NEOXALINE, A NEW ALKALOID PRODUCED BY *ASPERGILLUS JAPONICUS*

PRODUCTION, ISOLATION AND PROPERTIES

ATSUSHI HIRANO, YUZURU IWAI, ROKUROU MASUMA,
KIYOIZUMI TEI and SATOSHI ŌMURA*

Kitasato University and The Kitasato Institute, Minato-ku, Tokyo 108, Japan

(Received for publication June 1, 1979)

A new alkaloid named neoxaline has been isolated from culture broth of *Aspergillus japonicus* Fg-551 by solvent extraction and silica gel chromatography. The compound does not possess antimicrobial activities, but weakly stimulates the central nervous system. The molecular formula of neoxaline has been determined as $C_{23}H_{25}N_5O_4$ on the basis of elemental analysis and its mass spectrometry.

We have previously reported on the isolation of new alkaloids from actinomycetes[1~4]. In the course of our continuing search of new alkaloid from microorganisms, a new alkaloid named neoxaline was isolated from a fermentation broth of a strain of *Aspergillus japonicus* by solvent extraction and silica gel chromatography.

The present paper deals with taxonomy of the producing strain, fermentation, isolation, and physicochemical and biological properties of neoxaline.

Taxonomy of the Producing Organism

The organism which produces neoxaline was obtained from a soil sample collected in Hiroshima Prefecture, Japan.

CZAPEK's agar, malt agar and potato dextrose agar were prepared for the identification of the fungus. A stock culture of the isolate was inoculated onto these media and incubated at 27°C and 37°C, and the growth was observed for about 10 days.

Cultural characteristics and morphology of the organism are summarized in Tables 1 and 2. The organism has been identified as *Aspergillus japonicus* by comparison of the cultural characteristics and morphology with those given by RAPER and FENNEL[6] for the type species and with *A. japonicus* SAITO IFO 4060 from Institute for Fermentation, Osaka, Japan. The specific strain has been placed on our file as *Aspergillus japonicus* Fg-551.

Table 1. Morphology of the organism.

Conidial head	radiate or split into few indistinct columns, 103~131 μ in diameter
Conidiophores	smooth, colorless, 500~850 $\mu \times$ 11~12 μ
Vesicles	30~40 μ in diameter and spherical
Sterigmata	uniseriate, 5.6~6.1 $\times$ 2.3~3.5 μ
Conidia	globose, echinulate, 3.0~3.7 μ in diameter

Fermentation

Aspergillus japonicus Fg-551 was maintained on potato-dextrose agar slants. A spore suspension was prepared by addition of 5 ml of sterilized distilled water to a slant, followed by vigorous agitation of the water over the slant surface with a sterile loop. One milliliter of

* To whom all correspondence should be addressed.

Table 2. Cultural characteristics at 27°C* after 3 and 7 days growth.

		Growth (colony size)	Color[5]	Remarks
CZAPEK's agars	3 days	19.7~20.9 mm floccose	Light brown (3 lg) Oyster white (gsb) in reverse	No soluble pigment
	7 days	50.9~60.1 mm floccose	Covert brown (2 nl) Putty ($1\frac{1}{2}$ ec) in reverse	
Malt agars	3 days	32.0~34.0 mm floccose	Chocolate brown (4 pn)	More quickly sporulating than on CZAPEK's
	7 days	40.0~49.0 mm floccose	Chocolate brown (4 pn) Dusty yellow ($1\frac{1}{2}$ gc) in reverse	No soluble pigment
Potato dextrose agar	7 days	30.5~37.1 mm floccose	Dark brown (2 pn) Pastel yellow (1 fb) in reverse	More slowly growing than other two media No soluble pigment

* Growth at 37°C was poor in these media.

suspension was used for the inoculation of a 1-liter ROUX flask containing 400 ml of the following medium: glucose 1.0%, sucrose 2.0%, $NaNO_3$ 0.2%, K_2HPO_4 0.1%, KCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, corn steep liquor 1.0% (pH adjusted to 6.0 prior to sterilization). The inoculated fermentation medium was incubated at 27°C for 7 days to obtain neoxaline. Neoxaline was not produced by submerged culture. The potency of the alkaloid accumulated in culture broth was determined by DRAGENDORFF's method described in our previous article[1].

Isolation

Cultured broth (18 liters) of *Aspergillus japonicus* Fg-551 obtained by incubation in 50 ROUX flasks (1 liter capacity) was used as starting material for isolation of the alkaloid neoxaline. The broth containing mycelia was adjusted to pH 10 with aqueous ammonia. The alkaloid produced was extracted with 8 liters *n*-butyl acetate and then transferred into 3 liters 0.1 N hydrochloric acid. The water layer was subsequently adjusted to pH 10 with aqueous ammonia and extracted twice with 0.8 liter chloroform. The combined extracts were dried over anhydrous sodium sulfate, concentrated *in vacuo* to a small volume, and then chromatographed on silica gel (60 g, Merck, Kieselgel G) eluting with a solvent mixture of chloroform and methanol (150: 1, v/v). Alkaloid fractions, which gave a positive test with DRAGENDORFF's reagent and of which the Rf value was 0.45 on silica gel thin-layer chromatography (chloroform - methanol, 10: 1), were collected and evaporated to dryness *in vacuo* to yield a pale yellowish powder (350 mg). The powder was crystallized from benzene to afford colorless needles (230 mg) of neoxaline.

Physical and Chemical Properties

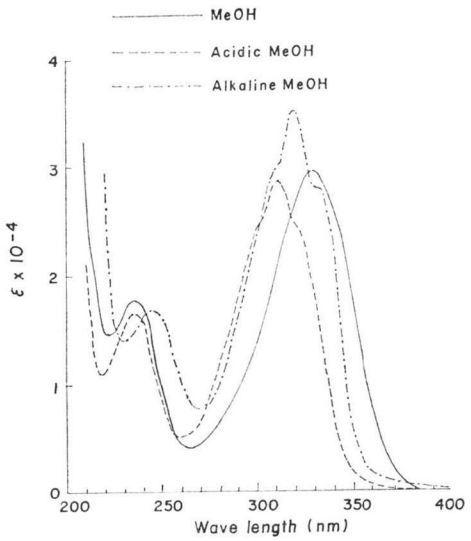
Neoxaline is obtained as basic and lipophilic crystals. Its physical and chemical properties are summarized in Table 3.

The molecular weight and the molecular formula of neoxaline were determined on the basis of its mass spectrum (Found, 435.1863; Calcd. for $C_{23}H_{25}N_5O_4$; 435.1907) and elemental analysis (Table 3). The formula indicated neoxaline to be highly unsaturated. The ultraviolet absorptions showed

Table 3. Physical and chemical properties of neoxaline.

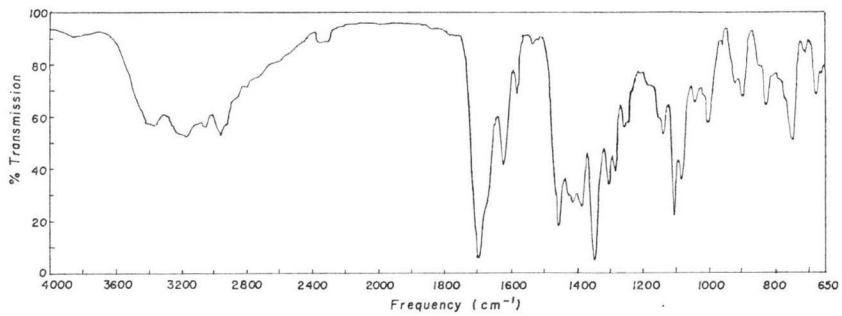
Appearance	Colorless needles
Melting point	202°C (decomp.)
Elemental analysis	C 63.23%, H 5.70%, N 15.90% no halogen, phosphorus, sulfur
Calcd. for $C_{23}H_{25}N_5O_4$	C 63.43%, H 5.79% N 16.08%
Molecular weight	435 (M^+, *m/e*)
Molecular formula	$C_{23}H_{25}N_5O_4$
Optical rotation	$[\alpha]_D^{24}$ −16.3° (*c* 1, $CHCl_3$)
UV absorption	λ_{max}^{MeOH} nm (ε), 237 (17,620), 330 (29,560)

Fig. 1. UV spectra of neoxaline.



up at 330 nm (ε 29,560) and 237 nm (ε 17,620) in methanol (Fig. 1). The infrared spectrum in KBr tablet is shown in Fig. 2, which exhibits characteristic bands of amine and/or hydroxyl group around 3400~3100 cm^{-1}, carbonyl and double bond groups at 1700 and 1623 cm^{-1} respectively, and alkanes around 2960 and 1365 cm^{-1}. The proton NMR spectrum of neoxaline suggested the presence of amine or hydroxyl group at δ 12.78, aromatic and olefinic protons at δ 7.6~6.6, a methoxyl group at δ 3.71, a methylene group at δ 2.35, and two methyl groups at δ 1.27 (Fig. 3).

Fig. 2. Infrared spectrum of neoxaline (KBr).



Biological Properties

Neoxaline was found to stimulate weakly the central nervous system by primary test (dose: 100 mg/kg, mice, p.o.).

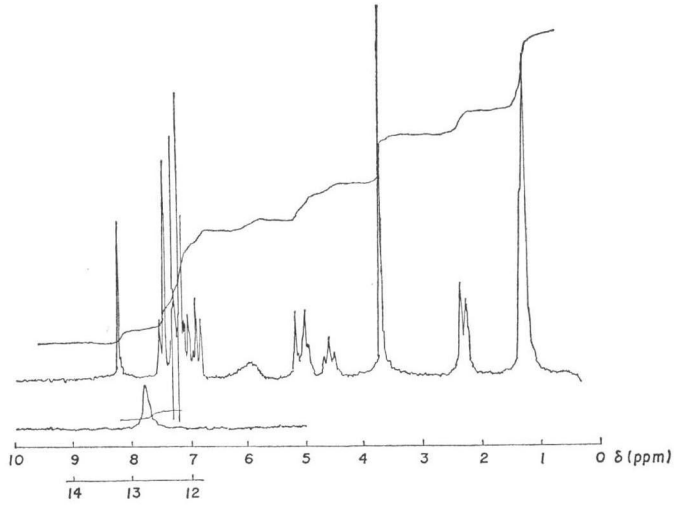
The acute toxicity (LD_{50}) of the compound by intraperitoneal administration in mice was greater than 200 mg/kg.

Neoxaline was found to have no antibacterial and antifungal activity at levels of 500 μg/ml as shown by the paper disc method. Further investigation on its pharmacological activities is now in progress.

Discussion

It is known that some alkaloids, such as echinulin[7], neoechinulins[8,9], fumitremorgin[10], ver-

Fig. 3. NMR spectra of neoxaline (100 MHz, $CDCl_3$).



ruculogen[11], tryptoguvaline[12], nigragilline[13] and austamide[14] are produced by *Aspergillus*. Neoxaline was differentiated from those alkaloids by comparison of UV spectra and molecular weight.

Among known alkaloids of microorganism origin, oxaline[15] produced by *Penicillium oxalicum* most closely resembles to neoxaline in physico-chemical properties, especially IR spectrum and NMR spectrum. However, neoxaline is apparently different from oxaline in molecular weight and elemental analysis. Consequently, it can be concluded that neoxaline is a novel alkaloid structurally similar to oxaline.

It is of interest that the compound, structurally closely related to oxaline which is produced by *Penicillium*, was isolated from *Aspergillus*. Structure determination is now in progress.

Acknowledgements

The authors wish to thank Asahi Chemical Industry Co., Ltd. and Toyo Jozo Co., Ltd. for assay of biological activities. Thanks are also due to Dr. Y. KONDA of Kitasato University for valuable suggestions, and Mr. T. YAZAKI and Miss M. KIMURA for their technical assistance.

References

1) ŌMURA, S.; H. TANAKA, J. AWAYA, Y. NARIMATSU, Y. KONDA & T. HATA: Pyrindicin, a new alkaloid from a *Streptomyces* strain. Taxonomy, fermentation, isolation and biological activity. Agr. Biol. Chem. 38: 899~906, 1974
2) ONDA, M.; Y. KONDA, Y. NARIMATSU, H. TANAKA, J. AWAYA & S. ŌMURA: Alkaloids from *Streptomyces* sp. NA-337. Chem. Pharm. Bull. 22: 2916~2920, 1974
3) ŌMURA, S.; Y. SUZUKI, C. KITAO, Y. TAKAHASHI & Y. KONDA: Isolation of a new sulfur-containing basic substance from a *Thermoactinomyces* species. J. Antibiotics 28: 609~610, 1975
4) ŌMURA, S.; Y. IWAI, A. HIRANO, A. NAKAGAWA, J. AWAYA, H. TSUCHIYA, Y. TAKAHASHI & R. MASUMA: A new alkaloid AM-2282 of *Streptomyces* origin. Taxonomy, fermentation, isolation and preliminary characterization. J. Antibiotics 30: 275~282, 1977
5) Container Corporation of America: Color Harmony Manual. 4th edition, Chicago, U.S.A., 1958
6) RAPER, K. B. & D. I. FENNEL: The genus *Aspergillus*. The Williams & Wilkins Co., Baltimore, 1965
7) NAKASHIMA, R. & G. P. SLATER: The configuration of echinulin. Tetrahedron Lett. 1967: 4433~4436, 1967
8) DOSSENA, A.; R. MARCHELLI & A. POCHINI: New metabolites of *Aspergillus amstelodami* related to the biogenesis of neoechinulin. J. Chem. Soc., Chem. Comm. 1974: 771~772, 1974
9) CARDILLO, R.; C. FUGANTI, G. GATTI, D. GHIRINGHELLI & P. GRASSELLI: Molecular structure of cryptoechinulin biosynthesis. Tetrahedron Lett. 1974: 3163~3166, 1974

10) YAMAZAKI, M.; K. SASAGO & K. MIYAKI: The structure of fumitremorgin B (FTB), a tremorgenic toxin from *Aspergillus fumigatus* FRES. J. Chem. Soc., Chem. Comm. 1974: 408~409, 1974
11) FAYOS, J.; D. LOKENSGARD, J. CLARDY, R. J. COLE & J. W. KIRKSEY: Structure of verruculogen, a tremor producing peroxide from *Penicillium verruculosum*. J. Am. Chem. Soc. 96: 6785~6787, 1974
12) CLARDY, J. & J. P. SPRINGER: Tryptoquivaline and tryptoquivalone, two tremorgenic metabolites of *Aspergillus clavatus*. J. Am. Chem. Soc. 97: 664~665, 1975
13) CAESER, VON F.; K. JANSSON & E. MUTSHLAR: Über Nigragillin, ein neues Alkaloid aus der *Aspergillus niger*-Gruppe. Pharm. Acta Helv. 44: 676~690, 1973
14) STEYN, P. S.: Austamide, a new toxic metabolite from *Aspergillus ustus*. Tetrahedron Lett. 1971: 3331~3334, 1971
15) NAGEL, D. W.; K. G. R. PACHLER, P. S. STEYN, R. VLEGGAAR & P. L. WESSELS: The chemistry and ^{13}C NMR assignments of oxaline, a novel alkaloid from *Penicillium oxalicum*. Tetrahedron 32: 2625~2631, 1976