STUDIES ON A SESQUITERPENE ANTIBIOTIC, COMPOUND A-2,MINOR COMPONENT FROM *MYROTHECIUM*. I

FERMENTATION, ISOLATION, PURIFICATION, PHYSICOCHEMICAL PROPERTIES AND THE STRUCTURE

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A new antifungal substance, compound A-2 was isolated from fermentation broth of a fungus which was identified as a new variety of *Myrothecium verrucaria* by the authors. It was obtained as colorless prisms melting at 147~148°C having the molecular formula $C_{19}H_{26}O_6$. From the physicochemical properties and the structure, compound A-2 was concluded to be a new antifungal substance of sesquiterpene, 4, 15-diacetylverrucarol. This substance was active against fungi, especially *Trychophyton rubrum*.

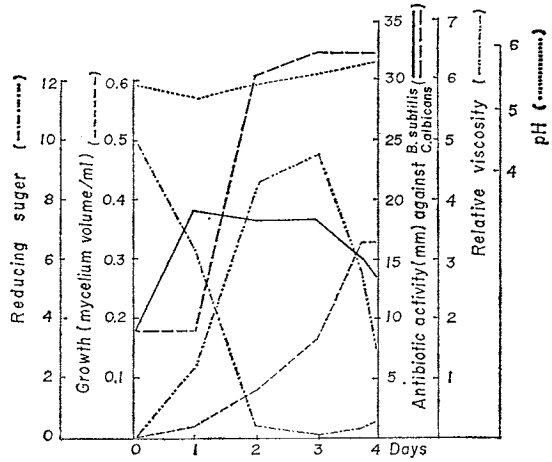
In a course of screening studies, applying multiple tests of biological activities to culture filtrate of isolated microorganisms, we found a new antifungal sesquiterpene, compound A-2 produced by strain 310-1 isolated from soil in Thailand. Among the known microbial products, the properties of compound A-2 were most closely related to trichodermin reported by S. VANGEDAL[1] as a product of *Trichoderma* sp.

In this paper, the fermentation, isolation, purification, physicochemical properties and chemical structure of the sesquiterpene were described.

Fermentation, Isolation and Purification of the Materials

When strain 310-1 was cultivated in a 20-liter fermentor containing 10 liters of a medium (glucose 1%, peptone 0.1% and yeast extract 0.1%, pH 5.4) at 30°C for 96 hours (Fig. 1), seven antifungal substances (A-1, A-2, B-1, B-2, C, D and E) and a butanol-extractable product with antimicrobial activity against *Bacillus subtilis* were found. A large quantity of viscous polysaccharide also accumulated in the culture fluid during the fermentation. The yields of the antibiotics were assayed by the conventional paper disc method with *Candida albicans* and *B. subtilis* as test organisms.

Fig. 1. Culture process of strain No. 310-1



The antifungal substances were isolated and

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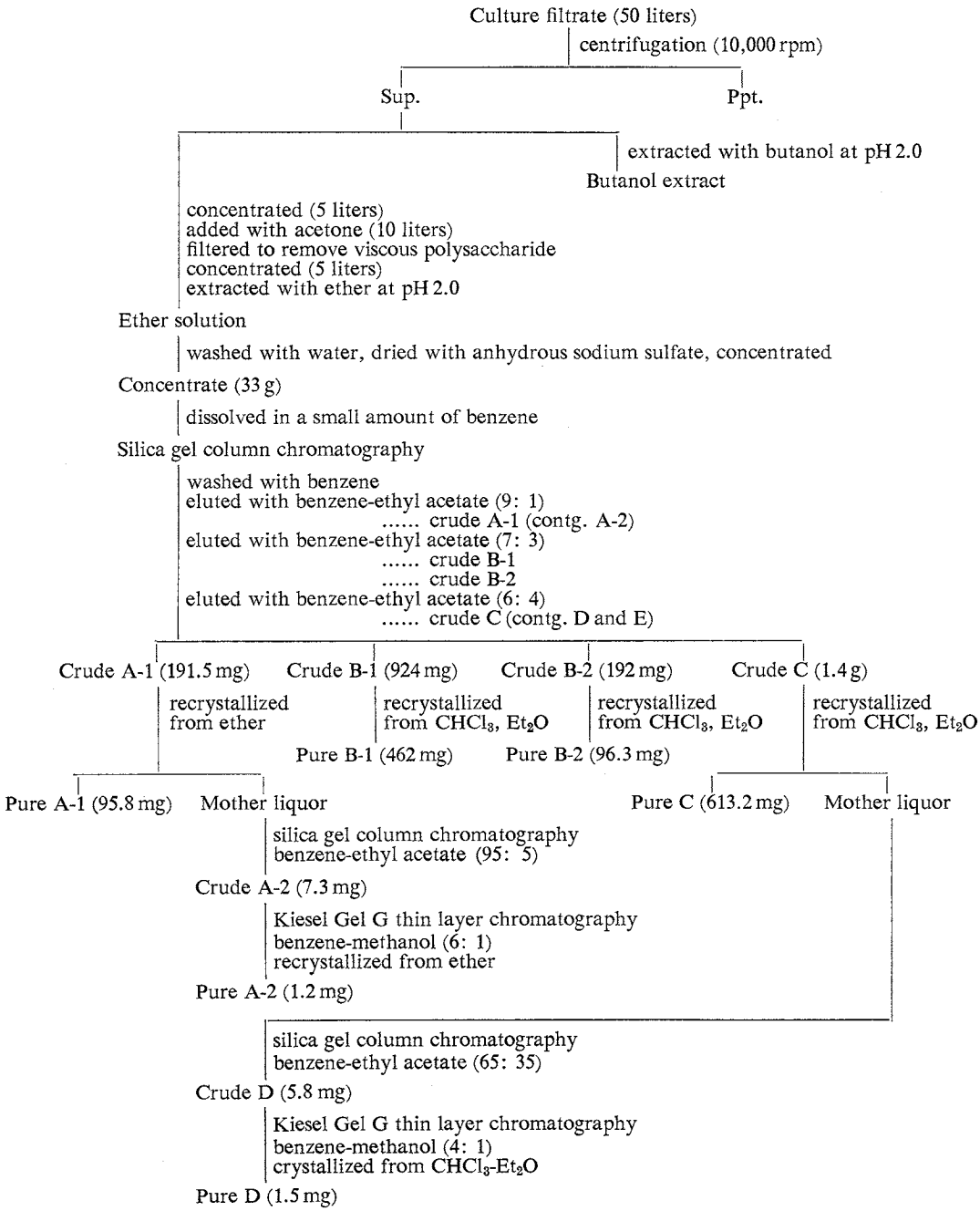
Fig. 2. Isolation and purification of seven antibiotics



purified by the following procedures (Fig. 2.). The culture fluid was centrifugated at 10,000 rpm to remove the mycelium. After the supernatant (50 liters) was concentrated *in vacuo* to 5 liters, acetone (10 liters) was added to precipitate and to remove the viscous polysaccharide, and the supernatant was again concentrated *in vacuo* to 5 liters. The concentrate was extracted several times with ether at pH 2.0. The combined extracts were washed with water, dried with anhydrous sodium sulfate and then evaporated *in vacuo* to yield an yellowish oily material (33 g), which was dissolved in a

small amount of benzene and adsorbed onto a silica gel column. The column was washed with 1 liter of benzene, and then developed with 1 liter of benzene-ethyl acetate (9: 1). The eluate active against *C. albicans* that was obtained was concentrated *in vacuo* to give yellowish oily compound A-1 (containing A-2). This material was crystallized and recrystallized from ether as white needles (95.8 mg) that melted at 360°C (decomp.).

Crude compounds B-1 and B-2 were next eluted from the column with benzene-ethyl acetate (7: 3). After removing the eluant the two materials were dissolved in ether and crystallized. They were respectively recrystallized from chloroform-ether as white plates (462 mg) and as white prisms (96.3 mg). Compounds B-1 and B-2 melted at 360°C (decomp.) and at 212.5~213.5°C, respectively.

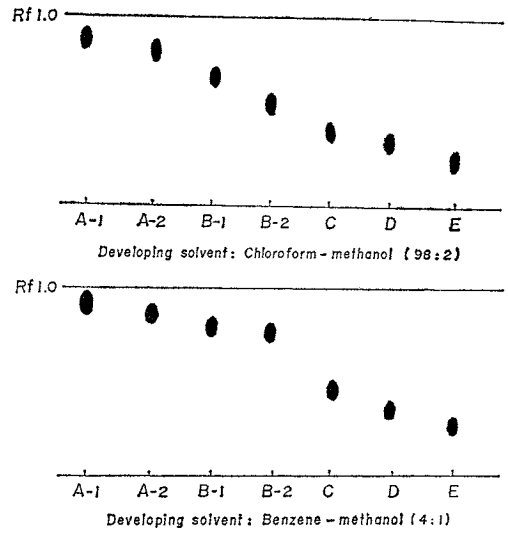
The chromatogram was subsequently developed with benzene-ethyl acetate (6: 4). Fractions active against *C. albicans* were combined and concentrated *in vacuo* to yield an yellowish oily compound C (containing D and E), which was crystallized and recrystallized from chloroform-ether as white prisms (613.2 mg); m.p. 183~184°C.

Compound A-2 was found in the mother liquor of compound A-1 and recovered from the solution by repeated silica-gel column chromatography employing benzene-ethyl acetate (95: 5) as eluant. Finally, the solution of compound A-2 was treated several times by thin-layer chromatography of Kiesel Gel G with benzene-methanol (6:1) as developing solvent. The purified zone of compound A-2 was eluted with ether. The solution was concentrated and crystals thus yielded were recrystallized from ether to white prisms (1.2 mg).

Compounds D and E were found in the mother liquor of compound C. The mother liquor was also submitted to silica-gel column chromatography and was eluted with benzene-ethyl acetate (65: 35). After several such treatments, solutions of compounds D and E were repeatedly developed with benzene-methanol (4: 1) on thin-layer chromatography of Kiesel Gel G. The purified zones of compounds D and E were eluted with ether. The eluate of compound D was concentrated and crystals of D thus obtained were recrystallized from chloroform-ether as white needles (1.5 mg) which melted at 126~127°C. The quantity of compound E contained in its eluate was so small that its ultimate purification and crystallization has not yet been successful.

The Rf values of compounds A-1, A-2, B-1, B-2, C, D and E on thin-layer chromatogram of Kiesel Gel G are shown in Fig. 3.

Fig. 3. Thin-layer chromatogram of seven antifungal substances



Compounds A-1 and B-1 were found to be verrucarin B and verrucarin A from their physicochemical properties, and compounds B-2, C and D appeared to be respectively verrucarin C, roridin A and verrucarin D as reported by CH. TAMM *et al.*[2~6] However, their complete chemical structures are still under investigation by us. Compound E also seems to belong to verrucarin-roridin group. The structure of the butanol extractable material from broth having antimicrobial activity against *B.*

subtilis will be elucidated later though its physicochemical properties resembled to those of the product of *Myrothecium verrucaria* A-H-14 reported by M. AOKI and co-workers.[7]

Physicochemical Properties of Compound A-2

Compound A-2 melted at 147~148°C. It is easily soluble in most organic solvents such as methanol, acetone, chloroform, ether and benzene, but sparingly soluble in water, hexane and petroleum ether.

The ultraviolet absorption spectrum had only end absorption. As shown in Fig. 4 the infrared absorption spectrum of compound A-2 in KBr tablet had the following characteristic bands; 1730 cm^{-1} (carbonyl), 1670 cm^{-1} (C=C), 1255 and 1245 cm^{-1} (ester) and 1085 cm^{-1} (ether).

Optical rotation in 1.0% methanol solution, $[\alpha]_D^{25}$ was −14°. The nuclear magnetic resonance spectrum of compound A-2 in deuterochloroform at 60 Mc is shown in Fig. 5. The singlet at δ 0.77

Fig. 4. IR spectra of A-2 (1) and acetylverrucarol (2) in KBr

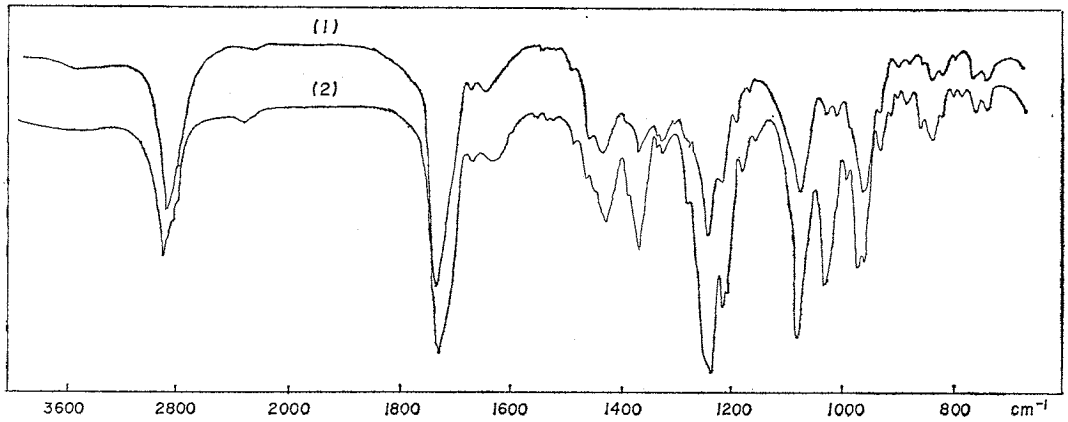
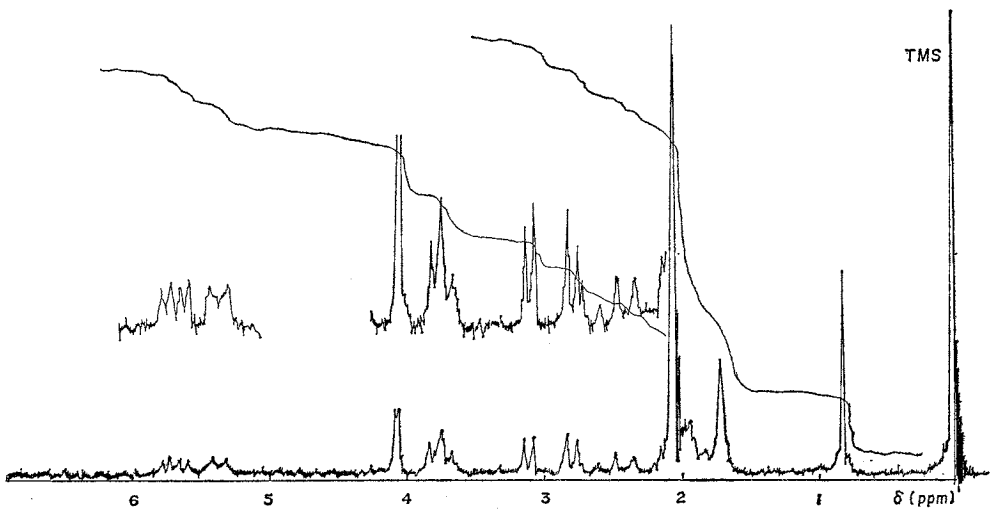


Fig. 5. NMR spectrum of A-2 in $CDCl_3$ (60 MHz)



revealed the presence of a quaternary methyl group, and the singlet at δ 1.70 could be attributed to a methyl group attached to a carbon-carbon double bond. Moreover the doublet at δ 1.90 revealed a presence of a quaternary $-CH_2-$ group, and the singlet at δ 2.0 demonstrated a presence of OAc. AB-system consisting of two doublets centered at δ 2.73 and δ 3.03 was characteristic of the grouping $\begin{matrix}CH_2\text{–}O\\ \diagdown\ \diagup\\ C\end{matrix}$. Of the three O–C–H signals (δ 3.66, δ 3.98 and δ 5.58) the quartet at δ 5.58 was due to the proton at the hydroxyl-bearing carbon atom. It should be noted that only one vinylic proton was present (signal at δ 5.25) which implied that the double bond in compound A-2 was tri-substituted. The six protons in compound A-2 spectrum which have not been accounted for were all in the region δ 1.2 to 2.6.

Elemental analysis of compound A-2 was as follows:

Found: C, 65.09, H, 7.45.
Calcd. for $C_{19}H_{26}O_6$: C, 65.12, H, 7.48. MW 350.40.

The mass spectral data of A-2 showed that mass number of its parent peak was 350 which precisely coincided with the theoretical molecular weight.

Antibiotic A-2 gave positive results to DRAGENDORFF, potassium permanganate, 2, 4-dinitrophenylhydrazine and ferric hydroxamate tests. But it gave negative results to potassium iodide, vanadium oxinate, DENIGÉS, periodic acid, methyl orange, fuchsin-sulfurous acid, sodium nitroprusside, chromotropic acid and EHRLICH tests.

No reduction of the antifungal activity was observed after heating at 60°C for 60 minutes in aqueous solution at pH 2.0~7.0, but the activity decreased after treating at 100°C for 30 minutes at pH 10.0.

The Rf values on paper chromatograms were as follows: Rf 1.0 with water-saturated *n*-butanol, with 50% acetone, with *n*-butanol-methanol-water (4: 1: 2), with benzene-methanol (4: 1) and with water; Rf 0.9 with 0.5% NH_4Cl; Rf 0.85 with 3.0% NH_4Cl; Rf 0.7 with 20% NH_4Cl.

On thin-layer chromatograms of Kiesel Gel G, the following Rf values were observed: Rf 0.9 with benzene-methanol (4:1); Rf 0.8 with chloroform-methanol (98: 2); Rf 0.2 with benzene-ethyl acetate (10:1).

Table 1. Antifungal spectrum of A-2 by agar dilution method

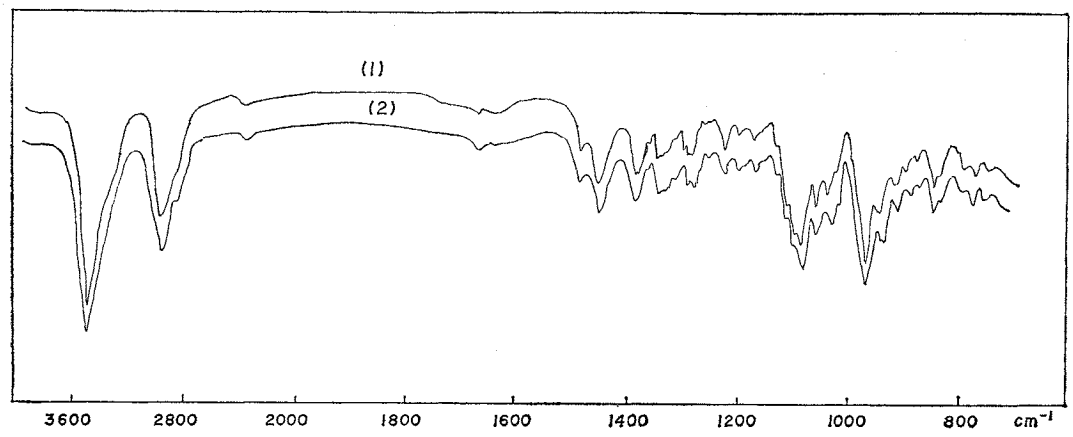
Test organism	M.I.C. (mcg/ml)
Gloeosporium kaki	10
Trichophyton rubrum	10
Trichophyton asteroides	50
Trichophyton interdigitale	50
Glomerella cingulata	50

As shown in Table 1, compound A-2 exhibits high antifungal activity against *Gloeosporium kaki* and *Trichophyton rubrum*. It is less active against yeast and other kinds of fungi such as *Candida albicans*, *Aspergillus niger*, *Penicillium chrysogenum*, *Botrytis cinerea*, *Sclerotium, hydrophilum*, *Trichophyton asteroides*, *T. interdigitale*, *Fusarium lini*, *F. roseum*, *Piricularia oryzae*, *Glomerella cingulata* and *Colletotrichum gloesporioides*, and inactive against bacteria including *Mycobacterium*.

Chemical Structure of Compound A-2

A solution of compound A-2 (10.2 mg) in a mixture of ethanol (0.5 ml) and 0.5 N aqueous sodium hydroxide (1 ml) was refluxed for 1 hour. After cooling, most of the ethanol was removed *in vacuo*, and the residue was extracted with chloroform. The extract was washed with water,

Fig. 6. IR spectra of hydrolysis product from A-2(1) and authentic verrucarol (2)



dried and evaporated *in vacuo*. The residue was crystallized and recrystallized from chloroform-methanol to white plate (6.1 mg), m.p. 157~158°C.

Physicochemical properties of this alkali-hydrolysis product were as followings: Optical rotation in 0.98% methanol solution, $[\alpha]_D^{25}$ was −54.9°. The ultraviolet absorption spectrum had only end absorption. The infrared absorption spectrum in KBr tablet was demonstrated in Fig. 6. The characteristic band, 3460 cm^{-1} (hydroxyl group) appeared instead of 1730 cm^{-1} (carbonyl group) of compound A-2. In NMR data of the hydrolysis product the singlet at δ 2.02 and the singlet at δ 2.28 (OH) appeared instead of the singlet at δ 2.0 ($-OCH_3CO$) of compound A-2. Its elemental analysis was as follows:

Found: C, 67.41; H, 7.98.
Calcd. for $C_{15}H_{22}O_4$: C, 67.64; H, 7.64. MW 266.33.

In its mass spectrum the parent peak occurred at *m/e* 266.

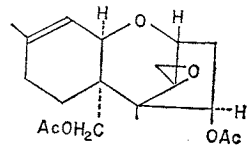
The above-mentioned data suggested a presence of two acetyl groups in compound A-2. Moreover, infrared absorption spectra as shown in Fig. 6 and other physicochemical properties of the above hydrolysis product and authentic verrucarol[8] prepared by alkali-hydrolysis of verrucarin B were in perfect agreement with each other. Besides, a mixture melting point test of the substance and the authentic specimen evidenced no depression.

Authentic verrucarol (260.3 mg) was dissolved in a mixture of pyridine (2.5 ml) and acetic anhydride (5 ml). After standing at room temperature for 15~20 hours, 50 ml of ice-water was added, and the mixture was repeatedly extracted with chloroform. The extracts were combined, washed with water, dried, and evaporated to a syrup, which was subsequently dissolved in benzene-methanol and crystallized. The crystal (305.4 mg) was again dissolved in the same solvent and recrystallized to white prisms, (196.7 mg), m.p. 147~148°C, $[\alpha]_D^{25}$ −15° (*c* 1.01, methanol). Its elemental analysis was as follows:

Found: C, 64.96; H, 7.50.
Calcd. for $C_{19}H_{26}O_6$: C, 65.12; H, 7.48. MW 350.40.

Infrared absorption spectra of this product and compound A-2 gave excellent agreement (Fig. 4). Furthermore, a mixture melting point test of both materials revealed no depression and in other physicochemical and biological properties they were thoroughly in accordance

Fig. 7. Structure of A-2



with each other.

Obviously, therefore, compound A-2 was defined to have the structure of 4, 15-diacetylverrucarol (Fig. 7) being a hitherto unknown antibiotic occurring in nature.

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