Naramycin B, an Antibiotic from *Streptomyces griseus* Strain 587 with Herbicidal Properties-Fermentation, Isolation, and Identification

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Dedicated to Prof. Dr. O. Bayer *in memoriam*

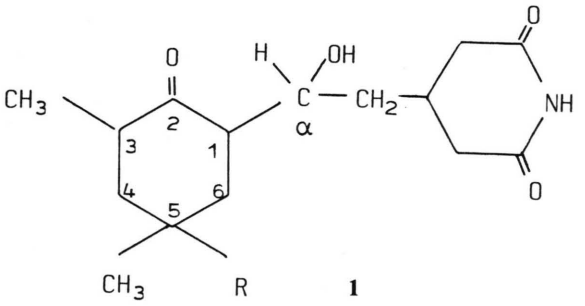
Naramycin B, Absolute Configuration, Herbicide, Abscission Agent

From the culture filtrate of *Streptomyces griseus* strain 587 a herbicidal active fraction was isolated by adsorption on Lewapol®, methanolic desorption, and chromatography on Sephadex® LH-20. Further purification was achieved by HPLC. The pure product was characterized by TLC and different colour reactions. By MS, ^{1}H-NMR, IR and ORD spectroscopy the herbicidal compound could be identified as naramycin B. Naramycin B is an optical isomer of cycloheximide (naramycin A, Actidione®, Acti-Aid®). This communication reports on the fermentative production of naramycin B with strain 587, its isolation, identification, and herbicidal activity.

Introduction

A group of antibiotics of historical importance has glutarimide as the common basic structure. So far more than twenty compounds of this type have been reported. The common structural principle is the 2-hydroxy-ethyl substitution in 3-position of the glutarimide ring and additionally a substitution by a cyclic or acyclic keton in the 2-hydroxy-ethyl region.

Examples for those compounds which generally are isolated from streptomycetes are cycloheximide



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[1]. (**1**, R=H, 1S, 3S, 5S, αR, 3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-2,6-piperidindione = naramycin A = Actidione®, Acti-Aid®), naramycin B (**1**, R=H, as naramycin A, but 1R-configuration) [2, 3], E-73 (**1**, R=$OCOCH_3$) [4]; streptovitacin A (**1**, R=OH) [5], streptimidone [6] (3,5-dimethyl-heptadien-(4,6)-one(2) instead of 3,5-dimethyl-cyclohexan-one(2) in naramycin A) and inactone [7] (1,6-dehydro-naramycin A).

Cycloheximide has been isolated from the culture filtrate of *Streptomyces griseus* [8, 9] and is sold under the name Actidione® by Upjohn Company as active ingredient of a fungicidal product against leaf blights and rust diseases. Under the name Acti-Aid® cycloheximide acts as an abscission agent and is applied in orange, grapefruit, and olive cultures.

For cycloheximide (naramycin A) the phytotoxic effects are well known [10]. From that reason the practical application as a fungicide is only possible in a few indications. Japanese authors described naramycin A [11] and its 5-acetoxyderivative (E-73) [12] as herbicides.

Nearly nothing is known about the biological activity of the stereoisomers of naramycin A. We describe the biological properties of the isomer naramycin B.

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Material and Methods

Culture conditions

Strain 587 was cultivated in the following medium: 5 g $(NH_4)_2SO_4$; 3 g KNO_3; 4 g soy meal; 4 ml corn steep liquor; 60 g starch; 5 g $CaCO_3$; 1 ml polyol filled up with water to one liter. The pH was adjusted to 7.0 with KOH. The medium was sterilized for 30 min at 121 °C. Small scale cultivations were carried out in 1 l shaking flasks containing 150 ml at 280 rpm, large scale cultivations in 200 l fermenters. The incubation temperature was 28 °C, the aeration rate 0.5 vvm. All cultures were inoculated with 5 vol-% of a preculture in the same medium cultivated for three days.

Ammonium-determination

0.1 ml test solution (0 to 0.1 µmol NH_4^+) was mixed with 1 ml 0.212 M phenol + 0.34 µM sodiumnitroprusside and 1.0 ml 22 µM sodiumhypochlorite +0.25 M NaOH and incubated for 30 min at 37 °C. The optical density was measured at 630 nm against a blank. The standard curve was constructed with ammonium sulfate.

Chemicals

All chemicals used were reagent grade. Lewapol® is a product of Bayer AG, Sephadex® LH-20 was purchased from Pharmacia and Lichrosorb® SI 100 (10 µm) from Fa. Merck.

Isolation and taxonomic description of strain 587

Streptomyces strain 587 was isolated in 1968 from a marine soil sample taken in the North Sea off the Scottish coast in a depth of 63 m. *Streptomyces* strain 587 was characterized as belonging to the genus *Streptomyces griseus* according to the following criteria: strain 587 formed sympodially branched aerial mycelium with spore chains of the "rectus flexibilis" type, carrying up to 60 spores. The spores were smooth and measured 0.4 to 0.8 µm × 0.8 to 1.8 µm. The colour of the young aerial mycelium was white, that of the mature aerial mycelum yellow ("*griseus*"). Strain 587 was not chromogenic. Strain 587 was of cell wall type I, the cell wall analysis led to the following results:

LL-DAP	+++
meso-DAP	(+)
hydroxy-DAP	–
glycin	+++
D,L-alanine	+++
D,L-glutamic acid	+++
D-glucosamine	+
D,L-valine	+
muramic acid	+
D,L-leucin/isoleucin	+
D-glucose	++
D-galactose	–
D-mannose	–
L-arabinose	–
D-xylose	–
L-rhamnose	–
D-fucose	–

The following carbon sources were utilized by strain 587:

D-glucose	+++
D-xylose	+
L-arabinose	++
L-rhamnose	++
D-fructose	+++
D-galactose	++
raffinose	–
D-mannitol	++
meso-inosit	–
sucrose	–
sorbitol	(–)
glycerol	+++
starch	+++
chitin	–

Growth on yeast extract – malt extract agar at increasing salt concentrations and temperatures:

NaCl	
0%	++++
4%	++
7%	++
10%	–
13%	–

Temperature	
8 °C	+++
18 °C	+++
28 °C	++++
37 °C	–

Results and Discussion

Kinetics of naramycin B-formation and regulation by NH_4^+-ions

Fig. 1 shows the result of a typical fermentation. Naramycin B was formed between the fifth and the seventh day when the NH_4^+-concentration in the culture broth had dropped below 10 mM. That the initial NH_4^+-concentration in the medium severely influenced the yield of naramycin B is demonstrated in the experiment of Fig. 2. Lowering of the initial NH_4^+-concentration led to an increasing naramycin B yield.

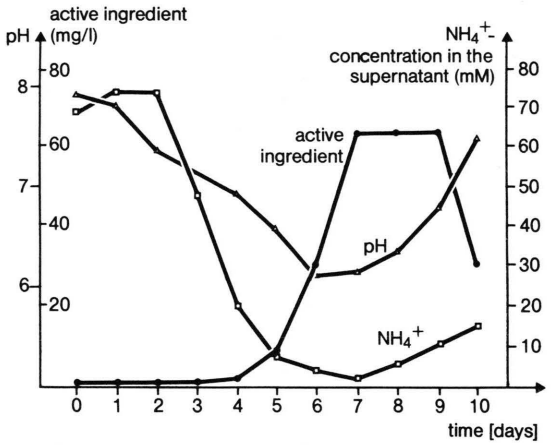


Fig. 1. Kinetics of naramycin B formation. Strain 587 was cultivated in a 200 l fermenter, the conditions were: 200 rpm, 0.5 vvm air, 28 °C.

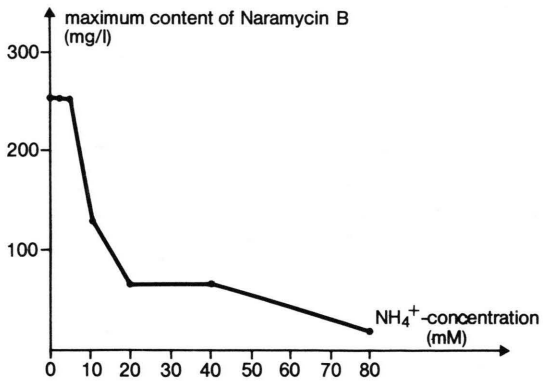


Fig. 2. Dependence of naramycin B yield on initial NH_4^+-concentration. Strain 587 was cultivated in shaking flasks; the initial $(NH_4)_2SO_4$ concentration of the medium was varried between 0 and 80 mmol NH_4^+.

Isolation

The isolation of the active ingredient is shown in the following example: The culture broth of a 200 l fermentation harvested after 7 days was centrifuged. The culture filtrate (about 170 l) was applied to a Lewapol®-column (30 × 50 cm) with a flow rate of 20 l/h. After washing with 50 l water and 50 l 50% methanol, naramycin B was eluted with 50 l 100% methanol. The eluate which contained all the biological activity was evaporated to dryness, redissolved in 2 l water and lyophilized (yield: 40.2 g of 3.5% active ingredient). 4 g of this crude product was dissolved in 30 ml methanol, separated from undissolved material by centrifugation and applied to a Sephadex®-LH-20 column (5 × 100 cm) which had been equilibrated with methanol. Fig. 3 shows the elution profile of the separation. Naramycin B was detected by its antifungal activity in fraction D. The yield was 119 mg; the material was about 50% pure. The final clean up of the compound was achieved by preparative HPLC on 10 µm silicagel in $CHCl_3$ (column 1.6 × 23 cm). Fig. 4 shows the elution profile of the separation of 80 mg of fraction D. Fraction 7 contained 42 mg of the pure active ingredient. The isolated substance showed the following physico-chemical data: The elementary analysis gave values for C: 63.2%, H: 8.0%, N: 4.7% and O: 24.1%. The product is a white amorphous powder with a decomposition point of 220 °C. Fig. 5 shows the IR-KBr-spectrum. The ORD-spectrum which is shown in Fig. 6 showed a maximum at 270 nm (+ 380°, $c = 3$ mg in 10 ml $CHCl_3$). In the region from 350 nm to 200 nm the compound showed no absorption. The ^{1}H-NMR-spectrum is given in Fig. 7. In the mass-spectrum a

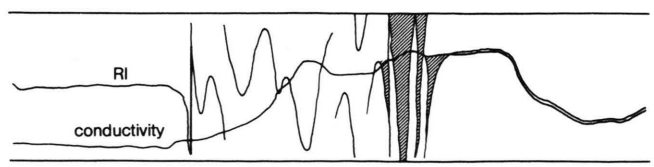


Fig. 3. Chromatography of 4 g rough material (desorbed from Lewapol® with 100% methanol) on Sephadex® LH-20. Column: 5 cm × 100 cm, equilibrated with methanol, flow rate: 100 ml/h, fractions 12', detection: RI 32× (Winopal), conductivity: 1 mS.

molecule-ion could not be observed. There were fragments at 263, 205, 204, 155, 152, 126, 112, 84, 69, 55 and 41.

The compound is soluble in methanol, ethanol, ethylacetate, benzene and $CHCl_3$, but hardly soluble in water. The compound can be hydrolysed with acids. After 14 h treatment with 6 N HCl at 110 °C the hydrolysate contains ammonium ions.

It was possible to stain the compound on silicagel plates after TLC. Some reactions which could be used for identification are listed in Table I. The R_f-values of the herbicide on neutral silicagel plates are given in Table II.

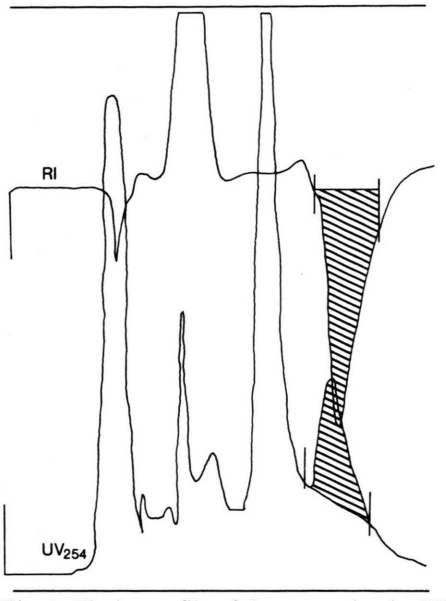


Fig. 4. Elutionprofile of the separation by HPLC. Column: 1.6 cm × 23 cm, Lichrosorb® Si 100 (10 µm), solvent: $CHCl_3$, flow rate: 8,0 ml/min injected: 30 mg active fraction D from LH-20 in 500 ml $CHCl_3$, detection: $UV_{254\,nm}$ span 2, RI 100×.

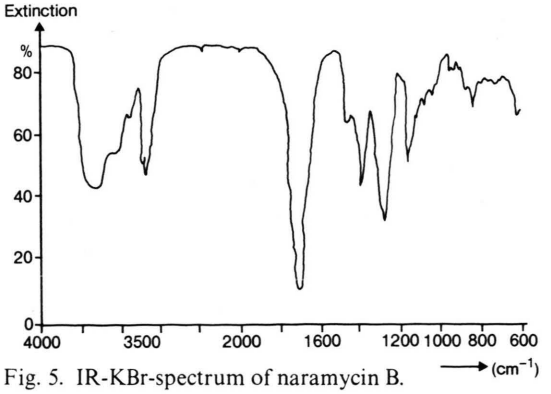


Fig. 5. IR-KBr-spectrum of naramycin B.

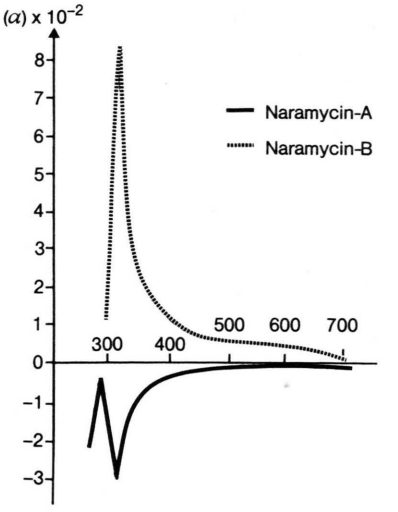


Fig. 6. ORD-spectra of naramycin A and B.

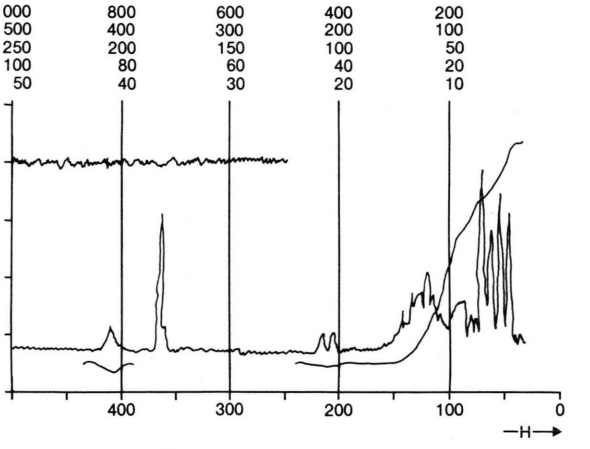


Fig. 7. 100 MHz-^{1}H-NMR-spectrum of naramycin B (solvent: CD_3OD).

Identification

Elementary analysis and mass-spectrum showed a strong similarity to cycloheximide. The direct comparison of the isolated herbicidal compound to cycloheximide by TLC showed no differences in the R_f-values. The IR- and ^{1}H-NMR-spectra again showed close similarities with the exception of marginal differences in the ^{1}H-NMR spectrum at about $\delta = 2$ ppm and in the IR-spectrum in the fingerprint region below 1500 cm^{-1}.

Because of the identity of the mass-spectrum with that of cycloheximide, which was reported by John-

Table I. Staining of naramycin B on neutral silicagel-plates (KG 60, Merck Co.).

Reagent/reaction according to*	Staining
ninhydrine	–
Morgan/Elson	–
4-dimethylaminobenzaldehyde	–
$KMnO_4$, alk.	+
thymole/H_2SO_4	+
anisaldehyde/H_2SO_4	+
perjodate	–
2,7-dichloro-fluorescein	–
J_2	–
bromokresole-green	–
rhodamine B	–
molybdatophosphoric acid	+
anilinphtalate	–
Tollen's reagent	–
$FeCl_3$/HCl	+
Hydroxylamine/$FeCl_3$	+

* The reagents were used according to [15].

Table II. Characterization of naramycin B by TLC on neutral silicagel-plates (KG 60, Merck Co.).

n-BuOH/HOAc/H_2O (50/25/25)	0.60
$CHCl_3$/CH_3OH/HOAc (90/8/2)	0.63
$CHCl_3$/CH_3OH/H_2O (80/20/2.5)	0.66
CH_3–CO–CH_3	0.55
$CHCl_3$/CH_3OH (80/20)	0.65
$CHCl_3$/CH_3OH (90/10)	0.37
$CHCl_3$	0.05
C_2H_5–O–C_2H_5	0.06
$CHCl_3$/CH_3OH/2N NH_3 (20/3/0.5)	0.62
n-BuOAc/*n*-BuOH/HOAc/0.1 M Sörensen-buffer pH 7 (50/10/25/15	0.62
i-C_3H_7OH/2N NH_3/H_2O (7/1/2)	0.71
CH_3OH	0.64

son [13], the compound had the same constitution. But the question remained, which of the possible stereo-isomeres of cycloheximide had been isolated. The identity with cycloheximide itself could be excluded, as the compound from strain 587 showed a strong positive cotton-effect at 297 nm, cycloheximide itself, however, showed a negative cotton-effect at the same wavelength. From the ^{1}H-NMR-spectrum the identity with iso-cycloheximide and neocycloheximide could also be excluded.

The ORD-spectrum for the compound from strain 587 on the other hand is totally identical with that of naramycin B [14] so that the absolute configura-

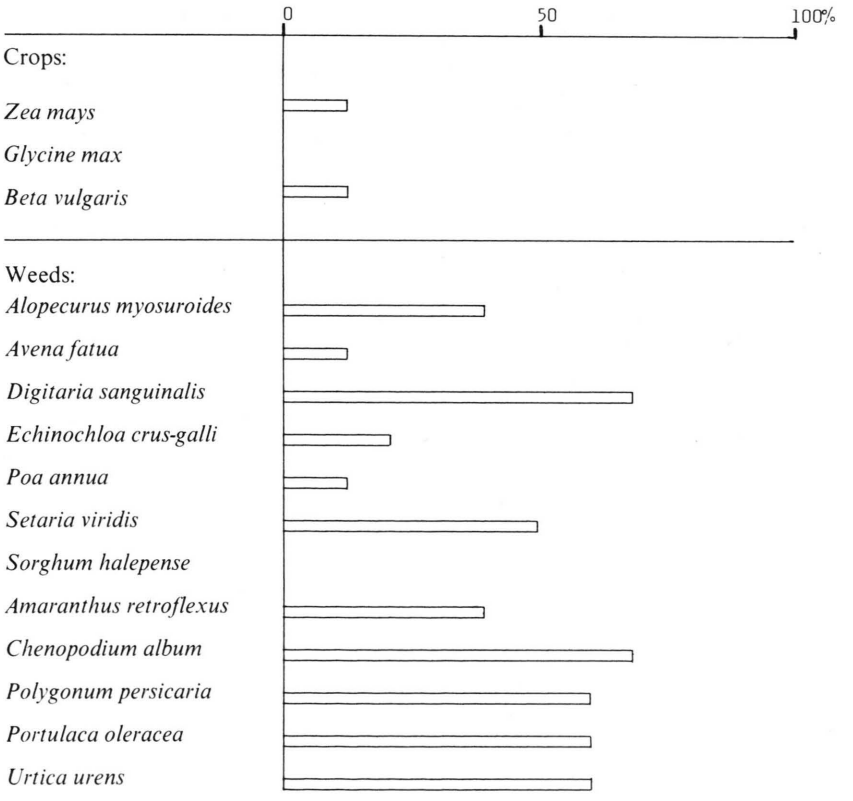


Fig. 8. Herbicidal activity of naramycin B in the green house, *pre-emergence application* (5 kg/ha, a.i., evaluation after 18 days).

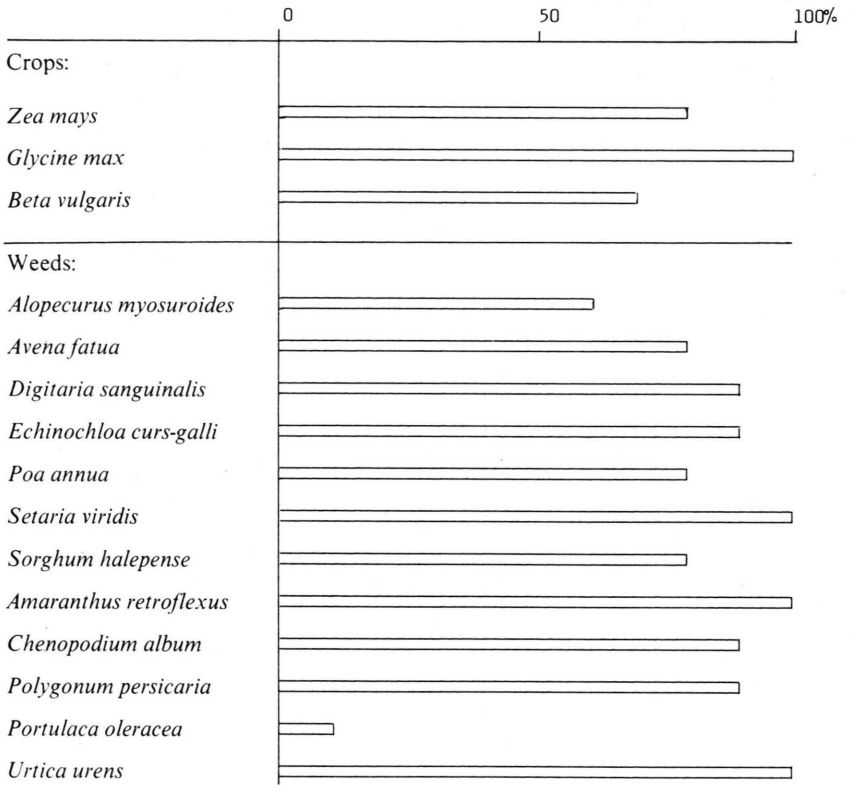
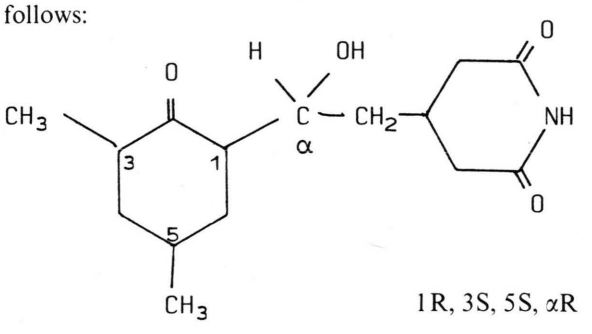


Fig. 9. Herbicidal activity of naramycin B in the green house, *post-emergence application* (0.05% a.i., evaluation after 14 days).

tion of the isolated compound can be given as follows:



1R, 3S, 5S, αR

Biological properties

The antibiotic activity of the isolated substance is shown in Table III.

The compound was active against yeasts and some plant pathogenic fungy, but had no activity against bacteria.

Fungicidal and insecticidal effects could not be shown in the greenhouse, however the compound is active as a herbicide (Fig. 8, Fig. 9).

Table III. Antibiotic effectiveness of naramycin B.

Organism	MIC [µg/ml]
lab-strain, presumably:	
Metschnikowia pulcherrima	≦ 0.5
Saccharomyces elipsodeus	≦ 0.5
Saccharomyces cerevisiae	≦ 1.0
Pyricularia oryzae	≦ 0.5
Pellicularia sasakii	≦ 1.0
Sclerotinia sclerotiorum	≦ 1.0
Botrytis cinerea	≦ 8.0
Xanthomonas oryzae	> 1000
E. coli	> 1000
Staph. aureus	> 1000

Table IV. Citrus abscission screen.

Compound	Dosage a.i. [ppm]	Pull Force [% of check]
untreated	–	100% (19 lb.)
naramycin B	1000	4%
naramycin A = cycloheximide	25	12%
Pickoff	250	6%
Release	250	13%

The herbicidal effectiveness of naramycin B could be established in field experiments as well. The best effects could be obtained with the post-emergence application. The effectiveness is relatively short in time and depends on the growth stadium of the plant. Unfortunately, the compound showed no selectivity. Thus, a technical application of naramycin B as a herbicide is not meaningful.

Another property, however, seemed very interesting. Naramycin B showed just like cycloheximide [16, 17] a high activity as a citrus abscission agent. The results of a typical field experiment are shown in Table IV. However, despite of its potent activity, naramycin B showed no advantage over cycloheximide.

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