# HYDANTOCIDIN: A NEW COMPOUND WITH HERBICIDAL ACTIVITY FROM *Streptomyces hygroscopicus*

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Hydantocidin†, a new compound with potent non-selective herbicidal activity, was found in a submerged culture of *Streptomyces hygroscopicus* SANK 63584. It was isolated from the culture filtrate by the following successive treatments comprised of activated carbon, Diaion HP-20, Dowex 50WX4, and Avicel column chromatographies. Finally it was crystallized as colorless needles from acetone.

The molecular formula, $C_7H_{10}N_2O_6$, was determined by analyses of HRFAB mass spectrum in conjunction with $^{13}C$ NMR spectrometry. The structural elucidation revealed that it has a unique structure with a spiro-bond between ribose and hydantoin moieties in the molecule. The characteristic herbicidal activities against annuals as well as perennials, including monocotyledonous and dicotyledonous weeds, were observed.

In the course of screening for new herbicidal substances from culture broths of microorganisms, we found a new compound, hydantocidin, produced by a streptomycete identified as *Streptomyces hygroscopicus* SANK 63584, which was isolated from a soil sample collected in Annaka, Gunma Prefecture, Japan.

In this paper, we report the taxonomy, fermentation of the producing strain, isolation, physico-chemical and biological properties of hydantocidin. The detailed herbicidal activity and the structural elucidation of the compound will be reported elsewhere (under preparation).

## Materials and Methods

Taxonomic Studies

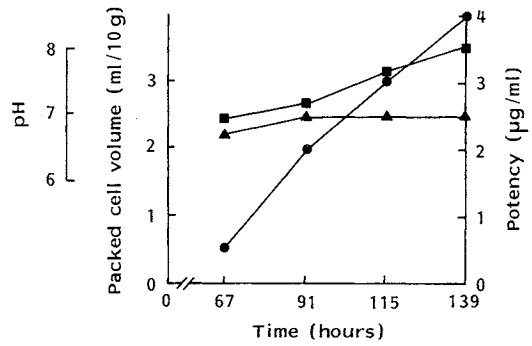
The producing organism, SANK 63584, was isolated from a soil sample collected in Annaka, Gunma Prefecture, Japan.

† Jpn. Kokai 12789 ('87) Jan. 21, 1987 [Eur. Pat. Appl. 232, 572 Aug. 19, 1989].

Taxonomic studies were carried out according to the procedure of the International Streptomyces Project[1]. The color recorded for the mature culture was described according to Guide to Color Standard[2]. Diaminopimelic acid in the whole-cell was analyzed by the method of BECKER *et al.*[3]. This strain was deposited as FERM BP-958.

Fig. 1. Fermentation of hydantocidin.

● Potency, ▲ packed cell volume, ■ pH.




## Preparation of a Spore Suspension of the Producing Organism and Fermentation

To maintain a constancy of the fermentation process for hydantocidin production, a spore suspension of SANK 63584 was conveniently used as a seed culture.

The spore suspension was prepared by the following method. A 2-liter Erlenmeyer flask containing 210 g of wet rice, which had been immersed in water for about 30 minutes and filtered to remove the excess amount of water, was autoclaved for 45 minutes at 120°C. A loopful of growth of the strain SANK 63584 was inoculated onto this medium and incubated at 28°C under an appropriately moistened atmosphere. After 3 weeks, it was suspended in 1,000 ml of sterilized water and transferred directly into a 600-liter fermenter containing 300 liters of the medium composed of glucose 3%, pressed yeast 1%, soybean meal 3%, $CaCO_3$ 0.4%, $MgSO_4$ 0.2%, and Nissan Disfoam CB-442 0.02%. To keep the D.O. level between 0.2~11 ppm, fermentation was carried out at 28°C for 139 hours with an air-flow rate of 150 liters/minute and an agitation rate adjusted between 100~150 rpm.

The packed cell volume was determined after centrifugation of 10 g of the culture broth at 3,000 rpm for 15 minutes. The time course of the fermentation was monitored by the potency of the germination-inhibitory activity, and the maximum potency of hydantocidin reached around 4 $\mu$g/ml at 139 hours, as shown in Fig. 1.

## Potency of Germination Inhibition of Plant Seeds

The potency of hydantocidin in culture broth or partially purified samples was determined by the effect on germination of seeds of Chinese cabbage. Eight to ten seeds were placed on an absorbent cotton bed (5 mm in height from the bottom of a 10 × 100 mm test tube) moistened with 0.5 ml of the specimens to be tested.

The MIC at a 2-fold dilution was determined by the term of the lowest concentration required for inhibition of germination of all of the seeds after incubation of the test tubes at 28°C for 3 days.

## Antimicrobial Activity

The antimicrobial activity of hydantocidin against a total of 30 species of Gram-positive and Gram-negative bacteria, as well as yeasts and fungi, was tested by conventional paper disc-agar diffusion method using various media, such as Nutrient agar (Eiken Co., Ltd., Japan) for bacteria at 37°C, Sabouraud-dextrose agar (Nissui Co., Ltd., Japan) for yeasts at 30°C, and Potato-dextrose agar (Nissui Co., Ltd., Japan) for fungi at 26°C.

## Herbicidal Activity

Seeds of crops and weeds, including mono- and di-cotyledonous plants, were sown on soil stuffed in flowerpots, and then covered with soil 5 to 10 mm in depth.

The pots were placed in a greenhouse for 2 weeks to allow the plant to grow well, and then the sample solutions to be tested were directly sprayed over the leaves and stems of the plants.

The herbicidal effect was determined on the 14th day after application of the samples and scored as a grade of effectiveness as follows: In comparison to an untreated control, a score of 0 indicates 0~5% damage to the area of the stems and leaves, 1 indicates 5~20% damage, 2 indicates 20~50% damage, 3 indicates 50~80% damage, 4 indicates 80~95% damage, and 5 indicates 95~100% damage.

Isolation

Three hundred liters of the culture broth from the 600-liter fermenter were filtered with Celite 545, and the cake was washed with water. Two hundred and ninety liters of the filtrate, including the washing, were passed through 30 liters of Diaion HP-20 column, and the effluent was applied onto 90 liters of an activated carbon column. After washing the column with 90 liters of water, the active principle was eluted with a 30% solution of aqueous methanol.

The pooled active fractions were evaporated *in vacuo* to a small volume and applied on 30 liters of a Diaion CHP-20P column, which was developed with deionized water. The active fractions thus obtained were concentrated *in vacuo* and lyophilized in the presence of Avicel. The powder thus obtained was suspended in acetonitrile and layered gently on the top of the Avicel column packed with acetonitrile. The column was developed with 100% acetonitrile, followed by aqueous 95% and 90% acetonitrile solutions, respectively, and the active principle was found in the 90% aqueous acetonitrile fraction. After concentration of the active eluate, the concentrate was applied onto 800 ml of a column of Dowex 50WX4 ($Ca^{++}$), and the column was developed with deionized water, and the eluate was collected in fractions of 10 ml. The active fractions thus obtained were concentrated to a small volume and applied onto 1 liter of a Diaion CHP-20P column and eluted with water.

By lyophilization of the active fraction, the purified hydantocidin was obtained as 408 mg of white amorphous powder. One hundred twenty mg of the sample were dissolved in a minimum amount of hot acetone and left for hours at room temperature to obtain 37 mg of colorless needles of hydantocidin.

## Results and Discussion

### Taxonomy of Strain SANK 63584

The vegetative hyphae of the microorganism are observed generally on various agar media. The strain forms spiral sporophores branching monopodially on aerial hyphae. Spores are covered with a capsule-like membrane with a fairly irregular or rugose, possibly warty surface (Plate 1). Special structures, such as sporangia, zoo-spores, ball-like bodies, or sclerotia were not observed on the media employed. The cultural characteristics on various agar media at 28°C for 14 days are shown in Table 1. The color of the vegetative mycelium is pale yellowish brown to pale yellowish orange. The strain forms a brownish white to brownish gray aerial mycelium. Sometimes, moist black, liquefied (hygroscopic) areas are found on the aerial mycelium of aging cultures. The strain grows within the temperature range of 11 to 46°C. Hydrolysis of starch and liquefaction of gelatin are positive.

Other physiological properties, including those properties described above, are shown in Table 2.

Since strain SANK 63584 can grow weakly on basal medium without any added carbon source, it is difficult to describe exactly its ability to utilize a carbon source. The relative utilizations are shown in Table 3, taking the utilization of the above basal medium as a negative control. The whole-cell analysis of the strain showed the presence of LL-diaminopimelic acid, and it was classified as chemotype I/NC.

Based on the taxonomic properties described above, the strain SANK 63584 is considered a member of the genus *Streptomyces*. By comparison

Plate 1. Scanning electron micrograph of spores of strain SANK 63584 on potato extract-carrot extract agar.

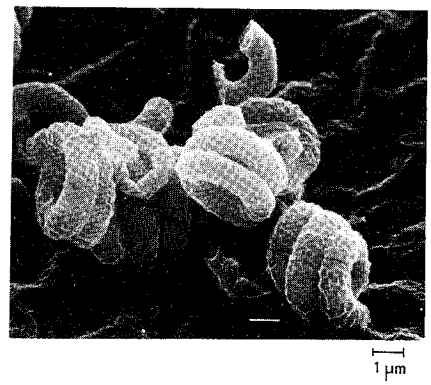


Table 1. Cultural characteristics of strains SANK 63584 and *Streptomyces hygroscopicus* NRRL 2387.

| | | SANK 63584 | NRRL 2387 |
|---|---|---|---|
| Yeast extract-malt extract agar (ISP 2) | G: | Abundant, flat, pale yellowish brown | Abundant, flat, pale yellowish brown |
| | AM: | Abundant, velvety, brownish gray | Abundant, velvety, brownish gray |
| | R: | Yellowish brown | Yellowish brown |
| | SP: | None | None |
| Oatmeal agar (ISP 3) | G: | Abundant, flat, pale yellowish brown | Abundant, flat, yellowish brown |
| | AM: | Abundant, velvety, brownish white | Abundant, velvety, brownish white |
| | R: | Yellowish brown | Yellowish brown |
| | SP: | None | None |
| Inorganic salts-starch agar (ISP 4) | G: | Abundant, flat, pale yellowish brown | Abundant, flat, pale yellowish brown |
| | AM: | Abundant, velvety, brownish gray | Abundant, velvety, brownish white |
| | R: | Yellowish brown | Pale yellowish brown |
| | SP: | None | None |
| Glycerol-asparagine agar (ISP 5) | G: | Abundant, flat, pale yellowish orange | Abundant, flat, pale yellowish brown |
| | AM: | Moderate, velvety, white | Moderate, velvety, white |
| | R: | Pale yellowish orange | Pale yellowish brown |
| | SP: | None | None |
| Peptone-yeast extract-iron agar (ISP 6) | G: | Abundant, flat, pale yellowish brown | Abundant, flat, pale yellowish brown |
| | AM: | Poor, velvety, white | Poor, velvety, white |
| | R: | Pale yellowish brown | Pale yellowish brown |
| | SP: | None | None |
| Tyrosine agar (ISP 7) | G: | Abundant, flat, pale yellowish brown | Abundant, flat, pale yellowish brown |
| | AM: | Moderate, velvety, white | Good, velvety, light brownish white |
| | R: | Yellowish brown | Yellowish brown |
| | SP: | None | None |
| Sucrose-nitrate agar | G: | Moderate, flat, pale yellowish orange | Moderate, flat, pale yellowish orange |
| | AM: | Poor, velvety, brownish white | Poor, velvety, brownish white |
| | R: | Pale brown | Pale yellowish brown |
| | SP: | None | None |
| Glucose-asparagine agar | G: | Good, flat, pale yellowish orange | Good, flat, pale yellowish orange |
| | AM: | Poor, velvety, white | Poor, velvety, white |
| | R: | Pale yellowish orange | Pale yellowish orange |
| | SP: | None | None |
| Nutrient agar (Difco) | G: | Good, flat, pale yellowish brown | Good, flat, pale yellowish brown |
| | AM: | Poor, velvety, white | Moderate, velvety, white |
| | R: | Pale yellowish brown | Pale yellowish brown |
| | SP: | None | None |
| Potato extract-carrot extract agar | G: | Poor, flat, pale yellowish orange | Poor, flat, pale yellowish orange |
| | AM: | Moderate, velvety, brownish gray | Moderate, velvety, brownish gray |
| | R: | Light brownish white | Light brownish white |
| | SP: | None | None |
| Water agar | G: | Poor, flat, pale yellowish orange | Poor, flat, pale yellowish orange |
| | AM: | Moderate, velvety, light brownish white | Moderate, velvety, light brownish white |
| | R: | Light brownish gray | Light brownish gray |
| | SP: | None | None |

G: Growth, AM: aerial mycelium, R: reverse, SP: soluble pigment.

Table 2. Physiological properties of strains SANK 63584 and *Streptomyces hygroscopicus* NRRL 2387.

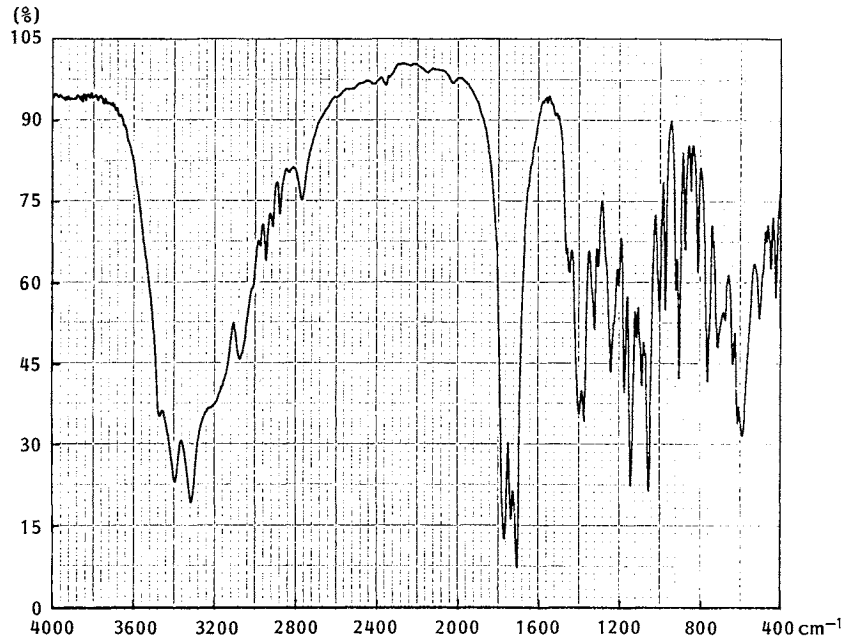
| | SANK 63584 | NRRL 2387 |
|---|---|---|
| Starch hydrolysis | Positive | Positive |
| Gelatin liquefaction | Positive | Positive |
| Nitrate reduction | Positive | Positive |
| Milk coagulation | Negative | Negative |
| Milk peptonization | Positive | Positive |
| Growth temperature | 11~46°C | 15~40°C |
| Optimum temperature | 26~38°C | 26~37°C |
| Sodium chloride tolerance | 7% | 7% |
| Casein decomposition | Positive | Positive |
| Tyrosine decomposition | Positive | Positive |
| Xanthine decomposition | Negative | Negative |
| Melanin formation | Negative | Negative |

Table 3. Carbohydrate utilization of strains SANK 63584 and *Streptomyces hygroscopicus* NRRL 2387.

| | SANK 63584 | NRRL 2387 |
|---|---|---|
| D-Glucose | + | + |
| L-Arabinose | ± | ± |
| D-Xylose | ± | ± |
| Inositol | − | − |
| D-Mannitol | + | + |
| D-Fructose | + | + |
| L-Rhamnose | − | ± |
| Sucrose | − | − |
| Raffinose | − | − |
| Control | − | − |

+: Positive utilization, ±: doubtful utilization, −: negative utilization.

Fig. 2. IR spectrum of hydantocidin (KBr).




of the description of strain SANK 63584 with those of the *Streptomyces* species and by direct comparison with *S. hygroscopicus* NRRL 2387 as the most related strain, the strain was identified as *S. hygroscopicus* and designated *S. hygroscopicus* SANK 63584.

## Physico-chemical Properties

Hydantocidin is neutral, water soluble, colorless needles with a mp of 187~189°C, and is insoluble in ethyl acetate, and chloroform, sparingly soluble in acetone, and soluble in water and methanol. It is positive to sulfuric acid (brown-yellow) on silica gel plate, and optical rotation in water was $[\alpha]_D^{25}$ +28.8° (*c* 1.04, $H_2O$). The MW and molecular formula were determined to be 218 (FAB-MS $(M+H)^+$ 219) and $C_7H_{10}N_2O_6$ (Obsd 219.06175, Calcd 219.06175), respectively by analyses of the HR mass spectrum as well as the number of carbons obtained from the $^{13}C$ NMR spectrum. The IR, $^1H$ and $^{13}C$ NMR spectra are

Fig. 3. $^{1}H$ NMR spectrum of hydantocidin in $CD_3OD$ (400 MHz).

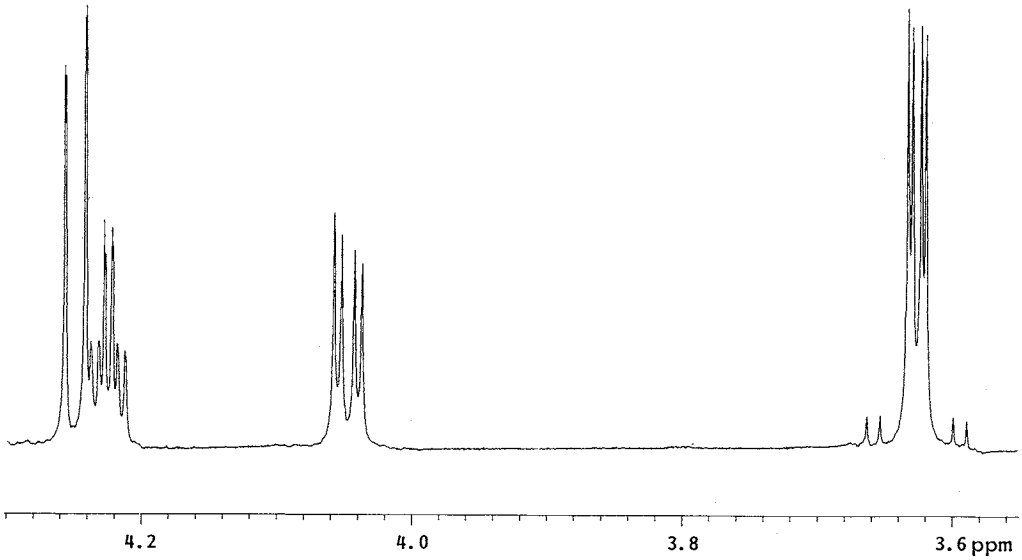
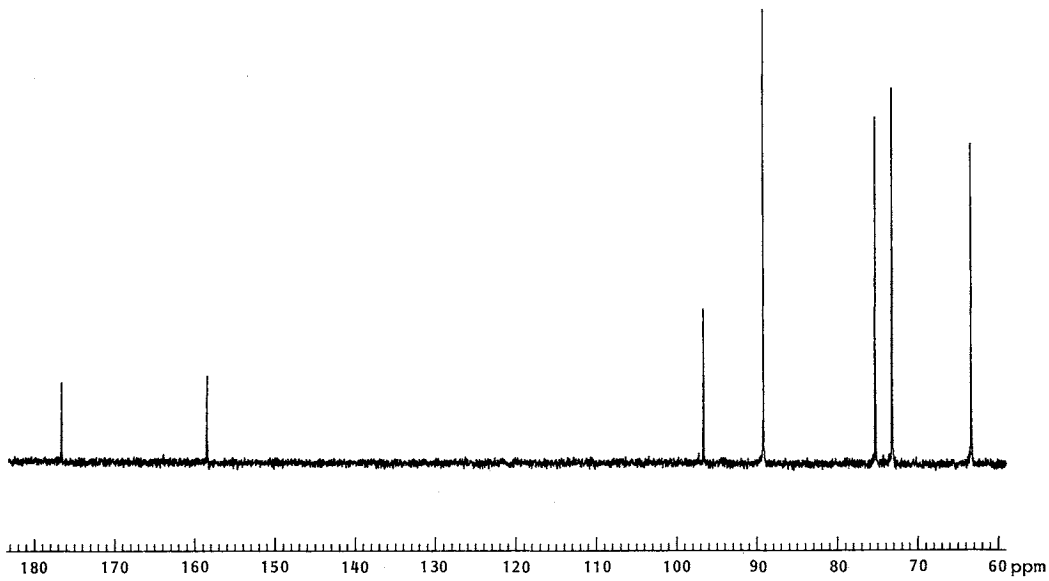


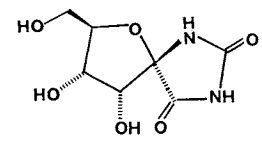

Fig. 4. $^{13}C$ NMR spectrum of hydantocidin in $CD_3OD$ (100 MHz).




shown in Figs. 2, 3 and 4, respectively.

Detailed analyses of these physico-chemical data revealed that hydantocidin possessed a unique structure, with a spiro-bond between C-1 of the ribose five-membered ring and C-5 of the hydantoin ring, as shown in Fig. 5.

Fig. 5. Structure of hydantocidin.



## Herbicidal Activities

As shown in Table 4, the effectiveness of hydantocidin against both monocotyledonous and dicotyledonous annuals was slightly higher than that of bialaphos[4] and was the same as that of glyphosate; these two reference herbicides have been marketed in Japan.

The activities of the hydantocidin against troublesome perennials, such as purple nutsedge, field bindweed, horsenettle, and yellow nutsedge were also observed to be quite similar or slightly superior to those of the reference herbicides as shown in Table 5. The characteistic of the activities was thought to be systemic in the plants. As such, the perennials were completely killed or seriously damaged by the compound, even if it was applied only on their leaf edges. The compound also suppressed the growth of tubers and root stocks of the perennials by the foliar application.

## Antimicrobial Activity

Hydantocidin did not have any antimicrobial activity against about 30 species of bacteria, 5 species of yeasts and fungi when tested at a concentration of 1,000 $\mu$g/ml by conventional paper disc-agar diffusion assay.

Table 4. Herbicidal activity of hydantocidin against annual weeds (500 ppm).

| Plant | Annual weed | Hydantocidin | Glyphosate | Bialaphos |
|---|---|---|---|---|
| Monocotyledon | Barnyardgrass | 5 | 5 | 3 |
| | Black grass | 4 | 4 | 2 |
| | Crabgrass, large | 5 | 5 | 5 |
| | Foxtail, giant | 5 | 5 | 4 |
| | Foxtail, green | 5 | 5 | 5 |
| | Johnsongrass | 5 | 5 | 3 |
| | Wild oat | 4 | 4 | 3 |
| Dicotyledon | Cocklebur, common | 5 | 5 | 5 |
| | Jimson weed | 5 | 5 | 5 |
| | Lambsquarters, common | 4 | 5 | 3 |
| | Morninggiory, tall | 4 | 4 | 4 |
| | Nightshade, black | 5 | 5 | 5 |
| | Pigweed, redroot | 4 | 5 | 5 |
| | Prickly sida | 5 | 5 | 5 |
| | Ragweed, common | 5 | 4 | 5 |
| | Velvet leaf | 5 | 4 | 3 |
| | Wild mustard | 5 | 5 | 5 |

Control rating (0; no effect, 5; 100% kill).

Table 5. Herbicidal activity of hydantocidin against perennial weeds.

| Perennial weed | Hydantocidin | | Glyphosate | | Bialaphos | |
|---|---|---|---|---|---|---|
| | 500[a] | 250 | 500 | 250 | 500 | 250 |
| Bermudagrass | 1 | 0 | 3 | 2 | 3 | 2 |
| Quack grass | 3 | 1 | 3 | 1 | 1 | 0 |
| Field bindweed | 5 | 5 | 3 | 2 | 2 | 1 |
| Horsenettle | 5 | 3 | 3 | 3 | 5 | 3 |
| Nutsedge, purple | 4 | 3 | 3 | 1 | 3 | 2 |
| Nutsedge, yellow | 5 | 5 | 5 | 5 | 4 | 2 |

Control rating (0; no effect, 5; 100% kill).

[a] ppm.

## Acute Toxicities

Hydantocidin is a low-toxicity substance, with an $LD_0$ value of more than 1,000 mg/kg when given orally and more than 100 mg/kg intravenously in mice. Toxicity to fish was determined using the guppy as a test fish; each group of ten fishes in a 500-ml conical beaker containing various concentrations of hydantocidin. $LC_{50}$ value was calculated to be 23 ppm, showing this substance to be a low-toxicity substance to fish.

There are several herbicidal antibiotics from actinomycetes, such as gluterimide group antibiotics, herbicidins[5], bialaphos and phosalacine[6] which belong to the phosphinothricin antibiotic group, herbimycin[7], oxetin[8] and homoalanocine[9]. Among these hydantocidin showed the most potent contact herbicidal activity against annual and perennial weeds, and monocotyledonous and dicotyledonous weeds.

As described above, the many characteristics of hydantocidin, such as strong contact herbicidal activity and excellent systemic permeability into the whole body of plants, particularly into the roots, are very promising as a natural herbicide with high-selective toxicity between plants and animals.

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