Citreobenzofurans A, B, and C, First Isolation of Sesquiterpene-Type Metabolites of a Hybrid Strain KO 0031 Derived from *Penicillium Citreo-viride* B. IFO 4692 and 6200

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Synopsis. Three new sesquiterpene-type metabolites, citreobenzofurans A, B, and C, have been isolated from the mycelium of the hybrid strain KO 0031 derived from *Penicillium citreo-viride* B. IFO 4692 and 6200. Their stereostructures have been also elucidated on the basis of their spectral data.

In connection with citreoviridin, a potent inhibitor of ATP-synthesis and ATP-hydrolysis catalyzed by mitocondrial enzyme system, a number of novel metabolites have been isolated from the mycelium of *Penicillium citreo-viride* B. Particularly, citreoviridin and related pyrones have been mainly produced by *P. citreo-viride* B. (IFO 6200).[1] In the case of another strain IFO 4692, however, citreoviranol and related phenols have been obtained as main products.[2] In the light of these results, more than ten hybrid strains have been produced by means of cell fusion technique using two different strains of IFO 6200 and 4692.[3] Among them, the hybrid strain KO 0031 was used for the present study.

Recently, we could isolate six terpenoid-type metabolites, which are quite attractive because of their novel structures as well as biological activities.[4,5] Particularly, citreohybridone B and isocitreohybridone B, which have already been obtained from the mycelium of the hybrid strain KO 0031,[6] show high antifeedant activity against *Plutella xylostella*. Further investigation of other metabolites in the same mycelium of the hybrid strain KO 0031 resulted in the first isolation of three new sesquiterpenes.

According to essentially the same procedure as described in the previous paper,[6,7] the EtOAc extract was chromatographed on silica gel. After elution of higher fatty acids and their esters with $CHCl_3$, further elution with $CHCl_3$–MeOH (10 : 1) afforded a pale yellow oil, which was further separated by repeated preparative TLC to give three new sesquiterpenes, named citreobenzofurans A(**1**), B(**2**), and C(**3**) in 0.0014, 0.0011, and 0.003% yields, respectively.

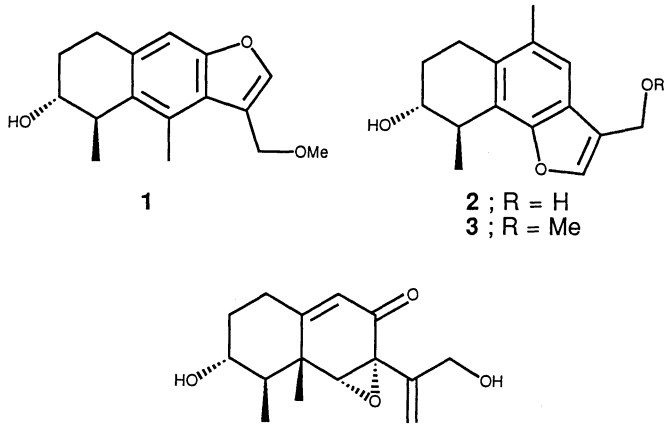

Fig. 1.

Citreobenzofuran A(**1**) with a molecular formula $C_{16}H_{20}O_3$ has the IR absorption band at 3440 cm^{-1}, and its ^1H NMR spectrum has the singlet due to a methoxyl group at δ=3.40, one singlet methyl at δ=2.59, and a methyl doublet at δ=1.17. As seen in Fig. 2, a detailled analysis of the ^1H NMR spectrum of **1** by homonuclear spin decoupling experiments indicated the presence of partial structures [**A**], [**B**], and [**C**]. Especially, long range couplings have been observed between C_1-methylene group (δ=2.90, and 3.12) and C_9-proton (δ=7.10) (allyl type coupling) and between C_2-α-proton (δ=1.93) and C_4-α-proton (δ=3.25) (W-type coupling). On the basis of NOE difference experiments, furthermore, not only gross structure but also stereostructure of **1** have been elucidated. Irradiation of the methoxyl group (δ=3.40) of **1** resulted in 0.85% NOE of the C_{12}-proton (δ=7.48), 0.66% NOE of the C_6-methyl group (δ=2.59), and 1.5% NOE of C_{13}-methylene group (δ=4.59 and 4.60), irradiation of the methylene group (C_{13}, δ=4.59 and 4.60) resulted in 8.4% NOE of the C_{12}-proton, 2.6% NOE of the C_6-methyl group, and 3.7% NOE of the methoxyl group (δ=3.40), irradiation of the C_6-methyl group (δ=2.59) resulted in 13.5% NOE of the C_4-proton (δ=3.25), 1.5% NOE of the C_4-methyl group, 2.9% NOE of the C_{13}-methylene group, and 0.79% NOE of the methoxyl group (δ=3.40), and irradiation of the C_4-methyl group (δ=1.77) also resulted in 2.1% NOE of the C_6-methyl group, 10.6% NOE of the C_4-proton (δ=3.25), 7.93% NOE of the C_3-proton (δ=4.14), and 7.15% NOE of the C_2-β-proton (δ=2.08). Other NOE interactions are shown in Fig. 3.

On detailed comparisons of the ^1H NMR spectra between citreobenzofurans B(**2**) and C(**3**), their structures are quite similar to each other except for the following point. The former with a molecular formula $C_{15}H_{18}O_3$ has two OH groups (ν_{max} 3370 cm^{-1}).[8] On the other hand, **3** with a molecular formula $C_{16}H_{20}O_3$ has a MeO group (δ=3.38) instead of one of the two OH groups, indicating that the latter must be the methoxy derivative of **2** at C_3 or C_{13}-position. On the basis of detailed ^1H NMR decoupling experiments, **3** has a methine group (C_4) at δ=3.37 which has a W-type long-range coupling with the C_2-proton at δ=2.04, and an isolated methylene group at δ=4.57, and two isolated sp^2 protons at δ=7.31 and 7.53. Finally, the gross structure

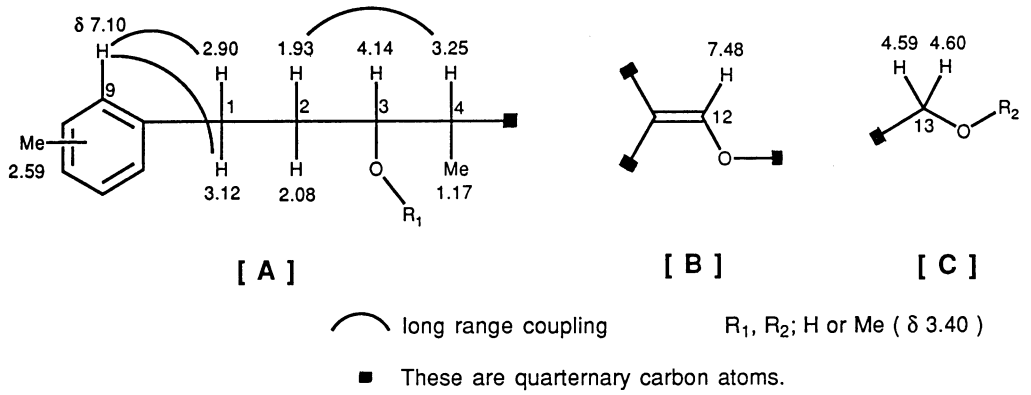

Fig. 2. Partial structures of citreobenzofuran A (**1**).

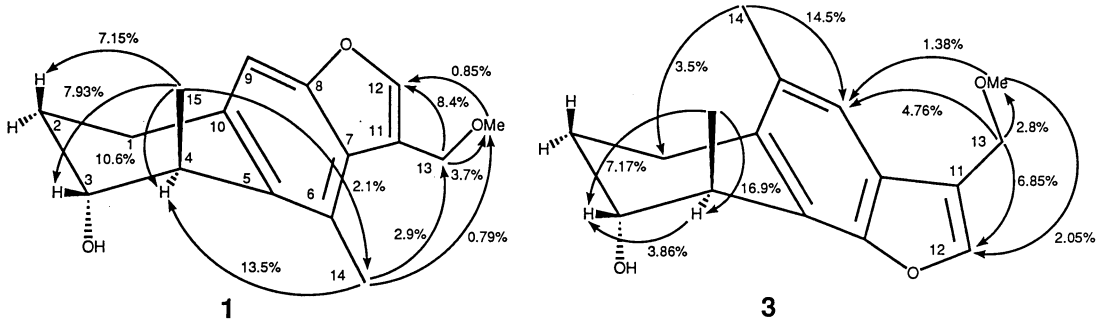

Fig. 3.

and the stereochemistry of **3** were elucidated by the NOE experiments. Irradiation of the C_{14}-proton (δ=2.32) of **3** resulted in 14.5% NOE of the C_8-proton (δ=7.31), and 3.5% NOE of the C_1-methylene group (δ=2.73 and 2.82), and irradiation of the C_{13}-methylene group resulted in 6.8% NOE of the C_{12}-proton (δ=7.53), 4.8% NOE of the C_8-proton (δ=7.31), and 2.8% NOE of the methoxyl group (δ=3.38). Other interactions are shown in Fig 3.

Recently, R. Capasso et al.[9] reported that phomenone (**4**),[10] a known phytotoxin and mycotoxic sesquiterpene, afforded a new substituted benzofuran by treatment with 10% H_2SO_4 in MeOH. Citreobenzofuran C(**3**) isolated by us was identical with Capasso's benzofuran. Therefore, we cannot rule out a possibility in which **3** is an artifact of phomenone (**4**). However, when **4**[11] was treated with SiO_2 in MeOH–$CHCl_3$ (1 : 10) at room temperature for 5 d, any amount of **3** was not detected, suggesting that **3** is not an artifact of phomenone.

From a biological point of view, citreobenzofurans A(**1**), B(**2**), and C(**3**) as well as citreohybridone B and phomenone are quite interesting. Further studies on biological properties of these compounds are in progress.

Experimental

All the melting points were uncorrected. Optical rotations were determined with a JASCO A-202 spectrophotometer. ^1H and ^{13}C NMR spectra were taken on a JEOL JNM-GX 400 NMR spectrometer in $CDCl_3$ with tetramethylsilane as an internal standard. Coupling constants are given in Hz (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet), unless otherwise noted. Mass spectra were obtained on a Hitachi M-80 mass spectrometer operating with an ionization energy at 70 eV. Preparative and analytical TLC were carried out on Kieselgel 60 PF_{254} (E. Merck A. G. Germany), unless otherwise stated.

Cell Fusion Technique. Each protoplast corresponding to *Penicillium citreo-viride* B. IFO 6200 and 4692 was prepared by enzymatic treatment of these two strains, which were incubated on potato sucrose agar (25°C, 7 d), using cellulase, chitinase, pectolyase and sulfatase (30°C, 60 min). And then, these two protoplasts in 0.05 M Ca solution (pH 10.5) were subjected to cell fusion experiments using polyethylene glycol (PEG 6000) as usual and incubated on potato sucrose agar (25°C, 3 d) to give a number of colonies, from which many new hybrid strains including *Penicillium citreo-viride* KO 0031 were obtained. These experiments were reported briefly (H. Furukawa, K. Kawai, M. Niwa, M. Yogo, S. Yamamura, and Y. Shizuri, 109 th National Meeting of The Pharmaceutical Society of Japan, Nagoya, April 1988, Abstr., No. 4FF 1-6.).

Incubation. Polished rice (ca. 4.5 Kg) in deionized water (ca. 6 l) was cooked using an electric cooker (99°C, ca. 20 min) and transfered into thirty-five Erlenmyer flasks (3 l), which were pasteurized at 121°C for 20 min at 2.1 atm. After inoculated with a suspension of the mycelium of the hybrid strain KO 0031 in a sterilized water, the rice was incubated stationarily at 25°C for 30 days and extracted with acetone (160 l).

Isolation and Separation. The acetone extract suspended in water (800 ml) was extracted with EtOAc (1.0 l×5). The EtOAc extract (dark brown syrup, 64.2 g) was chromatographed on silica gel (600 g, silica gel 60 K070, 70—230 mesh, Katayama Chemical). After elution of higher fatty acids and their esters with $CHCl_3$, further elution with $CHCl_3$–MeOH

(10 : 1) afforded a pale yellow oil (6.06 g), which was further separated by repeated preparative TLC (Kieselgel PF_{254}) using acetone–hexane (1 : 1), acetone–hexane (1 : 1.5), and then EtOAc–benzene (1 : 1.5) to give three new sesquiterpenes, named citreobenzofurans A (**1**), B (**2**), and C (**3**) [**1**: 0.9 mg (0.0014%), **2**: 0.70 mg (0.0011%), and **3**: 2.5 mg (0.0030%)] together with phomenone (32 mg, 0.050%).

Citreobenzofuran A(1) as a Colorless Oil: $[\alpha]_D^{28}$ +0.67° (*c* 0.045, $CHCl_3$); IR (film) 3440 cm^{-1}; ^1H NMR ($CDCl_3$) δ=1.17 (3H, d, *J*=7.1 Hz), 1.93 (1H, m), 2.08 (1H, m), 2.59 (3H, s), 2.90 (1H, ddd, *J*=17.9, 6.8. and 2.1 Hz), 3.12 (1H, ddd, *J*=17.9, 12.2, 7.2 Hz), 3.25 (1H, ddd, *J*=2.1, 1.0, 7.1 Hz), 3.40 (3H, s), 4.14 (1H, ddd, *J*=4.7, 2.5, 2.5 Hz), 4.59 (1H, d, *J*=11.8 Hz), 4.60 (1H, d, *J*=11.8 Hz), 7.10 (1H, s), and 7.48 (1H, s). MS *m/z* 260 (M^+), 242 (M^+-H_2O), and 210 (M^+-H_2O–MeOH). Found: *m/z* 260.1425. Calcd for $C_{16}H_{20}O_3$: M, 260.1411.

Citreobenzofuran B(2) as a Colorless Oil: $[\alpha]_D^{28}$ +19.4° (*c* 0.035, $CHCl_3$); IR (film) 3370 cm^{-1}; ^1H NMR ($CDCl_3$) δ=1.38 (3H, d, *J*=7.2 Hz), 2.05 (2H, m) 2.32 (3H, s), 2.77 (2H, m), 3.36 (1H, dq, *J*=2.6, 7.2 Hz), 4.07 (1H, ddd, *J*=4.7, 2.6, 2.5 Hz), 4.80 (2H, s), 7.33 (1H, s), and 7.55 (1H, s). MS *m/z* 246 (M^+) and 228 (M^+-H_2O). Found: *m/z* 246.1244. Calcd for $C_{15}H_{18}O_3$: M, 246.1254.

Citreobenzofuran C(3) as a Colorless Oil: $[\alpha]_D^{28}$ +9.0° (*c* 0.125, $CHCl_3$); IR (film) 3440 cm^{-1}; ^1H NMR ($CDCl_3$) δ=1.38 (3H, d, *J*=7.1 Hz) 2.04 (2H, m), 2.32 (3H, s), 2.73 (1H, ddd, *J*=17.6, 6.2, 2.7 Hz), 2.82 (1H, ddd, *J*=17.6, 10.1, 6.8 Hz), 3.37 (1H, m), 3.38 (3H, s), 4.06 (1H, ddd, *J*=5.3, 2.8, 2.8 Hz), 4.57 (2H, s), 7.31 (1H, s), and 7.53 (1H, s). ^{13}C NMR ($CDCl_3$) δ=19.9 (q), 20.0 (q), 22.0 (t), 25.3 (t), 36.4 (d), 57.8 (q), 65.0 (t), 70.6 (d), 117.3 (s), 118.2 (d), 122.8 (s), 124.4 (s), 130.2 (s), 131.1 (s), 142.0 (d), and 153.7 (d). MS *m/z* 260 (M^+), 242 (M^+-H_2O), and 210 (M^+-H_2O–MeOH). Found: *m/z* 260.1429. Calcd for $C_{16}H_{20}O_3$: M, 260.1411.

Conversion of phomenone (4) into Citreobenzofuran C(3). (1) Phomenone (14.5 mg) was added to a 5% solution of concd H_2SO_4 (0.025 ml) in MeOH–H_2O (0.375 ml, 0.1 ml). The reaction was stirred at 70—80 °C for 1 h and at room temp for 13 h. TLC analysis indicated the presence of the starting material (R_f 0.16), new UV-active component with an R_f of 0.58 (EtOAc–benzene 1 : 15 (v/v)) and too many components. The reaction mixture was neutralized with sat. aq $NaHCO_3$, diluted with H_2O (5 ml) and extracted with EtOAc (3×6 ml). The combined organic layers were dried on anhydrous Na_2SO_4 and evaporated under reduced pressure giving a crude residue (14.0 mg) which was purified by preparative TLC (EtOAc–benzene 1 : 1.5 (v/v)) to afford **3** (0.62 mg, 4.3% yield, 8.2% conversion yield) as a main product in addition to the recovered phomenone (6.95 mg, 48% yield).

(2) Phomenone (3.0 mg) in MeOH–$CHCl_3$ (0.1 ml, 1.0 ml) was absorbed on SiO_2 (100 mg) at room temp for 5 d. After addition of EtOAc (15 ml), the reaction mixture was washed with water (10 ml×2). The EtOAc solution was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to leave an oil, whereupon TLC analysis did not show any spot of **3** except for the unchanged starting material.

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3) Each protoplast corresponding to *P. citreo-viride* B. IFO 6200 and 4692 was prepared by enzymatic treatment of these two strains, which were incubated on potato sucrose agar (25 °C, 7 d), using cellulase, chitinase, pectolyase and sulfatase (30 °C, 60 min). And then, these two protoplasts in 0.05 M Ca solution (pH 10.5) were subjected to cell fusion experiments using polyethylene glycol (PEG 6000) as usual manner and incubated on potato sucrose agar (25 °C, 3 d) to give a number of colonies, from which many new hybrid strains including *P. citreo-viride* ME 0004 and 0005 were obtained. These experiments were reported briefly (H. Furukawa, K. Kawai, M. Niwa, M. Yogo, S. Yamamura, and Y. Shizuri, 109th National Meeting of The Pharmaceutical Society of Japan, Nagoya, April 1988, Abstr., No. 4FF 1-6.).

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8) The number of hydroxyl groups were confirmed by acetylation experiment affording the corresponding diacetate [δ=1.98 and 2.08 in $CDCl_3$, IR (film) 1735 cm^{-1} and no OH absorption band].

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11) This metabolite (**4**) has also been isolated from the mycelium of a hybrid strain KO 0031.