# OXETIN, A NEW ANTIMETABOLITE FROM AN ACTINOMYCETE

## FERMENTATION, ISOLATION, STRUCTURE AND BIOLOGICAL ACTIVITY

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A new amino acid-antimetabolite, oxetin, was isolated from a fermentation broth of a *Streptomyces* sp. OM-2317, a soil isolate. The chemical structure was elucidated as (2*R*,3*S*)-3-amino-2-oxetane carboxylic acid by analysis of the spectral data and by X-ray diffraction methods. The antibiotic is the first natural product possessing an oxetane ring. Certain microorganisms were inhibited by oxetin only when cultivated in minimal media. The inhibitory action was reversed by several amino acids such as L-isoleucine, L-methionine, L-valine and L-glutamine. It also exhibited herbicidal activity and inhibited glutamine synthetase from spinach leaves.

In the course of screening for new antimetabolites from actinomycetes, a new antibiotic was obtained from the culture filtrate of strain OM-2317, which was isolated from a soil sample collected at Jōgasaki, Izu peninsula, Japan and identified as *Streptomyces* sp. This antibiotic inhibited *Bacillus subtilis* and *Piricularia oryzae* in minimal media, and exhibited a herbicidal effect. The structure elucidation revealed that it is 3-amino-2-oxetane carboxylic acid. Since it was a novel compound, it was named oxetin after the structure.

The present paper deals with the taxonomy of the producing strain OM-2317 and the fermentation, isolation, physico-chemical and biological properties and structure of oxetin.

## Taxonomy of the Producing Strain

### Morphology

The vegetative mycelium grows abundantly on both synthetic and complex agar media, and does not show fragmentation into coccoid or bacillary elements. Good cottony growth of aerial mycelium was observed on oatmeal agar, inorganic salts - starch agar, glucose - asparagine agar and tyrosine agar.

The electron micrographs of strain OM-2317 were taken with a scanning electron microscope (Model S-430, Hitachi). The sporophores grown on most of agar media show type *Rectus-Frexibilis*. Mature spore chains on oatmeal agar have more than ten spores per chain, which are cylindrical in shape and have a smooth surface, $1.2 \times 0.7$ $\mu$m in size as shown in Plate 1. It was observed that the sporophore was type *Spira* and the spore was immature on glucose - asparagine agar (Plate 2). Scle-

Plate 1. Scanning electron micrograph of aerial mycelia of strain OM-2317 grown on oatmeal agar.
The bar represents 1 $\mu$m.

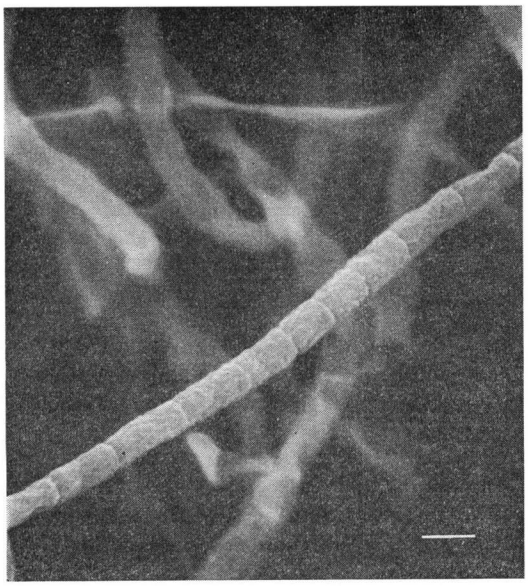
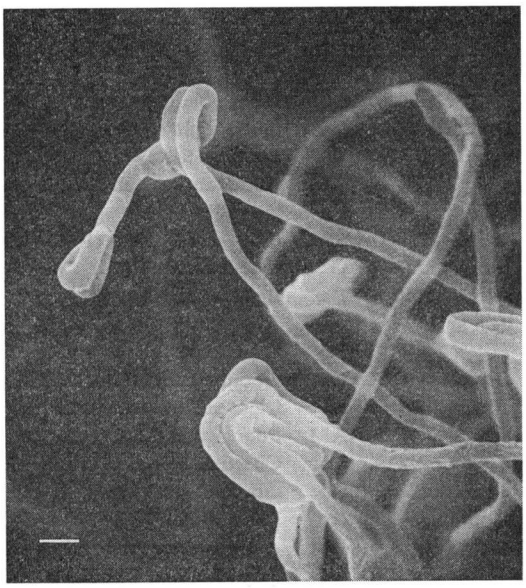


Plate 2. Scanning electron micrograph of aerial mycelia of strain OM-2317 grown on glucose-asparagine agar.
The bar represents 1 $\mu$m.



rotic granules, sporangia and flagellated spores were not observed.

Chemical Compositions

The chemical analyses of sugars in whole cell and amino acids in cell wall were carried out by the method of BECKER *et al.*[1] and LECHEVALIER & LECHEVALIER,[2] respectively. Strain OM-2317 shows no characteristic sugar pattern and contains LL-diaminopimelic acid ($A_2$pm) in the cell wall.

Cultural and Physiological Characteristics

The International Streptomyces Project (ISP) media recommended by SHIRLING & GOTTLIEB[3] and those recommended by WAKSMAN[4] were used for these experiments. Culture were observed after incubation at 27°C for two weeks. Color names and hue numbers indicated are those of the Color Harmony Manual (4th edition) published by the Container Cooperation of America. The utilization of carbon sources was tested by growth on PRIDHAM & GOTTLIEB's medium[5] containing 1% each carbon source at 27°C. The cultural and physiological characteristics are shown in Tables 1 and 2, respectively. The utilization of carbons of strain OM-2317 is shown in Table 3.

The cultural and physiological characteristics of strain OM-2317 are summarized as follows: sporophore is *Rectus-Frexibilis* and has long spore chains; the spore is cylindrical and has a smooth surface; color of aerial mycelium is white to gray; color of vegetative mycelium is yellow; $A_2$pm in the cell wall is LL-type. Based on the taxonomic properties described above, strain OM-2317 is considered to belong to the genus *Streptomyces* and is a strain of the gray or white series of PRIDHAM & TRESNER's grouping.[6] The strain has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, as *Streptomyces* sp. OM-2317 with the accession number FERM-P 7529.

Table 1. Cultural characteristics of strain OM-2317.

| Medium | | Cultural characteristics |
|---|---|---|
| Yeast extract - malt extract agar* | G: | Good, pearl pink (3ca) |
| | R: | Light amber |
| | AM: | Moderate, cottony, beige gray (3ih) |
| | SP: | Light gold (1½kb) |
| Oatmeal agar* | G: | Good, light ivory (2ca) |
| | R: | Bamboo (agc) |
| | AM: | Abundant, cottony, beige gray (3ih) |
| | SP: | Chartreuse yellow (1mb) |
| Inorganic salts - starch agar* | G: | Good, cream (1½ca) |
| | R: | Light antique gold (1½ic) |
| | AM: | Abundant, cottony, ashes |
| | SP: | Chartreuse yellow (1mb) |
| Glycerol - asparagine agar* | G: | Good, cream (1½ca) |
| | R: | Light yellow (1½ea) |
| | AM: | Poor, white (a) |
| | SP: | Chartreuse yellow (1mb) |
| Glucose - asparagine agar* | G: | Good, cream (1½ca) |
| | R: | Dusty yellow (1½gc) |
| | AM: | Abundant, cottony, silver gray (3fe) and white (a) |
| | SP: | Chartreuse yellow (1mb) |
| Peptone - yeast extract iron agar* | G: | Poor, bisque (3ec) |
| | R: | Camel (3ie) |
| | AM: | None |
| | SP: | None |
| Tyrosine agar* | G: | Good, camel (3ie) |
| | R: | Deep brown (3pl) |
| | AM: | Abundant, cottony, beige gray (3ih), partially pearl (2ba) |
| | SP: | Chartreuse yellow (1mb), a little dark brown (3nl) |
| Sucrose - nitrate agar** | G: | Thin, colorless |
| | R: | Light ivory (2ca) |
| | AM: | Poor, silver gray (3fe) |
| | SP: | None |
| Glucose - nitrate agar** | G: | Good, light yellow (1½ca) |
| | R: | Light yellow (1½ea) |
| | AM: | White (a) |
| | SP: | Chartreuse yellow (1mb) |
| Glycerol - calcium malate agar** | G: | Moderate, butter yellow (1½ga) |
| | R: | Butter yellow (1½ga) |
| | AM: | None |
| | SP: | Chartreuse yellow (1mb) |
| Glucose - peptone agar** | G: | Good, bamboo (2gc) |
| | R: | Gold (2lc) |
| | AM: | None |
| | SP: | Light gold (1½kb) |
| Nutrient agar** | G: | Thin, bisque (3ec) |
| | R: | Bisque (3ec) |
| | AM: | None |
| | SP: | None |

* Medium recommended by International Streptomyces Project.

** Medium recommended by S. A. WAKSMAN.

Abbreviation: G, growth of vegetative mycelium; R, reverse color; AM, aerial mycelium; SP, soluble pigment.

Table 2. Physiological properties of strain OM-2317.

| | Response* |
|---|---|
| Melanin formation | − |
| Tyrosinase reaction | − |
| Nitrate reduction | + |
| Liquefaction of gelatin | − (22°C) |
| Hydrolysis of starch | + |
| Coagulation of milk | + (37°C) |
| Peptonization of milk | − (37°C) |
| $H_2S$ formation | − |
| Cellulolytic activity | − |
| Temperature range for growth | 15~40°C |

* + Active, − not active.

Table 3. Utilization of carbon sources by strain OM-2317.

| Carbon source | Utilization* |
|---|---|
| D-Glucose | + |
| L-Arabinose | − |
| D-Xylose | − |
| D-Fructose | − |
| Rhamnose | − |
| D-Mannitol | − |
| *i*-Inositol | − |
| Sucrose | + |
| Melibiose | − |
| Raffinose | − |
| Cellulose | − |

* + Utilized, − not utilized.

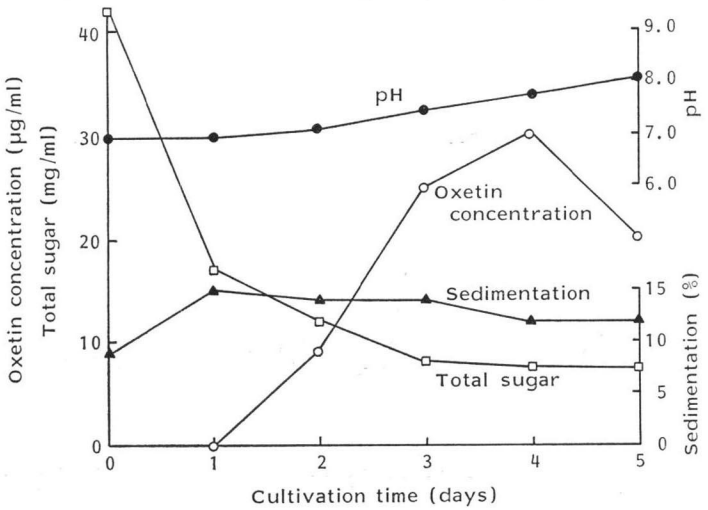
## Fermentation and Isolation

A loopful of the aerial mycelia of a slant culture of *Streptomyces* sp. OM-2317 was transferred into a seed medium (presterile pH 7.0, 100 ml) containing 0.1% glucose, 2.4% starch, 0.5% yeast extract and 0.4% $CaCO_3$ in a 500-ml Sakaguchi flask, and incubated with reciprocal shaking for 3 days at 27°C to give a seed culture for production of oxetin. The seed culture was transferred at the rate of 2% into the production medium (presterile pH 7.0, 100 ml) containing 2.0% dextrin, 0.2% glucose, 1.5% soybean meal, 0.3% yeast extract and 0.3% $CaCO_3$ in a 500-ml Sakaguchi flask, and then incubated with reciprocal shaking at 27°C.

A typical time course of oxetin production in a 500-ml Sakaguchi flask by strain OM-2317 is shown in Fig. 1. Oxetin production was monitored by paper disk assay using *B. subtilis* PCI 219 cultivated in a Davis minimal medium as a test organism and reached 30 $\mu$g/ml at day 4.

The cultured broth (70 liters) of *Streptomyces* sp. OM-2317, obtained by incubation in a 100-liter tank at 27°C for 4 days with 10 liters of air per minute and agitation of 200 rpm, was used as a starting material for the isolation of oxetin. The broth was adjusted at pH 2.0 with 6 N HCl. The culture supernatant obtained by centrifugation was passed through a column of Amberlite IR-120 ($H^+$) (3 liters) and after washing with water, the adsorbed material was eluted with 0.5 N $NH_4OH$. The active eluate was concentrated *in vacuo* until all the $NH_4OH$ contained was evaporated off, and applied to Amberlite IRA-410 ($OH^-$) column (1 liter). After washing the column with water, the adsorbed material was eluted with 0.5 N AcOH. The active eluate was concentrated *in vacuo* to 65 ml. Six volumes of MeOH was added to the concentrate, and the flocculated material was removed. The supernatant was further concentrated *in vacuo*, decolorized with activated carbon and then excess $Me_2CO$ was added. The precipitate was applied to a silica gel column (1.5 liters), and developed with BuOH - AcOH - $H_2O$ (13: 1: 1). The active fraction was concentrated *in vacuo*, lyophilized and applied to a silica gel column (60 ml) and developed with PrOH - $H_2O$ (11: 1, 1% $NH_3$). The active fraction was concentrated *in vacuo* and crystallized from water to give colorless cubes (163 mg, yield 10.7%).

Fig. 1. A typical time course of oxetin production by *Streptomyces* sp. OM-2317.



## Physico-chemical Properties and Structure

Oxetin was obtained as colorless cubes: mp 185~190°C (dec); $[\alpha]_D^{25}$ +56.4° (*c* 1.0, $H_2O$); UV end absorption; *Anal* Calcd for $C_4H_7NO_3$: C 41.00, H 6.03, N 12.00. Found: C 40.73, H 6.16, N 11.69. FD-MS *m/z* 118 $(M+H)^+$, 73 $(M-44)^+$. It is soluble in water but hardly soluble in MeOH, EtOH, EtOAc, chloroform and other common organic solvents. It shows positive color reaction to ninhydrin. The molecular formula $C_4H_7NO_3$ was determined from the above elemental analysis and FD-MS data.

The structure of oxetin was indicated to be 3-amino-2-oxetane carboxylic acid by the following spectral data. The IR spectrum (Fig. 2) indicated that the antibiotic was a typical zwitterionic amino acid. The $^{13}C$ NMR spectrum (25.1 MHz, $D_2O$, internal standard; dioxane) showed signals for a carboxylic acid (174.8 ppm), a methylene carbon (72.2) and two methyne carbons (80.2 and 47.4) and the $^{13}C$-$^1H$ coupling constants of protonated carbons were relatively large for $sp^3$-carbon, *i.e.* 156.2, 162.3 and 157.4 Hz, respectively. Since the presence of the carboxylic acid was confirmed by FD-MS data (*m/z* 73), the remaining structure must contain an oxetane or azetidine ring.

Trimethylsilyl (TMS) derivatives of oxetin obtained by treatment with bis(trimethylsilyl)trifluoroacetamide in acetonitrile were analyzed with GC-MS (Jeol JMS-DX300) and the main peak was identified as di-TMS-oxetin. A series of the fragment ion peaks are shown in Fig. 3. These results suggested the existence of an oxetane ring. From all the above results, the most plausible structure for oxetin was considered to be 3-amino-2-oxetane carboxylic acid.

The complete molecular structure of oxetin including the absolute configuration was established by X-ray crystallographic analysis. The crystal data were as follows: $C_4H_7NO_3$, MW=117.10, orthorhombic, space group $P2_12_12_1$, $a$=8.993(4) Å, $b$=9.921(7) Å, $c$=5.464(2) Å, U=487.5 Å$^3$, $Z$=4, D=1.595 g cm$^{-3}$. A single crystal having dimensions *ca.* 0.5×0.4×0.3 mm was used for the X-ray experiment. The unit-cell dimensions and diffraction intensities were measured on a Rigaku automated four-circle diffractometer with graphite-monochromated MoK$\alpha$ radiation. The $\omega-2\theta$ scanning technique was applied at an $\omega$ scan rate for 4° minute$^{-1}$; the background was measured for 20 seconds at each end of the scan range. The intensities were corrected for the Lorentz and polarization factors,

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

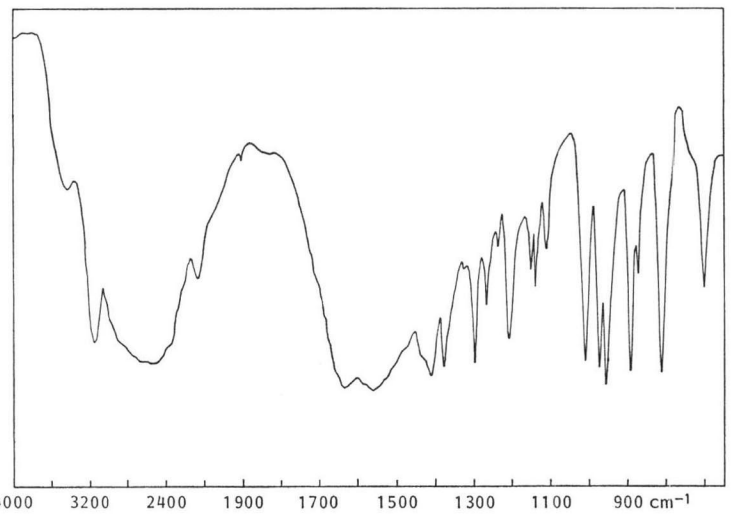
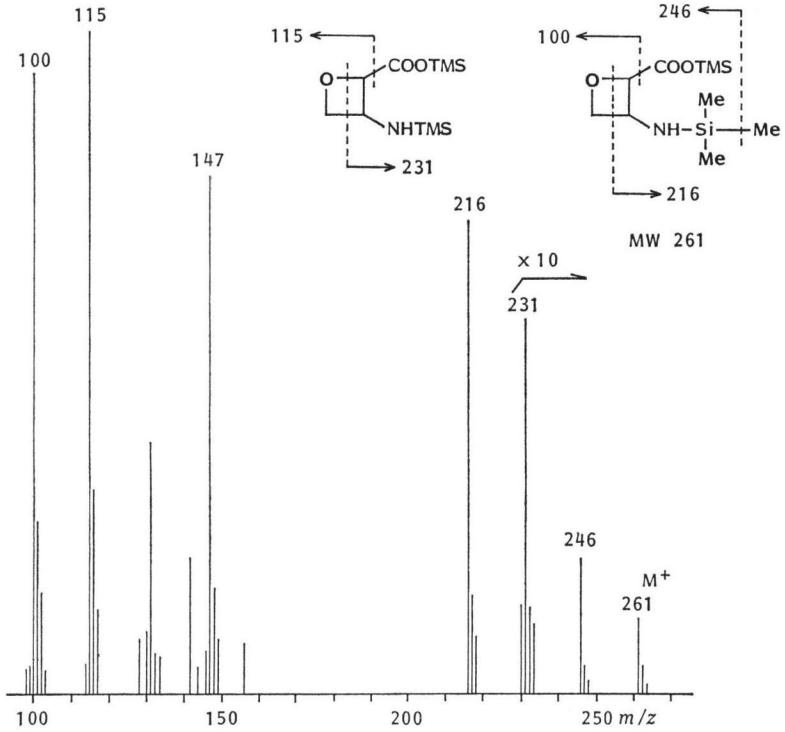
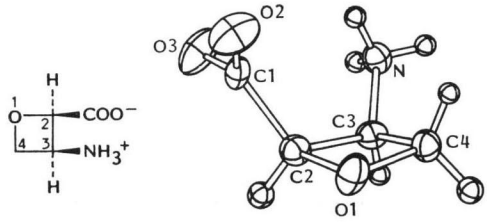


Fig. 3. MS of di-TMS-oxetin.




but not for absorption or extinction effects. In the range of $2\theta$ values up to 55°, 657 independent structure factors above the $\sigma$(F) level were selected for the structure determination. The structure was solved by the Monte Carlo direct method,[7] using the 10 strongest reflections as the starting set. An E-map based on the 14th random phase set afforded the positions of all the non-hydrogen atoms. After the structure had been refined by the block-diagonal least-squares methods with anisotropic thermal parameters, all the hydrogen atoms were located in a difference Fourier map. Further full-matrix least-squares refinements were carried out including the hydrogen atoms with isotropic temperature factors. The final R value was 0.034. The absolute configuration was determined by taking account of the anomalous dispersion effects of the oxygen, nitrogen and carbon atoms for CuK$\alpha$ radiation. For the application of the Bijvoet method, 15 pairs of hkl and hk$\bar{l}$ reflections with large values of $||F_c(hkl)|^2-|F_c(hk\bar{l})|^2|/(|F_c(hkl)|^2+|F_c(hk\bar{l})|^2)$ were selected. The sample used for ordinary structure determination was employed as it was. The reflection intensities were measured on the diffractometer, using Ni-filtered CuK$\alpha$ radiation which was passed through a collimator 0.8 mm in inside diameter; the $\omega-2\theta$ scan mode was applied. As a result of the measurements, the $|F_o(hk\bar{l})|$ values were found to be always greater than the $|F_o(hkl)|$ values by 1~14%; this was probably due to the absorption of the sample. Therefore, further intensity measurements were

Fig. 4. A perspective view of the oxetin molecule.



performed on 13 pairs of standard reflections in order that the ENGEL correction[8] might be applied. The results are obtained from the Crystallographic Data Center. The corresponding $\Delta F_o$ and $\Delta F_c$ values do not necessarily show a quantitative agreement, but their signs agree with each other for 14 of the 15 pairs of reflections. The molecular structure thus determined is shown in Fig. 4.

## Biological Properties

The antimicrobial spectrum of oxetin is shown in Table 4. It inhibited *B. subtilis* and *P. oryzae* in minimal media although showing no activity in a complete medium. The reversion by L-isoleucine, L-methionine or L-valine of the inhibition against *B. subtilis* was observed in paper disk assay. The partial reversion was also observed with either L-glutamine, L-glutamic acid or L-alanine.

Herbicidal activity of oxetin was assayed as described previously.[9] Oxetin exhibited herbicidal activity against alfalfa (*Medicago sativa*) and turnip (*Brassica rapa*) at the concentration of 125 $\mu$g/ml as shown in Table 5.

No toxicity of oxetin was observed even when it was administered ip to mice at a dose of 200 mg/kg.

Table 4. Antimicrobial spectrum of oxetin.

| Test organism | MIC* ($\mu$g/ml) |
|---|---|
| *Staphylococcus aureus* FDA 209P | >100 |
| *Bacillus subtilis* PIC 219 | 25 |
| *Micrococcus luteus* ATCC 9341 | >100 |
| *Mycobacterium smegmatis* ATCC 607 | >100 |
| *Escherichia coli* NIHJ | >100 |
| *Pseudomonas aeruginosa* IFO 3080 | >100 |
| *Proteus vulgaris* IFO 3167 | >100 |
| *Candida albicans* KF 1 | >100 |
| *Saccharomyces cerevisiae* ATCC 9763 | >100 |
| *Aspergillus niger* KF 105 | >100 |
| *Piricularia oryzae* KF 180 | 12.5 |
| *Fusarium oxysporum* KF 166 | >100 |
| *Mucor racemosus* IFO 4581 | >100 |

Medium: Davis minimal medium pH 7.0 for bacteria (37°C, 48 hours), dextrose Czapek medium pH 6.4 for fungi (27°C, 48~96 hours).

Fig. 5. The inhibition of glutamine synthetase from spinach leaves by oxetin.

*Km* 111 mM, *Ki* 3.4 mM.

○ Oxetin 4.3 mM, □ 2.1 mM, ● 1.1 mM, △ none.

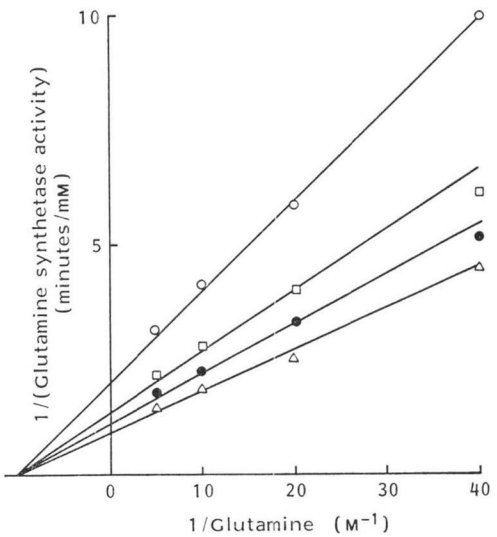



Table 5. Herbicidal effects of oxetin and phosphinothricin.

| Compound (ppm) | 4 day | | 9 day | |
|---|---|---|---|---|
| | *Medicago sativa* | *Brassica rapa* | *Medicago sativa* | *Brassica rapa* |
| None | − | − | − | − |
| Oxetin | | | | |
| 125 | − | + | + | + |
| 250 | − | + | + | ++ |
| 500 | − | + | ++ | +++ |
| 1,000 | − | + | +++ | +++ |
| Phosphinothricin | | | | |
| 125 | + | ++ | +++ | +++ |
| 250 | + | ++ | +++ | +++ |
| 500 | +++ | ++ | +++ | +++ |

Herbicidal effect: − No effect, + low activity, ++ medium activity, +++ high activity.

### Inhibitory Effect of Oxetin on Glutamine Synthetase

The preparation and assay of glutamine synthetases from *B. subtilis* and spinach (*Spinacia oleracea*) leaves were carried out according to the methods of DEUEL *et al.*[10] and LEASON *et al.*,[11] respectively, with some modifications. The details of the preparation method have been described previously.[9]

Oxetin inhibited noncompetitively the glutamine synthetases from *B. subtilis* and spinach leaves: the *Ki* values were 1.2 mM and 3.4 mM, and the *Km* values were 22.2 mM and 111 mM, respectively. Fig. 5 shows the inhibition of glutamine synthetase from spinach leaves by oxetin.

## Discussion

As described above, it was found that oxetin isolated from the cultured broth of a soil isolate named *Streptomyces* sp. OM-2317 is a new type of antimetabolite possessing an oxetane ring and herbicidal activity. Oxetin is the first natural product consisting of a oxetane ring.

The antimicrobial activity of oxetin on a minimal agar medium was reversed by several amino acids such as L-isoleucine, L-methionine or L-valine. However, the mechanism of reversion remains to be clarified.

Glutamine antimetabolites such as tabtoxin,[12] methionine sulfoximine,[13] phosphinothricin,[14] bialaphos[15] and phosalacine[9] are known to give damage to plants by the inhibition of glutamine synthetase. This suggests that the herbicidal activity of oxetin is due to the inhibition of glutamine synthetase. Although phosphinothricin, methionine sulfoximine, bialaphos and phosalacine are competitive inhibitors of the enzyme, oxetin is noncompetitive one like tabtoxin.

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