PRODUCTION OF NITRAMINOACETIC ACID BY *STREPTOMYCES NOURSEI* 8054-MC3

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Nitraminoacetic acid ($C_2H_4N_2O_4$) which showed partial inhibition of some gram-negative bacteria was produced by *Streptomyces noursei* 8054-MC_3. It is extracted from culture filtrate with butanol and after chromatographic purification it is recrystallized from mixed solvent of ethylacetate and chloroform as colorless needles. It is acidic and melts at 106°C. Physicochemical properties including n.m.r., infrared and ultraviolet spectra, and its chemical synthesis are also reported.

Nitraminoacetic acid, a substance with bacteriostatic activity, was isolated from the culture filtrate of *Streptomyces noursei* 8054-MC_3. It showed a partial inhibition against some gram-negative bacteria, *Escherichia coli*, *Xanthomonas oryzae* and *Pseudomonas tabaci*, and exhibited inhibition against *Mycobacterium phlei*. It had been synthesized by HANTZSCH and METCALF[1], but there were no reports on its biological properties. Studies on the taxonomical properties of the producing organism, fermentation, isolation, physicochemical and biological properties and chemical synthesis of the active substance are described in this paper.

1. Producing Organism

Streptomyces sp. 8054-MC_3, isolated from the soil sample collected on the Tokyo University campus, produced both nitraminoacetic acid and cycloheximide in the same fermentation medium.

The strain was found to resemble to one of cycloheximide-producing strains. FURUMAI, HAMADA and OKAMI[2] reported that cycloheximide-producing strains isolated from soil collected from many places of Japan could be classified into A, B and C groups by taxonomical studies. The strain 8054-MC_3 belongs to the A-group designated as *Streptomyces noursei*. The summarized characteristics of strain 8054-MC_3 and those of *Streptomyces noursei* are described in Table 1. Both strains are very similar except for minor differences such as soluble pigments produced on organic media.

2. Production and Isolation

The strain of *Streptomyces noursei* 8054-MC_3 produced nitraminoacetic acid under aerated, submerged fermentation in a medium consisting of soluble starch 25 g,

Table 1. Summarized characteristics of *Streptomyces* sp. 8054-MC_3 and *S. noursei*

	8054-MC_3	*S. noursei*
Sporophores	spirals	spirals
Spore surface	long spines	long spines
Synthetic media G	colorless to pale yellowish brown or reddish brown	colorless to tan with dark gray reverse
A	white, later becoming to purplish or reddish gray	white, later becoming to reddish gray, finally becoming to ash gray
S	on some media, reddish brown or pale pink	on some media, shell pink
Organic media G	pale yellow to dull yellow to yellowish brown	on some media, brown
A	white to white with yellowish tinge	brown to chalky white
S	pale yellow to dull yellow to yellowish brown	brown to reddish brown purple
Melanin formation	negative	negative
Starch hydrolysis	positive	positive
Nitrate reduction	positive	positive
Gelatin liquefaction	positive	positive
Milk peptonization	positive	positive
Products	nitraminoacetic acid	nystatin
	cycloheximide	cycloheximide

G : growth A : aerial mycelium S : soluble pigment

soybean meal 15 g, $(NH_4)_2SO_4$ 2 g, NaCl 5 g, $CaCO_3$ 4 g in 1 liter of water which was adjusted to pH 6.8. The activity reached 100 mcg/ml after 96 hours fermentation at 27°C in a jar fermentor. The culture broths and samples obtained during purification could be assayed microbiologically by a filter paper disc method using a monolayer plate inoculated with *Escherichia coli*.

The active substance was extracted and purified by the following procedure. The culture filtrate on the alkaline side was decolorized by stirring with active carbon (1.0 % w/v), and filtered. Thirty liters of filtrate was acidified with hydrochloric acid to pH 2.0 and extracted with butanol. The extract was concentrated to 500 ml, and transferred to an equal volume of alkaline water. The aqueous layer was acidified and reextracted with 100 ml of ether ten times. The ether extract was evaporated *in vacuo* to dryness. The active material was purified by silica gel column chromatography. The column was developed with mixed solvent of chloroform and ether (2 : 1, v/v). Active fractions were collected, combined and evaporated *in vacuo* to a small volume. The solution was allowed to stand overnight in a refrigerator and crude crystals were collected by filtration and washed with chloroform. They were further recrystallized from mixed solvent of ethylacetate and chloroform. Colorless needle crystals (1.8 g) were obtained.

3. Properties of Nitraminoacetic Acid

The substance crystallizes as colorless needles. It is acidic having pKa' values of 2.80 and 6.60, and melts at 106°C. It is soluble in water, methanol, and acetone; moderately soluble in ethylacetate and ether; and insoluble in benzene, chloroform, carbon tetrachloride and hexane. It is stable in aqueous solution over a wide pH

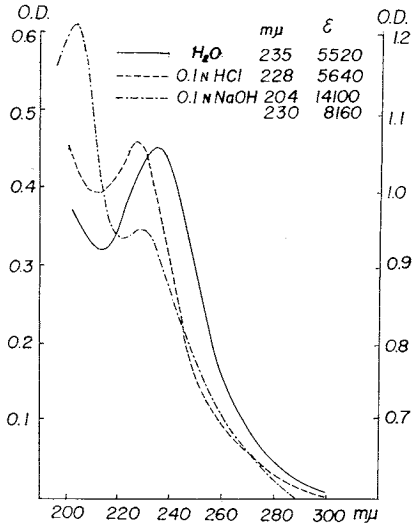
range (pH 2~9) at 100°C for 15 minutes, but gradually loses the activity when irradiated with ultraviolet light.

Elementary analysis of the crystals is as follows:

Calcd. for $C_2H_4N_2O_4$ (M. W. 120): C 20.00, H 3.36, N 23.33, O 53.30.
Found: C 20.16, H 3.26, N 23.23, O 53.00.

The molecular weight determination by the RAST method gave the values of 119 and 112, and by titration 121.7. The substance gives a positive LIEBERMAN test, ferric chloride and ferrous hydroxide reactions, oxidation of N,N'-diphenylbenzidine and test by conversion into the iron salts of hydroxamic acid, but gives negative ninhydrin, LEMIEUX, FEHLING and TOLLENS reactions.

Fig. 1. Ultraviolet absorption spectra of nitraminoacetic acid.

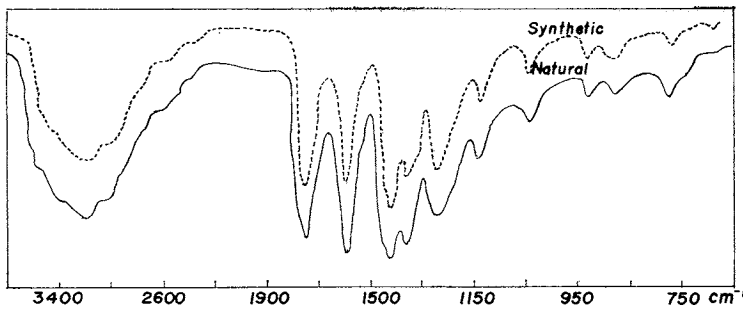


It shows a characteristic ultraviolet absorption with the maxima at 235 mμ (ε=5520) in water, 228 mμ (ε=5640) in 0.1 N hydrochloric acid, 204 mμ (ε=14100) and 230 mμ (ε=8160) in 0.1 N sodium hydroxide as shown in Fig. 1.

The color reaction and ultraviolet absorption suggest the presence of a nitramino group or an isonitramino group. If isonitramino group were present, it would have to show an additional peak at 360 mμ in ultraviolet absorption spectrum.

The infrared absorption spectrum in a KBr tablet is shown in Fig. 2. Absorption bands are seen at 1730 cm^{-1} corresponding to carboxyl,

Fig. 2. Infrared absorption spectra of nitraminoacetic acid (KBr).



and at 1575 cm^{-1} and 1240 cm^{-1} corresponding to nitramino group.

The n.m.r. spectrum of the substance, taken in deuteroacetone solution at 100 Mc with T.M.S. as internal reference exhibited a signal of unusual lower methylene at 4.25 p.p.m. in singlet and overlapped broad signals of carboxyl and NH of the nitramino group at 9.50 p.p.m.

Catalytic hydrogenation of the substance was carried out in glacial acetic acid with ADAM's catalyst in atmospheric pressure, and the hydrogen consumption was about 3 moles. The hydrogenated product was identical with glycine by the paper

chromatography and infrared absorption spectrum.

On the basis of the data given above, the antibiotic was suggested to be an isonitramino compound or nitramino compound by its color reactions, but the ultraviolet absorption spectra of the substance supported the presence of nitramino group rather than isonitramino group. The n.m.r. spectrum of the substance showed a sharp singlet of methylene protons which were not coupled and broad signals of carboxyl and nitramino groups at a very low field. The study of the reduced product indicated that the substance had a glycine skeleton. The studies above described showed the substance probably to be nitraminoacetic acid. The identity was confirmed by comparison with synthetic nitraminoacetic acid.

Fig. 3. Synthetic procedure of nitraminoacetic acid

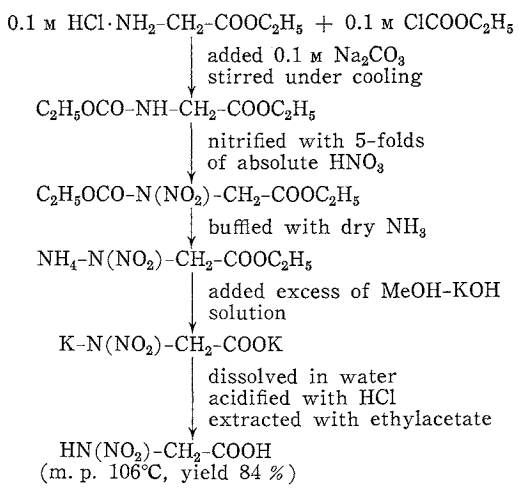


Table 2. Antimicrobial spectrum of nitraminoacetic acid (cup method)

Test organism	M.I.C. (mcg/ml)
Escherichia coli	0.18*
Xanthomonas oryzae	25*
Pseudomonas tabaci	0.78
Mycobacterium phlei	125
Bacillus subtilis	>100
Shigella sonnei	>100
Salmonella enteritidis	>100
Candida albicans	>100
Piricularia oryzae	>100

* partial inhibition

4. Chemical Synthesis of Nitraminoacetic Acid

The procedure of chemical synthesis of nitraminoacetic acid is shown schematically in Fig. 3. Synthetic nitraminoacetic acid was proved to be identical with natural product by melting point, ultraviolet absorption spectrum, infrared absorption spectrum and biological activity. The infrared absorption spectra of natural and synthetic nitraminoacetic acids are shown in Fig. 2.

5. Biological Properties

Nitraminoacetic acid is active against some gram-negative bacteria *in vitro* (Table 2). The determination of the minimum inhibitory concentration was performed by the cylinder plate method. Nitraminoacetic acid inhibited *Escherichia coli*, *Xanthomonas oryzae*, *Pseudomonas tabaci* at low concentrations, but was not active against *Bacillus subtilis*, *Shigella sonnei*, *Salmonella entiritidis* at 100 mcg/ml. Fungi and yeast grew at 100 mcg/ml.

The toxicity of nitraminoacetic acid in mice was also examined and the intravenous LD_{50} was estimated to be 32 mg/kg, the intraperitoneal and oral LD_{50} were 43 mg/kg and 40 mg/kg, respectively.

References

1) Hantzsch, A. & W. V. Metcalf: Ueber Nitraminoessigsäure. Berichte 29: 1680~1685, 1896
2) Furumai, T.; M. Hamada & Y. Okami: Studies on cycloheximide-producing strains. J. Antibiotics, Ser. B 17: 181~189, 1964