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Brefeldin A from a Strain of Fungi Imperfecti

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Nishimura and Kubo isolated a strain of Fungi Imperfecti which produces an antifungal metabolite. The metabolite was obtained in a crystalline state from the mycelia extract. The elementary analysis and spectral data implied that the compound is a dihydric alcohol containing a carbonyl group. A crude metabolite was purified by acetylation and a preparative TLC to give colorless needles, m. p. 129~130°C, of the molecular formula $C_{20}H_{28}O_6$. In the NMR spectrum of the acetate two acetoxyl groups and a secondary methyl group were inferred from the singlet signals at τ 7.91, 8.0, and 8.77 respectively. A couple of quartet at τ 2.8 (1H) and 4.35 (1H) were indicative of the partial structure (B). Based on the molecular formula and spectral data, it was deduced that the antifungal metabolite is a dihydroxy bicyclic diene ester (or lactone) or a dihydroxy monocyclic triene ester (or lactone).
The physical properties including IR and NMR data of the acetate agreed in every respect with those of brefeldin A diacetate.

INTRODUCTION

During the course of investigation on plant-injurious microbes, Nishimura and Kubo isolated a strain of Fungi Imperfecti which prevents the growth of *Helminthosporium oryzae* and produces needle-shaped crystals in the mycelia after 8~10 day's incubation (Nishimura and Kubo, 1970). After boiling the mycelia together with the culture fluid, the hot broth was filtered and cooled to afford crystalline precipitate. This preparation inhibited the growth of *H. oryzae* and also of *Pellicularia sasaki* (Nishimura and Kubo, 1971).

In this paper it is reported that the antifungal metabolite was assigned to brefeldin A which had been isolated from the culture filtrates of *Penicillium brefeldianum*, *P. cyaneum, P. decumbens,* and *Ascochyta imperfecta* (Härri *et al., 1963;* Sigg, 1964; Singleton et *al., 1958;* Singelton et *al.,* 1964; Suzuki et *al.,* 1970).

RESULTS AND DISCUSSION

The sample supplied by Nishimura et al. was a colorless crystalline material of m. p. 204~208°C. It was not soluble in water but in acetone and ethanol, and in chloroform it was hardly dissolved. According to the elementary analysis, the material did not contain nitrogen elements in the molecule.

In the mass spectrum of the sample (Fig. 1), the ion peak at m/e 280 was assumed to be the parent peak. Then the peaks at m/e *262* and *244* were assign-

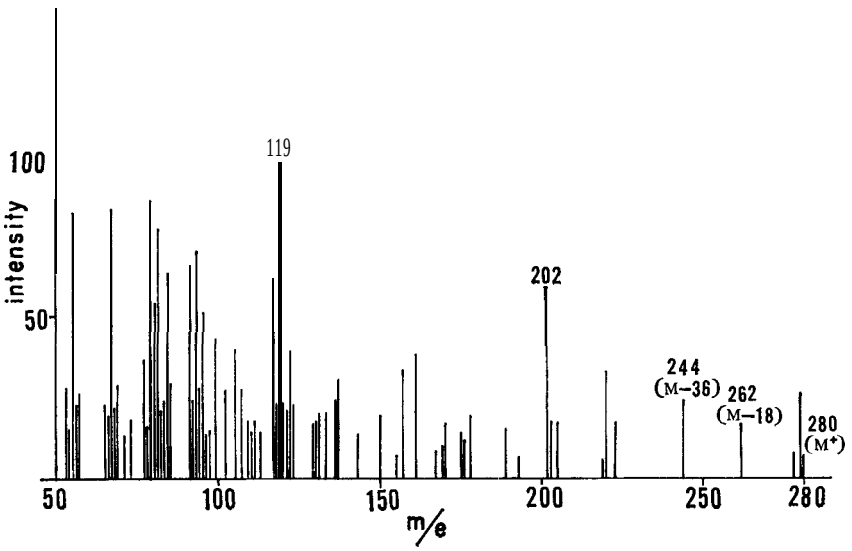


Fig. 1. Mass spectrum of an antifungal crystalline metabolite from a strain of Fungi Imperfecti (Brefeldin A).

able to the dehydrated ion peaks ($M-H_2O$ and $M-2H_2O$). Therefore, it was assumed that the material might be a dihydroxy-compound. The base peak appeared at m/e 119.

The hydroxy groups were inferred from the absorption band at 3300 cm-' in the IR spectrum (Fig. 2-l). In addition to the absorption bands at 1715 (carbonyl group), 1265 and 1080 cm^{-1} (ether-linkage), the fact that the material was soluble in a warm alkaline solution permitted to assume the presence of an ester or lactone-function in the molecule.

As the sample contained minor impurities, an acetylation procedure was employed for the purpose of purification. The acetylated material was purified by means of preparative TLC to afford colorless crystals of an acetate, m. p. 129-130" C. The molecular formula of the acetate was assigned to $C_{20}H_{28}O_6$ on the basis of the elementary analysis and the molecular weight (M^+ 364).

In the mass spectrum of the acetate (Fig. 3), the ion peaks at m/e 304 (M-60) and 244 (M-120) were assigned to deacetylated ion peaks. These corresponded to the dehydrated ion peaks in the spectrum of the original compound. The presence of two acetoxyl groups was confirmed by measuring the NMR spectrum as discussed later. The ion peak at m/e 119 was again the base peak of the acetate.

The IR spectrum of the acetate (Fig. 2-2) showed no absorption bands due to a hydroxy group, but instead of this, strong absorption bands at 1720, 1230 and 1085 cm^{-1} characteristic to an acetate.

Then all of the six oxygen atoms in the molecular formula of the acetate were assigned to four oxygens of the two acetoxyl groups and two of the ester

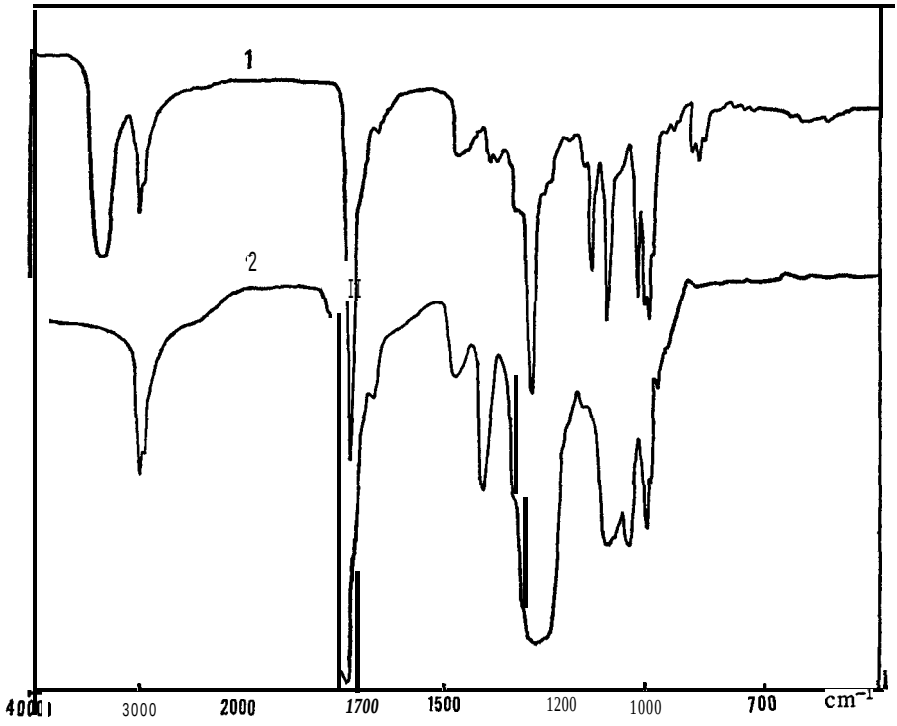


Fig. 2. IR Spectra of the antifungal metabolite and of its acetate. 1, Original sample: 2, Acetate.

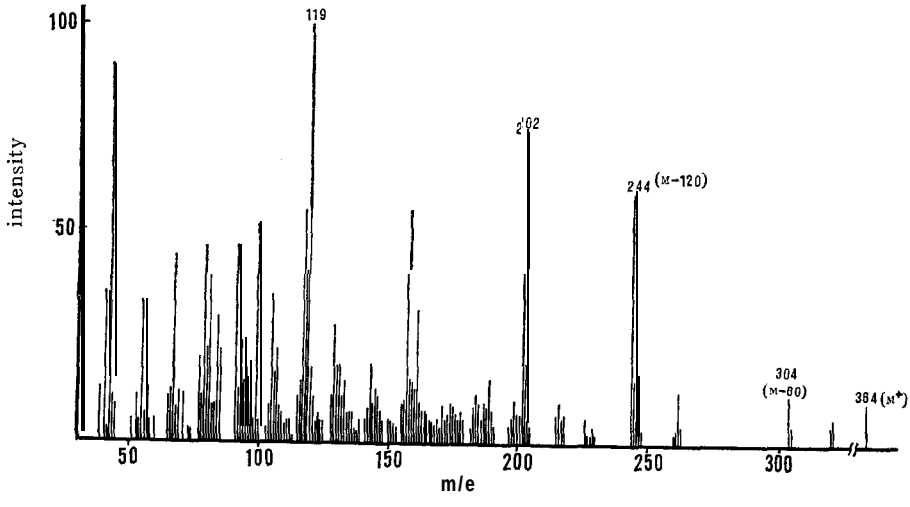


Fig. 3. Mass spectrum of the acetate of the preparation.

or lactone-function which was assumed to exist in the original dihydroxy-compound.

In the UV spectrum of the acetate (Fig. 4), the strong absorption at 213 nm (logε 3.9) suggested the presence of a carbonyl-conjugated double bond, but the presence of a conjugated dienenone structure was ruled out.

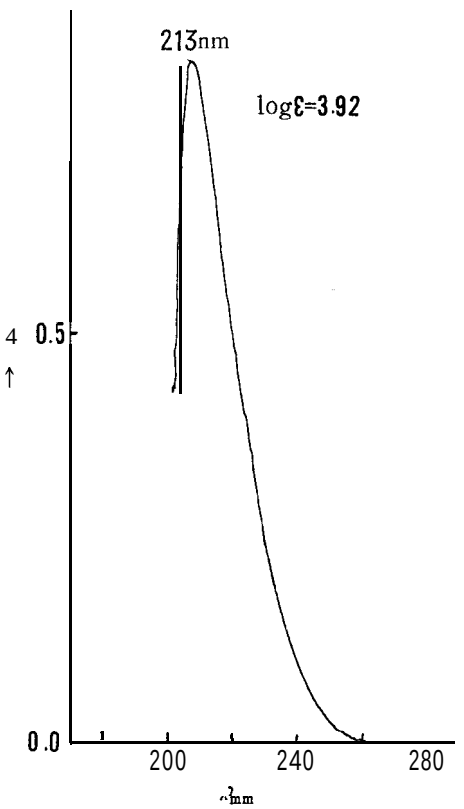
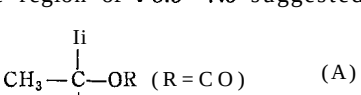


Fig. 4. UV Spectrum of the acetate of the preparation (ethanol solution).

In the NMR spectrum of the acetate (Fig. 5) there were no signals at the low magnetic field from τ 2.5. Therefore, the presence of an aldehyde group and a carboxylic acid function was excluded. Two singlet signals at τ 7.9 (3H) and 8.0 (3H) were assigned to acetoxyl groups, and the doublet signal at τ 8.8 (3H, J=6.3 cps) to a methyl group on a carbon atom bearing a hydrogen. The fact that the chemical shift position of the methyl-signal was shifted downfield (0.3 ppm) from the normal position for hydrocarbons implied that the carbon atom bearing the methyl group was linked to an oxygen atom. Then, the formula (A) was deducible.

The absence of signals at the region of τ 5.5~7.5 suggested that the methine



(A)

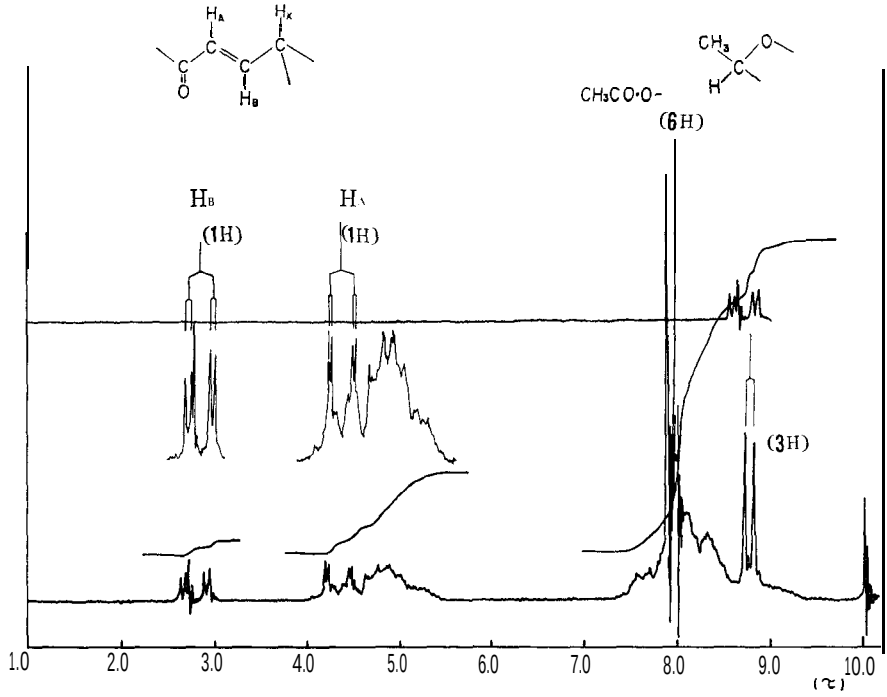


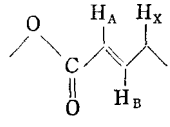
Fig. 5. **NMR** Spectrum of the acetate of the preparation.

hydrogen in the formula might be on a carbon atom bearing an ester group (namely R=CO) or on an acetal carbon atom. The latter possibility was ruled out, since the original alcohol did not give 2, 4-dinitrophenylhydrazone of acetaldehyde by reaction with a 2, 4-dinitrophenylhydrazine-sulfuric acid reagent.

The original dihydric alcohol was able to be acetylated with acetic anhydride by heating at 95°C for half an hour, therefore the hydroxy groups were not assumed to be tertiary.

The NMR spectrum of the acetate did not show any signals at the region of τ 5.5-6.5 where oxymethylene hydrogens of an ester of a primary alcohol resonate. With regard to these results, the hydroxy groups of the dihydric alcohol were assumed to be secondary.

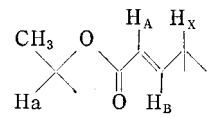
The couple of quartet at τ 2.8 (1H) and 4.35 (1H) was seemed to be the AB part of an ABX system ($J_{AM}=15, J_{AX}=3.5, J_{MX}=1.5$ cps). In addition to the coupling constants, the chemical shift values of these protons implied that the signals were displayed from the trans-hydrogen on a double bond conjugated with a carbonyl group. The presence of a trans-substituted double bond was also inferable from the IR absorption band at 980 cm-'. Then, the partial structure (B) was able to be postulated.



(B)

There were two possibilities, for the ester group in the formula A, whether the group originally existed in the moleule or was introduced by acetylation. No information was available to select the possibilities.

Based on the assumption that the ester group was not of acetate (formula A, $R \neq COCH_3$), the partial structures A and B could be linked and developed to the structure (C).



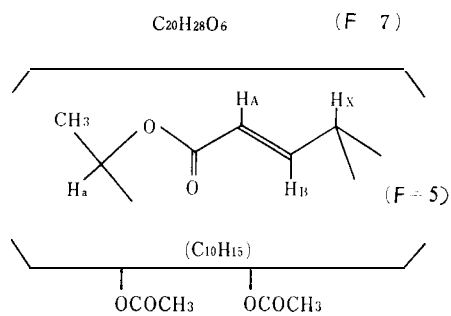
(C)

Then, among the six protons which resonated at the region of τ 4.0~5.5 in a complex multiplet, four protons were assignable to those on the carbon atoms bearing the ester groups (Ha and two methine hydrogens on the carbon atom bearing the acetoxyl groups) and the hydrogen H_A on the double bond in fromula C. Based on the chemical shift values, the remaining two protons also might be assigned to hydrogens on unsaturated carbon atoms. Therefore the existence of at least one more double bond was essential in the molecule.

τ : 4.0-5.5 (6H)	
$-\overset{\vert}{C}=\overset{\vert}{C}H_A$	1H
H_3C-CH_a-O-	1H
$>CH-OA_C$	2H
remained signal	2H

τ: 7.5-9.4 (21H)	
$H_3C-CO\cdot O-$	6H
H_3C-CH_a-O-	3H
remained signal	12H

The signals at the region of τ 7.5-9.4 corresponding to 21 protons should be assigned to the hydrogens on saturated carbon atoms. By subtracting the already assigned 9 protons which resonated at the region (2 x CH_3COO and $CH_3-CHOCO$) from 21 protons, then 12 protons remain. This indicated that at least 6 carbon atoms are necessary to be saturated.

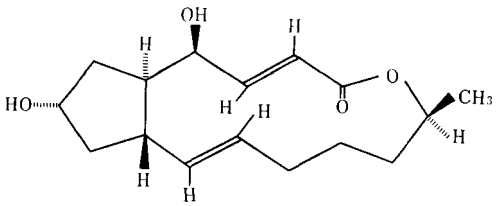


Therefore, the acetate possesses no more unsaturated bond aside from the two double bond mentioned above.

Referred to the degree of unsaturation of the molecular formula, the presence of three carbonyl groups and two double bonds in the mlecule permiotted the acetate to possess a two-ring structure.

Then, it was deducible that the antifungal metabolite might be a dihydroxy conjugated ester (or lactone) possessing two cyclic skeletons along with an isolated double bond bearing two hydrogens. And both of the hydroxy groups might be secondary.

On the assumption that the carbonyl group in formula A was introduced by acetylation, a dihydroxy monocyclic triene ester (or lactone) was deduced as another possible structure for the metabolite.



Brefeldin A

With these structural requirements for the metabolite, the physical properties including spectral data were referred to literatures and then permitted to assign the diacetate to brefeldin A diacetate, therefore the antifungal metabolite of the fungi to brefeldin A.

EXPERIMENTAL

Mps. were not corrected. IR spectra measured on a IR G-27 spectrometer, Shimazu, in a KBr pellet for brefeldin A and in a chloroform solution for brefeldin A diacetate. Mass spectra were measured on a JMS-01SG spectrometer at 75 eV by direct insertion of samples. NMR spectrum was measured on a JNM-C60-H spectrometer in a deuterochloroform solution with tetramethylsilane as internal standard.

Material was supplied by Nishimura *et al.* The material was sparingly soluble in water and chloroform, but soluble in ethanol and acetone. The sample contained a small amount of impurities. Colorless needles (240 mg), m. p. 204~208°C. Mass : m/e 280 (M^+). *Anal.* Found: C, 67.06; H, 8.13; N, zero. Calcd. for $C_{16}H_{24}O_4$: C, 68.54; H, 8.63 %.

Brefeldin A diacetate (a) Sixty milligrams of the sample were dissolved in 3 ml of acetic anhydride containing 100 mg of anhydrous sodium acetate. The mixture was heated on boiling water for 1 hr. Then the reaction mixture was poured into ice-water and the excess of acetic anhydride was decomposed. The precipitate was collected by centrifugation and washed with water. The acetate was purified by a preparative TLC (Rf 0.6; silica gel G/ benzene-ethyl acetate 9 : 1) to afford colorless needles, m. p. 129-130°C. *Anal.* Found: C, 66.05 ; H, 7.90

%. Mass : m/e (M^+) 364. Calcd. for $C_{20}H_{28}O_6$: C, 65.91; H, 7.74 %. Mol. wt. 364. Identical with brefeldin A diacetate on IR and NMR spectra.

On the preparative TLC, another compound of the Rf value 0.76 was detected. The compound is remained to study as only limited amount of sample was available.

(6) A crude preparation of brefeldin A (100 mg) was dissolved in 3 ml of acetic anhydride. The solution was heated on boiling water for 30 minutes, then the reaction mixture was poured into ice-water to decompose acetic anhydride. The precipitate was collected by centrifugation and dissolved in ether. The ether was washed with a dilute sodium bicarbonate solution and then water. The ether layer was separated and dried over anhydrous sodium sulfate, then the ether was removed. The residue was purified by a preparative TLC as in a to give brefeldin A diacetate along with the compound of Rf 0.76.

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