl-S and H-M-Bl-S is the presence in H-M-Bl-S of a hydroxy-methyl group on the cytosine ring, and it is known that hydroxylic groups can be easily involved in conjugation reactions in plant cells (Swetser 1985, Swetser et al. 1982). Since the biological activity of nucleoside antibiotics in microorganisms is due to protein synthesis inhibition (Misato 1967), we have thoroughly investigated to see if this mode of action also occurs in higher plant cells. Bl-S and H-M-Bl-S inhibited the incorporation of amino acids into proteins in both carrot and rice cells showing a higher activity in dicot species than monocot ones. More-

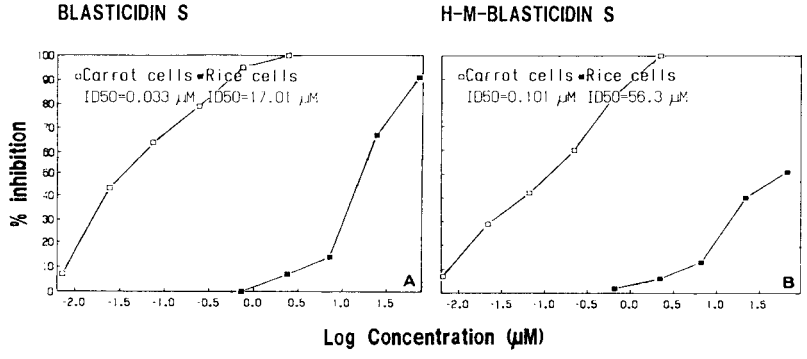

Fig. 5. Effect of Bl-S and H-M-Bl-S on the growth of cultured carrot and rice cells. Each value is the mean of four replicates. SE did not exceed ±5%.

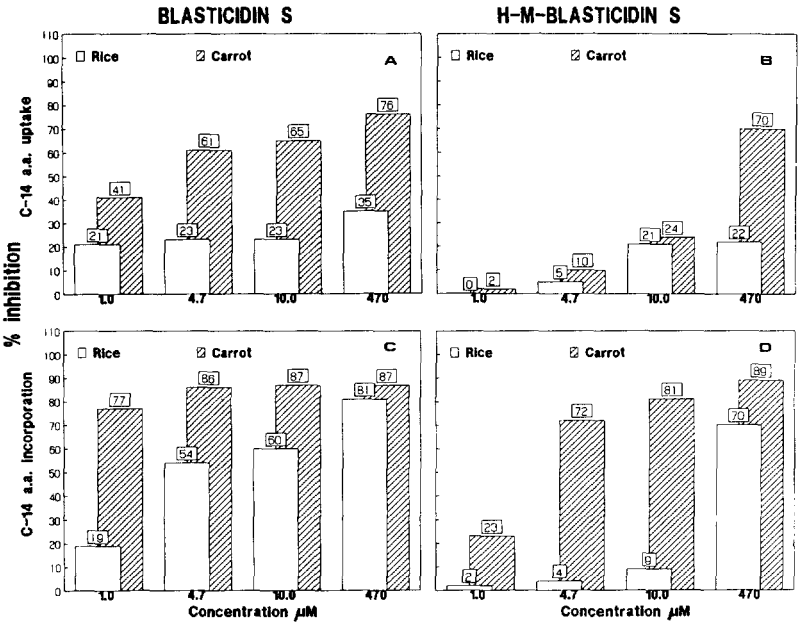

Fig. 6. Effect of Bl-S and H-M-Bl-S on uptake and net incorporation into proteins of [^{14}C]amino acids in rice and carrot cells. Each value is the mean of three replicates. SE did not exceed ±6%.

over, carrot and, to a lesser extent, rice cells also seem to be damaged by Bl-S and H-M-Bl-S at the amino acid uptake level. This could be a consequence of a progressive restriction in the availability of carrier proteins, which occurred during the 60-min preincubation of cells with the inhibitors. On the other hand, the lower uptake inhibition in rice cells can further contribute to explain its higher resistence to these compounds.

Further research on the molecular mode of action, activity spectrum, persistence, and nontarget effects of these antibiotics needs to be accomplished in order to define their development into practical use. Ideally, a herbicide should affect some plant-specific process not found in mammals and other nontarget organisms. Unfortunately, the aspecific mode of action of Bl-S and H-M-Bl-S makes them unsuitable for use in agriculture.

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