NEW ANTIVIRAL ANTIBIOTICS; XANTHOCILLIN X MONO- AND DIMETHYLETHER, AND METHOXY-XANTHOCILLIN X DIMETHYLETHER. I

ISOLATION AND CHARACTERIZATION

(STUDIES ON ANTIVIRAL AND ANTITUMOR ANTIBIOTICS. V)

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(Received for publication October 1, 1968)

The production, isolation, chemical and physical characteristics and biological properties of the new antiviral antibiotics, xanthocillin X mono- and dimethylether, and methoxy-xanthocillin X dimethylether are described. They were produced by two strains of *Aspergillus* sp. isolated from soil samples. The active principles were extracted from the mycelium, and isolated as yellowish crystals. They were identified as derivatives of xanthocillin X by physical methods. All three were effective against Newcastle disease, vaccinia and herpes simplex viruses in plate assays, and all inhibited the growth of *Bacillus subtilis*.

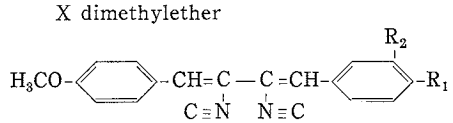
Since the development of the agar diffusion-plaque inhibition method by HERRMAN and his coworkers[1], many antivial antibiotics have been found using this method. During a screening programme of antiviral antibiotics employing the method, we found that some fungal metabolites such as trichothecin[2], brefeldin A[3] and geodin[4] exert antiviral activity against both RNA and DNA viruses.

Antiviral activity against Newcastle disease virus (NDV), vaccinia and herpes simplex viruses as well as antibacterial activity against *Bacillus subtilis* was demonstrated with an acetone extract of mycelium from two strains of *Aspergillus* sp. isolated from soil samples. The active principles from the mycelial extract were isolated in yellowish crystalline forms after passing through silicic acid column, and were found to be derivatives of xanthocillin X. Xanthocillin X was isolated by ROTHE in 1954 as an antimicrobial principle, and its structure was elucidated by HAGEDORN and TÖNJES[5,6] by chemical degradation studies and was confirmed by synthesis[7] to be 1, 4-di-(*p*-hydroxyphenyl)-2, 3-di-isonitrilo-1, 3-butadiene. ACHENBACH and GRISEBACH[8] studied the biogenesis of xanthocillin X and proved that tyrosine is an excellent precursor for the 1, 4-diaryl-butadiene portion of the antibiotic, but the origin of isonitrile group is obscure.

The structure of the antiviral antibiotics we isolated were determined spectrometrically to be 1-(*p*-hydroxyphenyl)-4-(*p*-methoxyphenyl)-2,3-di-isonitrilo-1,3-butadiene,

1, 4-di-(*p*-methoxyphenyl)-2, 3-di-isonitrilo-1, 3-butadiene and 1-(*p*-methoxyphenyl)-4-(*m*, *p*-dimethoxyphenyl)-2, 3-di-isonitrilo-1, 3-butadiene[9] (Fig. 1), and will be designated respectively as xanthocillin X monomethylether (XME), xanthocillin X dimethylether (XDE) and methoxy-xanthocillin X dimethylether (XTE). XME was also found to be produced by *Dichtomomyces albus*[10].

Fig. 1. Structures of xanthocillin X mono- and dimethylether, and methoxy-xanthocillin X dimethylether



	R_1	R_2
Xanthocillin X monomethylether	OH	H
Xanthocillin X dimethylether	OCH_3	H
Methoxy-xanthocillin X dimethylether	OCH_3	OCH_3

This paper deals with the isolation and chemical, physical and biological properties of these three antibiotics.

Cultivation and Assay Methods

Aspergillus sp. (strain No. 208 or No. 98) isolated from soil samples was cultivated in a medium containing 5 % glucose, 0.5 % Polypeptone, 0.2 % yeast extract, 0.1 % ammonium chloride, 0.06 % monobasic potassium phosphate, 0.02 % magnesium sulfate and 0.1 % calcium carbonate, adjusted to pH 6.0, at 26.5°C for 90 hours with aeration. They grew in a pulpy state and were harvested easily by filtration with the aid of Celite 545 (Johns-Manville Co., U. S. A.).

It was confirmed by silicic acid (Wakogel B-O, Wako Pure Chemical Industries, Japan) thin-layer chromatography that there were three antiviral principles in the mycelial acetone extract and that all had antibacterial activity against *Bacillus subtilis* I. A. M. No. 1026. Thus, production and purification were followed by antibacterial activity, and were checked by anti-NDV activity according to HERRMAN's method[1].

Purification and Isolation

Mycelial cake collected by filtration was immersed in acetone overnight with occasional stirring, and the active principles were isolated and purified according to the procedure shown in Fig. 2. The acetone extract was concentrated under reduced pressure at <45°C to remove acetone and 5 % (w/v) sodium chloride was added to the residual water. The concentrated aqueous solution was extracted twice with ethylacetate at pH 6.0, and solvent layers were pooled and concentrated under the conditions described above to yield a brownish gummy syrup. Cold methanol precooled to 4°C was added to the syrup.

After more than 10 hours at 4°C, yellowish precipitates were formed and collected on a glass filter. Most of the activity could be retained on the filter, and th precipitates were further purified by passing through a silicic acid (Mallinckrodt Chemical Works, U. S. A.) column. XDE was first eluted with benzene only, and XTE was eluted with benzene-methanol (97 : 3) followed by XME. XME, XDE and XTE were isolated and further purified by crystallizing from cold methanol.

Physical and Chemical Properties

XME, XDE and XTE were crystallized from methanol as pale yellow plates, greenish yellow needles and yellow plates, respectively. They were moderately stable at pH 3~10. They were all readily soluble in acetone, ethylacetate, ethylether and chloroform, and were hardly soluble in methanol, ethanol, water and hexane. They all showed no distinct melting point but decomposed at near 183°C.

Molecular weights of XME, XDE and XTE determined by mass spectrometry using Hitachi mass spectrometer RMU-6E were 302, 316 and 346, respectively. Elementary analyses of the three antibiotics were as follows:

Found:

XME	C 75.36, H 4.51, N 9.30.
XDE	C 75.81, H 4.95, N 8.73.
XTE	C 72.71, H 5.20, N 8.30.

Calculated for:

XME	$C_{19}H_{14}N_2O_2$,	C 75.48, H 4.67, N 9.27.
XDE	$C_{20}H_{16}N_2O_2$,	C 75.93, H 5.10, N 8.86.
XTE	$C_{21}H_{18}N_2O_3$,	C 72.82, H 5.24, N 8.09.

XME and XTE in methanol had ultraviolet absorption maxima at 243, 303, 368 and 385 mμ, and XDE at 230, 296, 363 and 380 mμ as shown in Fig. 3.

They all had similar infrared absorption spectra (Fig. 4 A, B and C), with characteristic bands near 2,150 cm^{-1} indicative of an X≡Y or X=Y=Z group, and also near 1,600, 1,560, 1,510, 1,300, 1,200, 1,050, 900 and 850 cm^{-1} suggestive of aromatic ring and ether linkage.

Fig. 2. Isolation procedure of xanthocillin X mono- and dimethylether, and methoxy-xanthocillin X dimethylether

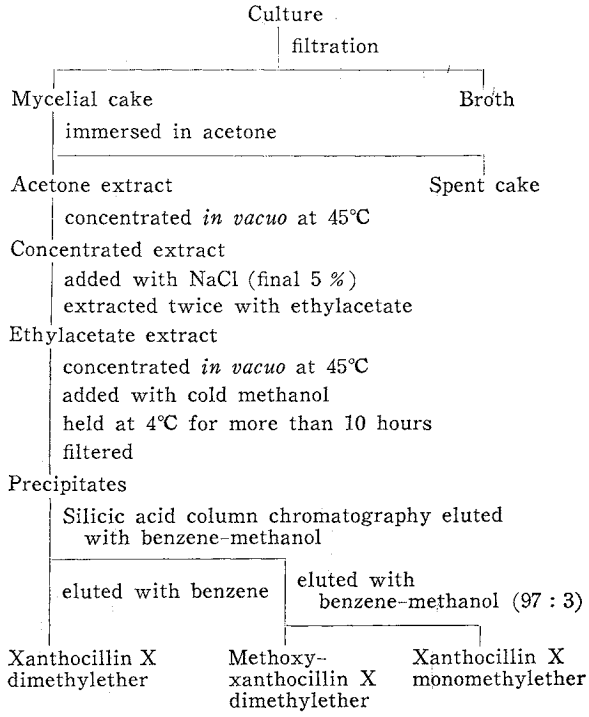
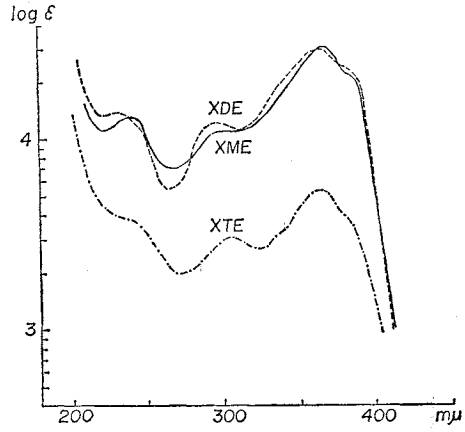


Fig. 3. Ultraviolet absorption spectra of xanthocillin X mono- and dimethylether, and methoxy xanthocillin X dimethylether (XME, XDE and XTE) in methanol.



The ultraviolet and infrared absorption spectra of the three newly isolated antibiotics were very similar to those of xanthocillin X suggesting that these antibiotics were derivatives of each other. That was the case confirmed by further interpretation of ultraviolet, infrared, magnetic resonance and mass spectra. The studies on structural elucidation will be reported elsewhere[9].

Fig. 4 A. Infrared absorption spectrum of xanthocillin X monomethylether (Nujol mull)

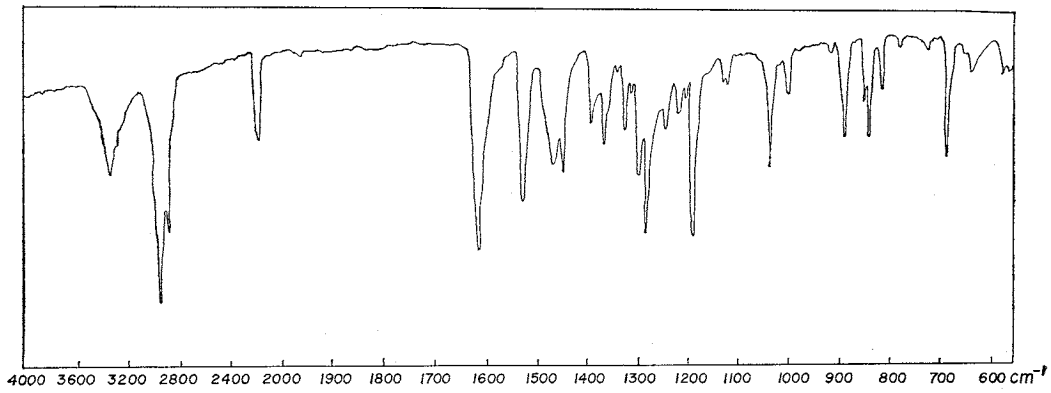


Fig. 4 B. Infrared absorption spectrum of xanthocillin X dimethylether (Nujol mull)

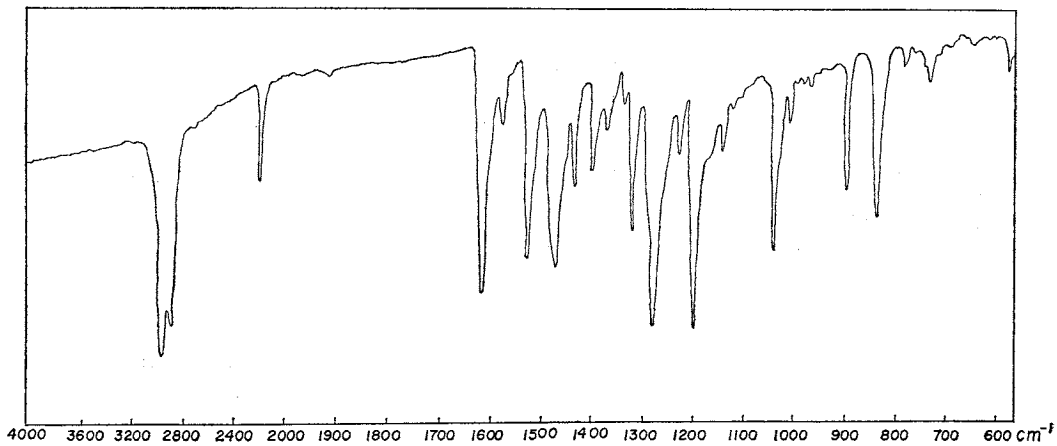
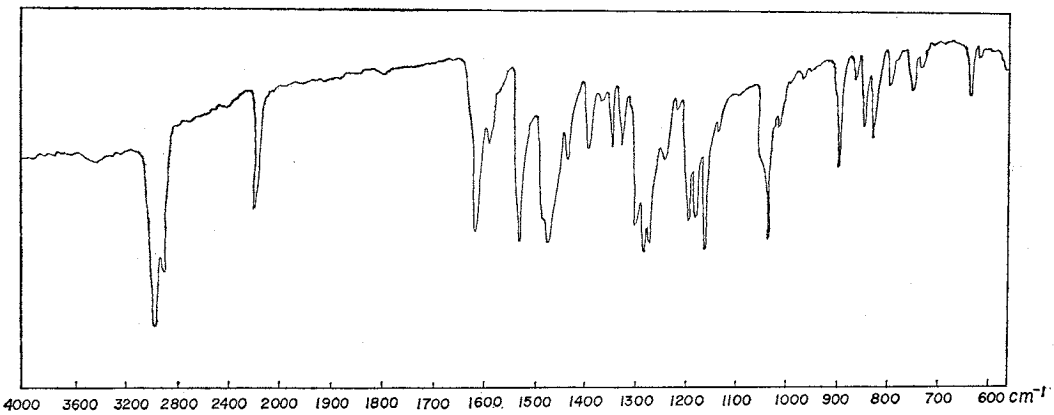


Fig. 4 C. Infrared absorption spectrum of methoxy-xanthocillin X dimethylether (Nujol mull)



Biological Properties

Table 1 shows the anti-NDV activity of the three antibitics determined by the plate assay method[1]. Because of their slight solubility in water, they diffused very

slightly and the plaque-free virus-inhibited zone was very narrow, especially with XDE and XTE. Besides NDV, they also effectively inhibited multiplication of herpes simplex and vaccinia viruses in cultured cells in the plate assay method. The biological aspects of the antiviral activity of the three antibiotics will be described in detail in later papers[11,12].

Table 1. Inhibitory activity of XME, XDE and XTE on plaque formation by NDV

Concentrations mcg/ml	XME		XDE		XTE	
	CTZ	AVZ	CTZ	AVZ	CTZ	AVZ
120	not determined		—	9.5	—	12.5
60	not determined		—	9.5	—	12.0
30	—	15.0	—	±	—	12.0
15	—	12.6	—	—	—	10.4
7.5	—	12.8	—	—	—	9.5
3.75	—	11.9	—	—	—	—
1.88	—	12.0	—	—	—	—
0.94	—	±	—	—	—	—

According to HERRMAN's method, confluent monolayer of CEF formed on Petri dish (90 mm in diameter) was infected by NDV Miyadera strain to form about 2,000 plaques per dish and soft agar medium was overlaid. Paper disks (Toyo Roshi Co., 8 mm in diameter) impregnated with samples dissolved at the concentrations indicated in the table were plated on hardened agar overlayer and incubated at 38.5°C. After 48-hour incubation, diameter of inhibition zone of plaque formation (AVZ) and cytotoxic zone caused by antibiotic (CTZ) was measured and expressed in mm.

Acknowledgment

The authors are indebted to Mr. K. AIZAWA of this department for measurement of UV, IR and NMR spectra, to Mr. I. SHIDA of the Institute of Applied Microbiology of this University for the measurement of mass spectra and also to Misses H. SASAKI and Y. HOSHINO of this department for the microanalyses.

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