THREE NEW PHENOLIC METABOLITES FROM *PENICILLIUM* SPECIES

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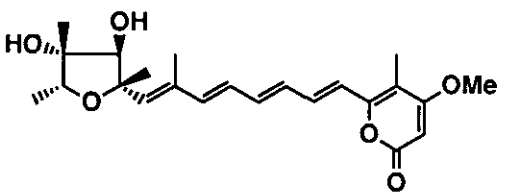
Abstract - Citreochlorol (**7**), citreoisocoumarin (**9**), and citreoisocoumarinol (**12**) have been isolated from the mycelia of *Penicillium citreo-viride* B. (IFO 4692), *P. citreo-viride* B. (IFO 4692) on addition of sodium bromide, and *P. citreovirens* (IFO 6030), respectively. Citreoisocoumarin was tentatively assigned as 3-(2S-hydroxy-4-oxopentyl)-6,8-dihydroxyisocoumarin and citreoisocoumarinol was identified as 3-(2S,4S-dihydroxypentyl)-6,8-dihydroxyisocoumarin. Both of them are new members of the small group of naturally occurring isocoumarins. The structure of the new phenolic metabolite citreochlorol has also been established on the basis of spectral data analysis.

Citreoviridin (**1**), a neurotoxic polyene-pyrone mycotoxin produced by *Penicillium citreo-viride* [1] and *P. pulvillorum*,[2] and related metabolites[3] are quite attractive to us because of its fascinating structure as well as remarkable physiological activities inhibiting ATP-synthesis and ATP-hydrolysis which are both catalyzed by the mitochondrial enzyme system.[4] In connection with citreoviridin, we have isolated a number of novel pyrone compounds from the metabolites of *P. citreo-viride* B. (IFO 6049, 6050, 6200) and *P. pedemontanum* (IFO 9583)[5] and two new phenolic compounds from a different strain of *P. citreo-viride* B. (IFO 4692).[6] On addition of sodium bromide to the polished rice inoculated with the latter (IFO 4692), furthermore, we could isolate four new phenolic metabolites including two novel compounds, citreoviranol (**2**) and demethylcitreoviranol (**3**), the first examples of naturally occurring spiro-dihydroisocoumarins.[7]



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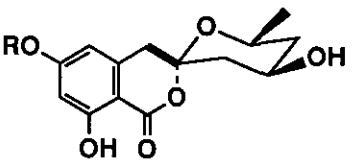
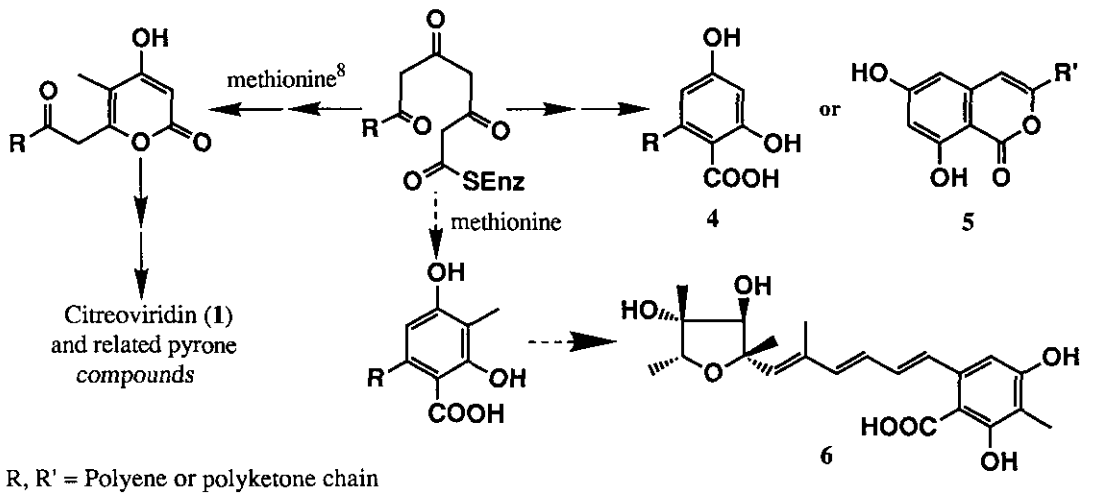


2 (**R= Me**)
3 (**R= H**)

From a biogenetic point of view, β-polyketo carboxylic acids are expected to convert into both the corresponding pyrones and phenolic compounds according to the mode of enzymatic cyclization. (Scheme 1)



Scheme 1 Biosynthetic pathways of pyrones and phenolic compounds

Thus, our further effort has been made on searching for phenolic compounds of both structural and physiological significance from the metabolites of *Penicillium* species, leading to the isolation and identification of three new phenolic compounds (Types **4** and **5**), citreochlorol (**7**), citreoisocoumarin (**9**), and citreoisocoumarinol (**12**), from the mycelia of *P. citreo-viride* B. (IFO 4692 with and without NaBr) and *P. citreovirens* (IFO 6030), respectively.

According to essentially the same procedure as described in the previous papers,[6,7] the EtOAc extracts (A, B, and C) were separated by chromatography on silica gel (Katayama Chemicals, Type 60) using a gradient solvent (1-10% MeOH in $CHCl_3$). Further separation and purification were achieved by recrystallization or preparative tlc with different solvent systems. 8% MeOH-$CHCl_3$ Elute of extract A afforded citreochlorol (**7**) in addition to the known precitreobiphenyl (**8**).[7] Extract B gave the new compound citreoisocoumarin (**9**) together with citreoviranol (**2**),[7] demethylcitreoviranol (**3**),[7] precitreobiphenyl (**8**),[7] citreovirenone (**10**),[6] and (+)-orthosporin (de-O-methyldiaporthin) (**11**)[9] in 5-7% MeOH-$CHCl_3$ elution fraction. 5-7% MeOH-$CHCl_3$ Elute of extract C afforded the new citreoisocoumarinol (**12**) in addition to (+)-orthosporin (**11**)[9] and phomenone (**13**),[10] as seen in Figure 1.

Citreochlorol (7) with a molecular formula $C_{12}H_{16}O_4Cl_2$ shows bands at 3400, 1600, and 1505 cm^{-1} in its ir spectrum, indicating the presence of at least one hydroxyl group and a benzene ring. The ^1H nmr spectrum suggests the presence of a 1,3,5-trisubstituted benzene ring [δ 6.21 (1H, t, J= 2.0 Hz), 6.28 (1H, t, J= 2.0 Hz), and 6.30 (1H, t, J= 2.0 Hz)], one methoxyl group [δ 3.73 (3H, s)], and a dichloromethyl group [δ 5.88 (1H, d, J= 3.4 Hz)]. Furthermore, the nmr signals at δ 4.02 (1H, m) and 4.13 (1H, m) indicate the presence of two methines bearing two hydroxyl groups or one hydroxyl and one methoxyl groups. Irradiation of the multiplet at δ 4.13 collapsed the doublet at δ 5.88 into a singlet and simplified the multiplet at δ 1.71 (2H, m). Irradiation of the multiplet at δ 4.02 also simplified the multiplet at δ 1.71 and changed both the signals at

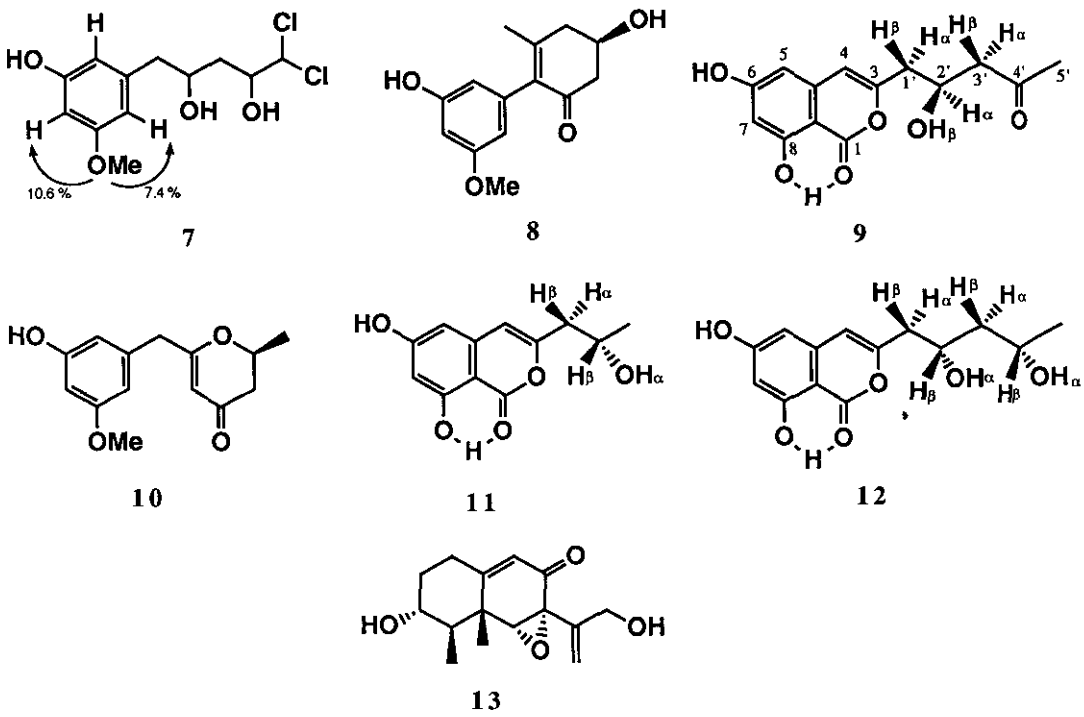
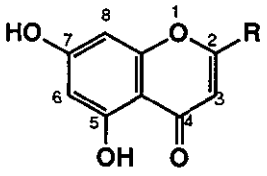


Figure 1 Some metabolites from *P. citreo-viride* B. (IFO 4692 with and without NaBr) and *P. citreovirens* (IFO 6030)

δ 2.62 (1H, dd, J= 13.2, 6.4 Hz) and δ 2.71 (1H, dd, J= 13.2, 7.3 Hz) into doublets (J= 13.2 Hz). Thus, citreochlorol was unambiguously established as structure (**7**) wherein the substitution pattern was supported by NOE experiment.

Citreoisocoumarin (**9**) has, in its high resolution mass spectrum, the highest peak at m/z 260.0669, corresponding to the composition of $C_{14}H_{12}O_5$ (260.0684). The ^1H nmr spectrum suggests the presence of a methyl group [δ 2.23 (3H, s)], two methylene groups [δ 2.67 (2H, d, J= 6.3 Hz), 2.71 (2H, d, J= 6.3 Hz)] coupling with a common methine group [δ 4.47 (1H, m)], and a chromone (**14**), a coumarin (**15**) or a isocoumarin (**16**) skeleton [δ 6.32 (1H, d, J= 2.2 Hz), 6.33 (1H, s), 6.39 (1H, d, J= 2.2 Hz)]. In the mass spectrum, citreoisocoumarin gives the same fragmentation pattern as that of (+)-orthosporin,[9] showing a base peak at m/z 192 (**18**) and predominent peaks at m/z 177, 164, 163, and 150. Absence of an ion at m/z 152 (**17**), the characteristic ion resulting from the retro-Diels-Alder reaction for the corresponding chromones, [11] excluded the possibility of the chromone structure (**14**). The ir spectrum shows absorptions of hydroxyl and two carbonyl groups (3200, 1710, and 1680 cm^{-1}) in addition to typical absorptions of a benzene ring (1615, 1585, and 1505 cm^{-1}). On acetylation, a triacetate was obtained and the band at 3200 cm^{-1} disappeared while the band at 1680 cm^{-1} shifted to nearby 1730 cm^{-1}, indicating the presence of three hydroxyl groups, one of which is hydrogen-bonded to the carbonyl group (1680 cm^{-1}). Thus, the coumarin skeleton (**15**) can also be



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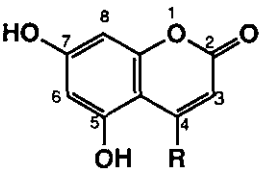

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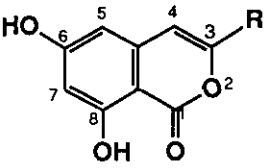

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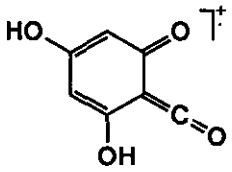
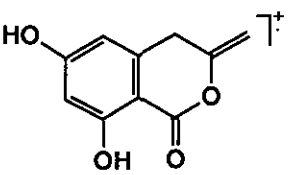


17 (m/z 152)



18 (m/z 192)

Table 1 Comparison of uv spectral data [λ_{max} nm (log ε)] of citreoisocoumarin (9) with some known compounds

9	11	14	15	16
(in EtOH)	(in MeOH)	(R= Me) (in MeOH)	(R= Me) (in MeOH)	(R= Me) (in EtOH)
240sh (4.03)	237 (4.31)	227 (4.08)	-	237 (4.62)
246 (4.08)	246 (4.38)	249 (4.13)	-	245 (4.69)
259sh (3.50)	-	256 (4.13)	258 (3.82)	-
278 (3.28)	275 (3.68)	-	-	278 (3.85)
289sh (3.14)	289 (3.53)	295 (3.65)	-	289 (3.72)
328 (3.22)	326 (3.52)	-	322 (4.10)	324 (3.79)

ruled out since in coumarins no hydrogen-bond can be formed between the C_2-ester carbonyl group and the C_5- or C_7-hydroxyl group. These observations were also supported by the uv spectral data which were quite different from both of 5,7-dihydroxy-4-methylchromone (**14**, R= Me)[12] and 5,7-dihydroxy-4-methylcoumarin (**15**, R= Me),[13] while very similar to those of 6,8-dihydroxy-3-methylisocoumarin (16, R= Me),[14] (+)-orthosporin (de-O-methyldiaporthin) (**11**)[9] (See Table 1) and other isocoumarin compounds.[15,16] Compared with (+)-orthosporin (**11**), citreoisocoumarin shows some similarities but significant differences in the ^{1}H nmr spectral data, particularly, the lack of a methyl signal at δ 1.24 (d, J= 6.3 Hz) and the additional presence of a methylene (δ 2.71, d, J= 6.3 Hz) and an isolated methyl groups (δ 2.23, s), as shown in Table 2. Hence,

citreoisocoumarin must be a 6,8-dihydroxyisocoumarin with a 2-hydroxy-4-oxopentyl group as the side chain at C_3-position and may be 3-(2S-hydroxy-4-oxopentyl)-6,8-dihydroxyisocoumarin (**9**) based on a comparison of the optical rotation ($[\alpha]_D$ -10°) with those of the synthetic (-)-orthosporin ($[\alpha]_D$ -22.4°) and the natural (+)-orthosporin (**11**, $[\alpha]_D$ +61.8°),[9] although the opposite 2'R-configuration is not necessarily ruled out.

Table 2 Comparison of the ^{1}H nmr data (δ, CD_3OD) of citreoisocoumarin, citreoisocoumarinol, and (+)-orthosporin*

Proton	Citreoisocoumarin (9)	(+)-Orthosporin (11)	Citreoisocoumarinol (12)
4 - H	6.33 (s)	6.30 (s)	6.30 (s)
5 - H	6.39 (d, J= 2.2)	6.37 (s)**	6.37 (s)**
7 - H	6.32 (d, J= 2.2)	6.30 (s)**	6.30 (s)**
1'- Hα		2.56 (dd, J= 15.1, 7.3)	2.55 (dd, J= 14.4, 8.3)
	2.67 (2H, d, J=6.3)		
1'- Hβ		2.61 (dd, J= 15.1, 5.9)	2.69 (dd, J= 14.4, 4.4)
2'- Hα	4.47 (m)	-	-
2'- Hβ	-	4.15 (m)	4.13 (m)
3'- Hα		-	1.70 (ddd, J= 13.7, 8.3, 7.3)
	2.71 (2H, d, J= 6.3)		
3'- Hβ		-	1.60 (ddd, J= 13.7, 5.4, 4.4)
3'- Me	-	1.24 (d, J=6.3)	-
4'- Hβ	-	-	3.98 (m)
5'- Me	2.23 (s)	-	1.19 (d, J= 5.9 Hz)

* Chemical shifts (δ) are given in ppm, and coupling constants (J) are given in Hz.

** Observed as doublets (J= 2.2 Hz) when measured on a JEOL FX 90 A spectrometer.

Citreoisocoumarinol (12) is somewhat similar to the new metabolite citreoisocoumarin (**9**) in both ir and ^{1}H nmr spectra except for that the former has only one carbonyl group (1680 cm^{-1}) and no isolated methyl group but instead has a tertiary methyl group [δ 1.19 (3H, d, J= 5.9 Hz)] and an additional hydroxyl-bearing methine group [δ 3.98 (1H, m)] as well as remarkable differences at the signals of 1'- and 2'-protons from those of the latter (**9**) (See Table 2). It gives the highest peak at m/z 280 in its mass spectrum in which the lack of an ion at m/z 152 (**17**) ruled out the chromone structure (**14**). The coumarin skeleton (**15**) was also excluded because the carbonyl group (1680 cm^{-1}) was hydrogen-bonded to the hydroxyl group (3350 cm^{-1}) in the molecule. Thus, the most plausible structure seems to be a 6,8-dihydroxyisocoumarin with a side chain of a 2,4-dihydroxypentyl group at C_3-position. As shown in Table 2, the signals at δ 1.70 (1H, ddd, J= 13.7, 8.3, 7.3 Hz) and 1.60 (1H, ddd, J= 13.7, 5.4, 4.4 Hz) should be assigned to 3'-Hα and 3'-Hβ or in reverse. The

proton at δ 1.60 is *syn* while the proton at δ 1.70 is *anti* to both 2'- and 4'-protons, clearly indicating that $C_{2'}$- and $C_{4'}$-hydroxyl groups exist on the same side, both have α (or β) -configurations. Furthermore, the protons at δ 4.13 (1H, m) and 3.98 (1H, m) may be attributed to 2'- and 4'-protons, respectively. This fact was confirmed by decoupling experiments as follows: Irradiation of the multiplet at δ 4.13 converted the signals at δ 1.60 and 1.70 into double doublets (dd, J= 13.7, 5.4 Hz; and dd, J=13.7, 7.3 Hz, respectively) in addition to collapsing each signal at δ 2.59 (1H, dd, J= 14.4, 4.4 Hz) and δ 2.55 (1H, dd, J= 14.4, 8.3 Hz) into a doublet (J= 14.4 Hz). Irradiation of the multiplet at δ 3.98 caused changes of the signals at δ 1.60 (into a dd, J= 13.7, 4.4 Hz), δ 1.70 (into a dd, J= 13.7, 8.3 Hz), as well as the doublet at δ 1.19 (3H, d, J= 5.9 Hz) into a singlet. Moreover, citreoisocoumarinol, like (+)-orthosporin, has a positive optical rotation ($[\alpha]_D$ +20.2°), strongly suggesting a 2'S absolute configuration in the molecule. From these results, citreoisocoumarinol was regarded as 3-(2S,4S-dihydroxypentyl)-6,8-dihydroxyisocoumarin (**12**).

Citreoisocoumarin (**9**) and citreoisocoumarinol (**12**) are new members of the small group of natural isocoumarins. They are probably the biosynthetic precursors of novel spiro-dihydroisocoumarin metabolites citreoviranol (**2**) and demethylcitreoviranol (**3**).

It is notable that the expected phenol (**6**) and related compounds may be, soon or late, found in nature and they are probably of some physiological importance as well as structural significance. A further study on this point is still in progress.

EXPERIMENTAL

Melting points were determined on a Mitamura Riken apparatus and uncorrected. Optical rotations were determined with a JASCO DIP-360 digital polarimeter. Uv spectrra were determined on a JASCO UVIDEC-610A spectrophotometer. And ir spectra were recorded on a JASCO A-202 spectrophotometer. ^{1}H Nmr spectra were recorded on a JEOL JNM-GX 400 NMR spectrometer in methanol-d_4 unless otherwise noted. Mass spectra were measured on a Hitachi M-80 GC-MS spectrometer, operating with an ionization energy at 70 eV.

Incubation and Extraction

1) *Penicillium citreo-viride* B. (IFO 4692): Polished rice (750 g) in deionized water (*ca.* 2000 ml) was cooked using an electric rice cooker (99 °C, *ca.* 20 min) and transfered into five Erlenmyer flasks (3 l) and then pasteurized at 120 °C for 20 min at 2 atm. Inoculated with a suspension of the mycelium of *P. citreo-viride* B. (IFO 4692) in sterilized water, the rice was further incubated at room temp (23- 25 °C) for three months and then extracted with acetone. Concentration of the acetone layer *in vacuo* gave an aqueous solution which was extracted with EtOAc, giving rise to a reddish brown oil (24.2 g) (Extract A).

2) *Penicillium citreo-viride* B. (IFO 4692) on addition of NaBr: Polished rice (300 g) in deionized water (*ca.* 800 ml) was allowed to stand at room temp for 30 min, then cooked using an electric rice cooker (99 °C, *ca.* 20 min) and transfered into an Erlenmyer flask (5 l), which was pasteurized at 120 °C for 20 min at 2 atm. After addition of sodium bromide (1 g), the polished rice so far treated was inoculated with a suspension of the mycelium of *P. citreo-viride* B. (IFO 4692) in sterilized water, incubated stationarily at 25 °C for 23 days and extracted with acetone and then with EtOAc. The combined extracts were partitioned between EtOAc and water. Concentration of the EtOAc layer *in vacuo* gave a reddish brown syrup (1.7 g) (Extract B)

3) ***Penicillium citreovirens*** **(IFO 6030):** Polished rice (450 g) in deionized water (*ca.* 1200 ml) was cooked using an electric rice cooker (99 °C, *ca.* 20 min), transfered into three Erlenmyer flasks (3 l) and pasteurized at 120 °C for 20 min at 2 atm. After inoculated with a suspension of the mycelium of *P. citreovirens* (IFO 6030) in sterilized water, the rice was further incubated at room temp (*ca.* 23- 25 °C) for four weeks and then extracted with acetone. The acetone layer was then concentrated *in vacuo* into an acetone-free aqueous solution which was extracted with EtOAc, giving an EtOAc extract as a dark brown oil (8.9 g) (Extract C).

Separation and Purification

1) Isolation of citreochlorol (7) from Extract A: Extract A was chromatographed on silica gel (800 g, Katayama Chemicals, Type 60), eluted with $CHCl_3$ (3000 ml) and a gradient solvent of MeOH-$CHCl_3$ (1-10% MeOH in $CHCl_3$, each 1000 ml). Elution with 8-10% MeOH-$CHCl_3$ afforded a brown solid which was further separated by repeated preparative tlc (Kieselgel 60 PF $_{254}$ and F $_{254}$) using $CHCl_3$-acetone (3 : 1), hexane-EtOAc-MeOH (6: 3 :1), and hexane-EtOAc (1 : 4), and recrystallization from $CHCl_3$-MeOH gave rise to citreochlorol (**7**, 0.043%) as pale brownish needles in addition to precitreobiphenyl (**8**, 0.026%).[7]

2) Isolation of citreoisocoumarin (9) from Extract B: Extract B was also chromatographed on silica gel (80 g, Katayama Chemicals, Type 60), using $CHCl_3$ (300 ml) and a gradient solvent of MeOH-$CHCl_3$ (1-10% MeOH in $CHCl_3$, each 200 ml). The brown oil from 5-7% MeOH-$CHCl_3$ eluant was then subjected to repeated preparative tlc (Kieselgel 60 PF $_{254}$ and F $_{254}$) using $CHCl_3$-MeOH [(10 : 1) x 2], hexane-EtOAc (1 : 1), hexane-acetone (5 : 1), and C_6H_6-EtOAc-MeOH (6 : 3 : 1), affording citreoisocoumarin (**9**, 0.26%) together with the known citreoviranol (**2**, 0.25%),[7] demethylcitreoviranol (**3**, 0.21%),[7] precitreobiphenyl (**8**, 0.15%),[7] citreovirenone (**10**, 0.18%),[6] and (+)-orthosporin (de-O-methyldiaporthin) (**11**, 0.14%).[9]

3) Isolation of citreoisocoumarinol (12) from Extract C: Extract C was separated by chromatography on silica gel (300 g, Katayama Chemicals, Type 60), using $CHCl_3$ (1000 ml) and then a gradient solvent of MeOH-$CHCl_3$ (1-10% MeOH in $CHCl_3$, each 1000 ml). The 5-7% MeOH-$CHCl_3$ elute gave citreoisocoumarinol (**12**, 0.015%) in addition to (+)-orthosporin (de-O-methyldiaporthin) (**11**, 0.03%)[9] and phomenone (**13**, 0.17%),[10] when further separation was carried out by repeated preparative tlc (Kieselgel 60 PF $_{254}$ and F $_{254}$) using $CHCl_3$-MeOH (5 : 1), hexane-acetone-MeOH (6: 3 :1), EtOAc, and $CHCl_3$-acetone-MeOH (10 : 4 : 1).

Citreochlorol (7), pale brown needles: mp 78-80 °C (from $CHCl_3$-MeOH). $[\alpha]_D^{30}$ +2.4° (c 0.672, MeOH). Ir (film) υ_{max} cm^{-1}: 3400, 2950, 2850, 1600, 1505, 1460, 1440, 1340, 1300, 1200, 1150, 1065, 935, 840, 780, and 760. ^1H Nmr: δ 1.71 (2H, m), 2.62 (1H, dd, J= 13.2, 6.4 Hz), 2.71 (1H, dd, J= 13.2, 7.3 Hz), 3.73 (3H, s), 4.02 (1H, m), 4.13 (1H, m), 5.88 (1H, d, J= 3.4 Hz), 6.21 (1H, t, J= 2.0 Hz), 6.28 (1H, t, J= 2.0 Hz), and 6.30 (1H, t, J= 2.0 Hz). Eims m/z: 296, 295, 294 (M^+), 278, 276 (M^+-H_2O), 259, 257, 242, 240, 223, 222, 205, 204, 194, 193, 177, 176,175, 167, 165, 163, 139, 138, 137, 125, 123,109, 107, and 77. Hrms Found: 296.0401. Calcd for $C_{12}H_{16}O_4{}^{35}Cl^{37}Cl$, M^+, 296.0440; Found: 295.0496. Calcd for $C_{12}H_{17}O_4Cl_2$, M^++1, 295.0502.

Citreoisocoumarin (9), yellowish powder: $[\alpha]_D^{29}$ -10.0° (c 0.1, MeOH). Uv (EtOH) λ_{max} nm (log ε): 240sh (4.03), 246 (4.08), 259sh (3.50), 278 (3.28), 289sh (3.14), and 328 (3.22), with bathochromic shift on addition of NaOH (256, 310, and 332 nm). Ir (film) υ_{max} cm^{-1}: 3200, 2925, 2855, 1710, 1680, 1615, 1585, 1505, 1455, 1345, 1290, 1230, 1160, 1070, 965, and 845. ^1H Nmr: δ 2.23 (3H, s), 2.67 (2H, d, J=

6.3 Hz), 2.71 (2H, d , J= 6.3 Hz), 4.47 (1H, m), 6.32 (1H, d, J= 2.2 Hz), 6.33 (1H, s), and 6.39 (1H, d, J= 2.2 Hz). Eims m/z: 260 (M^+-H_2O), 218, 192 (base peak), 177, 164, 163, 150, 146, and 121. Hrms Found: 260.0669. Calcd for $C_{14}H_{12}O_5$: M^+-H_2O, 260.0684.

Citreoisocoumarinol (12), yellowish powder: $[\alpha]_D^{26}$ +20.2° (c 0.12, MeOH). Ir (film) υ_{max} cm^{-1}: 3350, 2975, 2935, 2860, 1680, 1625, 1580, 1510, 1460, 1360, 1240, 1170, 1060, 970 and 850. ^1H Nmr: δ 1.19 (3H, d, J= 5.9 Hz), 1.60 (1H, ddd, J= 13.7, 5.4, 4.4 Hz), 1.70 (1H, ddd, J= 13.7, 8.3, 7.3 Hz), 2.55 (1H, dd, J= 14.4, 8.3 Hz), 2.69 (1H, dd, J= 14.4, 4.4 Hz), 3.98 (1H, m), 4.13 (1H, m), 6.30 (2H, s), and 6.37 (1H, s), Eims m/z: 280 (M^+), 263 (M^+-OH), 168, 167, 150, 149, and 129. Hrms Found: 263.0926. Calcd for $C_{14}H_{15}O_5$: M^+-OH, 263.0919.

Acetylation of citreoisocoumarin (9)

Citreoisocoumarin (**9**) (2.23 mg) in pyridine (0.2 ml) was treated with Ac_2O (0.2 ml) at room temp for 2.5 h, and then the reaction mixture was evaporated *in vacuo* to dryness. The residue was then subjected to preparative tlc (Kieselgel 60 F_{254}) developed with hexane-acetone (1 : 1), yielding the triacetate of citreoisocoumarin (2 mg) as yellowish powder. Ir (film) υ_{max} cm^{-1}: 2930, 2855, 1775, 1730 (br), 1610, 1565, 1365, 1235, 1185, 1130, 1020, 970, and 895. ^1H Nmr ($CDCl_3$): δ 2.03, 2.19, 2.34, 2.41 (each 3H, s), 2.83 (2H, complex), 2.84 (2H, complex), 5.48 (1H, m), 6.28 (1H, s), 6.93 (1H, d, J= 2.2 Hz), and 7.06 (1H, d, J= 2.2 Hz). Eims m/z: 344 (M^+-HOAc), 302, 260 (base peak), 218, 192, 189, 177, 163, and 121. Hrms Found: m/z 344.0912. Calcd for $C_{18}H_{16}O_7$: M^+- HOAc, 344.0894.

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