Organochlorine Compounds from a Terrestrial Higher Plant: Structures and Origin of Chlorinated Orcinol Derivatives from Diseased Bulbs of *Lilium maximowiczii*†

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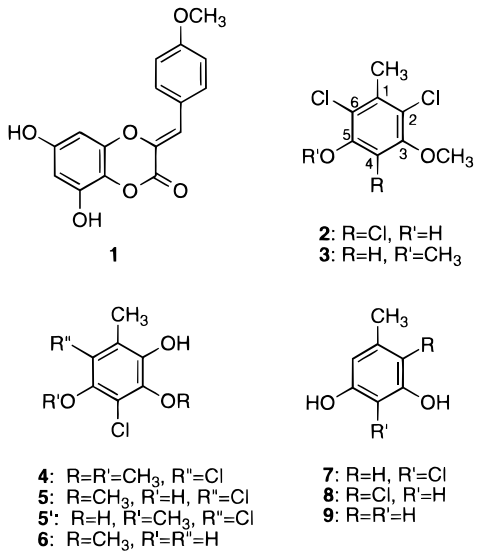
Seven chlorine-containing orcinol derivatives (**2**−**8**) and orcinol (**9**) have been isolated from diseased bulbs of the edible lily *Lilium maximowiczii*, and their structures have been elucidated. Six of the chlorinated orcinol derivatives (**2**, **4**−**8**) showed antifungal activity. Because organochlorine compounds are rare in terrestrial higher plants, their biosynthetic origin was examined. These compounds were shown to be induced in intact bulb scales by UV irradiation or by inoculation with the pathogenic fungus *Fusarium oxysporum f.* sp. *lilii*. Biosynthetic studies suggested that these "natural organochlorine pesticides" are produced by enzymatic chlorination of orcinol (**9**) with chloroperoxidase and hydrogen peroxide, which are both induced in the plant tissue under stress conditions.

The soil-borne fungal pathogen *Fusarium oxysporum f.* sp. *lilii*[1] is an economically important plant pathogen causing basal and bulb rot in lilies, and this seriously threatens the cultivation of the edible lily *Lilium maximowiczii* cv. Hakugin in Hokkaido, Japan.[2] Development of lesions in the bulbs is usually limited to the outermost scales. This restriction of the lesions suggests a dynamic resistance mechanism of the plant to invading pathogens. In a continuation of our studies on phytoalexins,[3] antimicrobial compounds synthesized by plants *de novo* after their exposure to microorganisms,[4] we examined *L. maximowiczii* cv. Hakugin and reported the isolation of a novel 3-benzylidene-1,4-benzodioxin-2(3*H*)-one derivative, yurinelide (**1**),[5] as the first lily phytoalexin. Further attempts to isolate additional antifungal constituents of this species, as indicated by a 2D TLC bioassay with *Bipolaris leersiae*,[6] have been hampered by compound lability. Careful separation using degassed solvents and the antioxidant BHT (2,6-di-*tert*-butyl-4-methylphenol)[7] has now resulted in the isolation of seven organochlorine compounds (**2**−**8**) together with orcinol (**9**).

Naturally occurring halogenated compounds have been isolated mainly from the marine environment and microorganisms.[8] Many chlorine- and bromine-containing metabolites from marine sources have been investigated because of recent interest in various biological activities as well as in their structural diversity.[9] However, only a few organochlorine or organobromine compounds have been isolated from terrestrial higher plants, though some organofluorine compounds are known to occur.[10] Considering the distinct differences in environmental factors between the land and the sea, the limited occurrence of halogenated compounds in terrestrial higher plants is not surprising. The isolation of appreciable amounts of organochlorine compounds from infected edible lily bulbs posed a significant question about their origin and has stimulated further examination. We wish to report herein the structures, origin, and biosynthetic studies of seven organochlorine compounds (**2**−**8**) obtained from infected lily bulb scales. Our results indicated that these unusual "natural organochlorine pesticides" are derived from orcinol (**9**) by induced enzymatic chlorination in the plant under stress conditions.



Results and Discussion

Diseased lily bulb scales (4.9 kg) collected from a field infested with *Fusarium oxysporum* f. sp. *lilii* were homogenized in EtOAc and centrifuged, and the combined EtOAc layer was evaporated. The extract (22 g)

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Table 1. ^{1}H NMR Chemical Shifts of Compounds **2**−**9**[a]

proton(s)	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
CH_3-1	2.47	2.49	2.29	2.30	2.21	2.24	2.31	2.23
H-2 or OH			5.80[c]	5.50[c]	5.30[c]	6.43		6.24[h]
OH-3 or OCH_3	3.88[b]	3.91[b]	3.91[b]	3.91[b]	3.90[b]	5.53[c,d]	5.18[c,d]	5.26[c]
H-4		6.46					6.32[e,f]	6.17[i]
OH-5 or OCH_3	5.91[c]	3.91[b]	3.84[b]	5.50[c]	5.03[c]	5.53[c,d]	5.18[c,d]	5.26[c]
H-6					6.61	6.43	6.39[e,g]	6.24[h]

[a] Recorded in $CDCl_3$ at room temperature, δ in ppm relative to external TMS. [b] $-OCH_3$ signal. [c] $-OH$ signal. [d] Broad singlet. [e] Doublet (J = 2.5 Hz). [f,g] Assignments may be reversed. [h] Doublet (J = 2.0 Hz). [i] Triplet (J = 2.0 Hz).

Table 2. ^{13}C NMR Chemical Shifts of Compounds **2**−**9**[a]

carbon	**2**[b]	**3**[b]	**4**[b]	**5**[b,c]	**6**[b,d]	**7**[b,c]	**8**[e]	**9**[b]
CH_3-1	17.9	17.9	12.9	12.9	15	21.3	20.5	21.4
OCH_3	60.7	56.5	60.7	61.2	61			
			61.2					
C-1	134.0	136.3	125.4	122.1	124	138.8	138.3	141.0
C-2	121.2	115.2	145.8	142.0[f]	142[f]	108.9	112.1	108.7
C-3	151.5	154.1	141.6[f]	141.5[f]	141[f]	151.5	154.7	156.6
C-4	113.8	95.2	119.2		109		102.4	99.9
C-5	147.2	154.1	141.8[f]	141.7[f]	144	151.5	157.4	156.6
C-6	117.2	115.2	122.4	117.0	112	108.9	110.2	108.7

[a] Assignments was based on comparison between observed and calculated chemical shifts. [b] Recorded in $CDCl_3$ at room temperature by a proton noise decoupled method, δ in ppm relative to external TMS. [c] One carbon signal was not observed. [d] Read from HMBC cross peaks. [e] Recorded in Me_2CO-d_6. [f] Assignments may be reversed.

was separated successively, guided by TLC bioassays using *B. leersiae*,[6] on Si gel (EtOAc, 0.005% BHT), Sephadex LH-20 (MeOH, 0.005% BHT), and Sephadex LH-20 (MeOH−CH_2Cl_2, 1:4, 0.005% BHT) columns, and finally by flash chromatography or HPLC to give three major (**2**, 34 mg; **4**, 33 mg; **7**, 23 mg) and four minor (**3**, 7 mg; **5**, 8 mg; **6**, 4 mg; **8**, 11 mg) organochlorine compounds, together with orcinol (**9**, 16 mg). These were eluted from the first Sephadex LH-20 column as constituents less polar than the previously reported yurinelide (**1**).

Compound **2**, mp 119−121 °C, showed characteristic molecular ion peaks at *m/z* 240, 242, and 244 in the ratio of 3:3:1. The HRMS gave a molecular formula, $C_8H_7O_2Cl_3$ (*m/z* 239.9500, calcd 239.9483). The ^{1}H NMR spectrum (Table 1) showed only three singlet signals at δ 2.47 (3H), 3.88 (3H), and 5.91 (1H, D_2O exchangeable). The co-occurrence of **2** with orcinol (**9**) suggested that it could be a trichloroorcinol derivative with one methyl, one methoxyl, and one phenolic hydroxyl group. Methylation of **2** with CH_2N_2 gave a symmetrical ether (δ 2.48, 3H, s; 3.88, 6H, s), which was identified as 2,4,6-trichloro-3,5-dimethoxytoluene by direct comparison with a synthetic sample derived from 2,4,6-trichloro-3,5-dihydroxytoluene (**10**).[11a] Therefore, the structure of this natural product is represented by the formula shown in **2**. An organochlorine compound assigned the same structure has been isolated[12] from a steam-volatile fraction of healthy bulbs of another edible lily, *Lilium lancifolium*; however, the reported ^{13}C NMR spectral data were different from those of **2**.

Compound **3**, mp 134−135 °C, was found to contain two chlorine atoms (M^+, *m/z* 220, 222, 224; 9:6:1) and its ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) indicated that it has a symmetrical structure. An observed NOE between the methoxyl protons at δ 3.91 and the aromatic proton at δ 6.46 identified **3** as 2,6-dichloro-3,5-dimethoxytoluene.

Table 3. Inhibition of Conidial Germination of *B. leersiae*[a]

	10[c]	50	100	300	500	1000
2	+++[d]	+++	+++	+++[h]	+++[h]	
3			[g,h]	[h]	[h]	
4	++[e]	+++	+++	+++	+++	
5	++	+++	+++	+++	+++	
6[b]						
7			++	+++	+++	
8			++	+++	+++	
9			+[f]	+	++	+++

[a] Germination was examined under a microscope after 24 h of incubation at 25 °C; blank columns were not tested. [b] No sample was available. [c] Concentration in ppm. [d] Complete inhibition. [e] Ca. 80% inhibition. [f] Ca. 30% inhibition. [g] No inhibition. [h] Not dissolved completely.

Compound **4**, mp 86−87 °C, has also two chlorine atoms (M^+, *m/z* 236, 238, 240; 9:6:1), and its ^{1}H NMR spectrum (Table 1) showed the presence of two methoxyls, one methyl, and one phenolic hydroxyl group. Methylation of **4** with CH_2N_2 gave a compound with three different methoxyl proton signals at δ 3.81, 3.86, and 3.89. The structure **4**, as suggested by an HMBC NMR experiment, was further confirmed by synthesis, in which hydroxylation of 3,5-dimethoxytoluene with *m*-chloroperbenzoic acid gave 2-hydroxy-3,5-dimethoxytoluene.[13] Chlorination with sulfuryl chloride[14] of 2-hydroxy-3,5-dimethoxytoluene gave a dichlorophenol, which was identical to **4**.

Compound **5**, mp 146−149 °C, has two chlorine atoms (M^+, *m/z* 222, 224, 226; 9:6:1). The ^{1}H NMR spectrum indicated the presence of one methyl, one methoxyl, and two phenolic hydroxyl groups (Table 1). Methylation of **5** with CH_2N_2 gave a trimethoxy compound, which was identical to the methylation product of **4**. Therefore, the oxygenation pattern of **5** should be the same as that of **4**. Because compound **5** was readily oxidized on a TLC plate to give a yellow compound, this *L. maximowiczii* constituent should have an *o*- or *p*-hydroquinone structure (**5**, **5′**). Further spectroscopic study could not be conducted due to the instability of **5**.

Compound **6**, mp 103−104 °C, has only one chlorine atom (M^+; *m/z* 188, 190; 3:1). The ^{1}H NMR spectrum indicated the presence of one methyl, one methoxyl, and two phenolic hydroxyl groups. Compound **6** was also oxidized readily to give a yellow compound, suggesting either an *o*- or *p*-hydroquinone structure. An HMBC NMR experiment (CH_3-1/C-1, C-2, C-6: OCH_3-3/C-3; H-6/C-2, C-4, C-5) showed the position of each substituent as shown in the formula of **6**.

Compounds **7** and **8** were identified as 4-chloro-3,5-dihydroxytoluene[11] and 2-chloro-3,5-dihydroxytoluene,[16] respectively, by comparison of their spectral data with those of reported synthetic samples. This is the first isolation of **7** and **8** from a natural source. A monomethyl ether of **7** and dimethyl ether of **8** were reported as constituents of oakmoss (*Evernia prunastri*) oleoresin as determined by GC−MS.[15] Compound **9** was identified as orcinol by direct comparison with an authentic sample.

The antifungal activity of these orcinol derivatives was tested against *B. leersiae* (Table 3). Inhibition of the conidial germination depended on the numbers of chlorine atoms in the compounds. Trichlorinated compound **2** showed the highest activity. Dichlorinated orcinols **4** and **5** were next, and the monochlorinated ones, **7** and **8**, were less active than **4** or **5**. The

nonchlorinated orcinol (**9**) showed the lowest activity. The lack of activity in compound **3** indicated that a free phenolic hydroxyl group is required for the activity.

Recently, three antimicrobial chlorinated orcinol derivatives were isolated from the mycelia of *Hericium erinaceum*.[17] In addition, polychlorinated benzene derivatives such as Daconil (1,2,3,5-tetrachloro-4,6-dicyanobenzene) have been used as pesticides.[18] Because chlorine-containing compounds are rare in terrestrial plants,[8] the present isolation of appreciable amounts of organochlorine compounds from *L. maximowiczii* cv. Hakugin bulbs has posed a question about the origin of these chlorinated orcinol derivatives. Several experiments were undertaken to answer this question.

A diseased lily bulb was extracted separately as follows: necrotic tissue of the outer scales, outer scales with fewer lesions, and inner intact scales. TLC bioassays of the respective EtOAc extracts revealed the localization of antifungal compounds in the necrotic tissue of the outer scales. The outer scales with fewer lesions contained much smaller amounts of the antifungal compounds, and the inner intact ones showed none of these compounds. These results might be explained if there were a microbial origin of these compounds; however, this possibility was ruled out inasmuch as the EtOAc extract from the pathogenic fungal culture of *F. oxysporum* f. sp. *lilii* showed the absence of these antifungal compounds based on the results of 2D TLC bioassay. Another possibility, that these were derived from organochlorine pesticides in the environment, is doubtful. If environmental organochlorine compounds were absorbed or adsorbed from the soil in which they were grown, these compounds would have distributed more evenly throughout the bulb tissues. Furthermore, no such metabolites of pesticide origin have been reported to the best of our knowledge. Consequently, the chlorinated compounds in the infected tissue appear to be produced by the host plant during microbial infection. TLC bioassays of the EtOAc extracts from incubated tissues of the fungus-inoculated or UV-irradiated lily bulb scales showed the antifungal spots of these compounds and also of yurinelide (**1**). They were, however, almost absent in untreated intact scales. These results indicated that the chlorinated compounds had originated from the plant itself.

Time−course studies provided further evidence for *de novo* production of the chlorinated orcinol derivatives in the plant tissue. Respective tissues of injured, UV-irradiated, and *F. oxysporum* f. sp. *lilii*-inoculated bulb scales were incubated at 25 °C for 14 h, and 1, 2, 4, and 7 days. Because direct confirmation by gas chromatography of these products in each extract was difficult due to the presence of large amounts of interfering substances, we enriched the extracts by successive chromatography and then analyzed by gas chromatography. Analysis of these samples at day 7 after each stress treatment (Figure 1) showed the presence of the chlorinated orcinol derivatives both in the inoculated and in the UV-irradiated samples. The sample from injured scales, however, lacked these compounds, except **8** and orcinol (**9**). Interestingly, orcinol (**9**) increased in the early stages of incubation. New GC peaks appeared after 14 h of incubation in the UV-irradiated or in the inoculated tissue (data not shown). GC−MS analysis

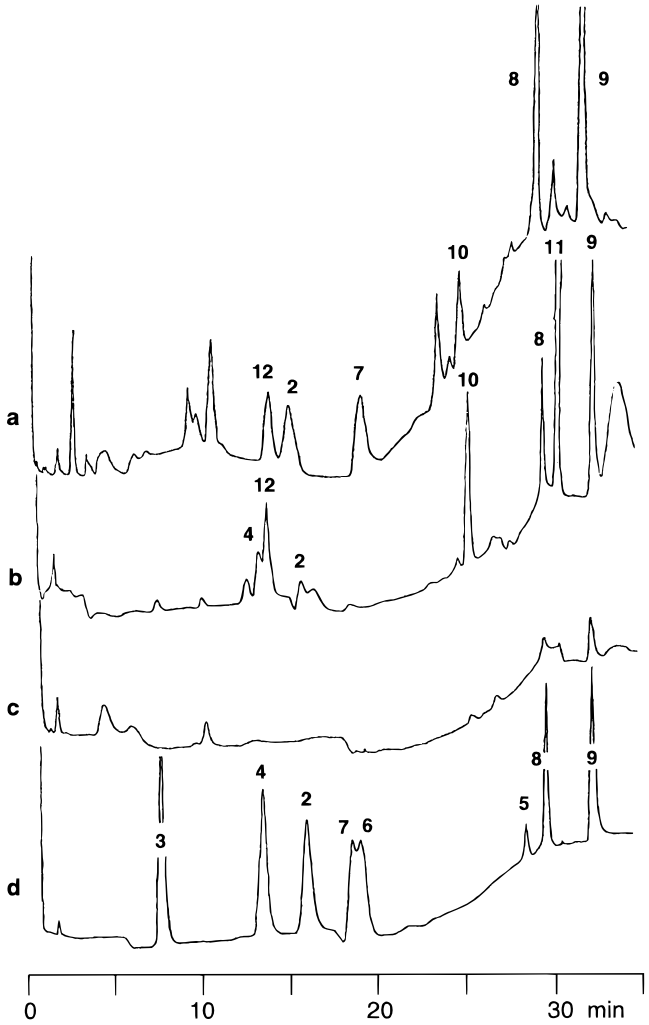
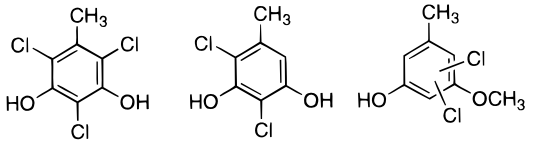


Figure 1. Gas chromatograms of extracts from differently treated lily bulb scales. All tissues were incubated at 25 °C for 7 days; (**a**) *Fusarium oxysporum*-inoculated tissue; (**b**) UV-irradiated tissue; (**c**) injured tissue; (**d**) authentic sample mixture. The bold numbers indicate each compound isolated or supposed in this study.

of the sample at day 7 after UV-irradiation showed the peaks corresponding to compounds **2**, **4**, **8**, and **9**, together with three unidentified ones. The GC−MS analysis of the unidentified peaks indicated that they could be represented by structures **10**, **11**, and **12**, if we assume an orcinol skeleton for them. Synthetic samples of **10**[11a] and **11**[11a] have the same respective retention times under the same analytical conditions. In the time−course study, peaks **10** and **11** increased in the early stages and then declined (data not shown). These results indicate that the lily tissue is able to produce organochlorine compounds and suggested *de novo* methylation of the chlorinated orcinols, yielding the aforementioned compounds such as **2**.



10 **11** **12**

Next, we carried out a number of biosynthetic studies. Biological chlorination of orcinol (**9**) would be catalyzed

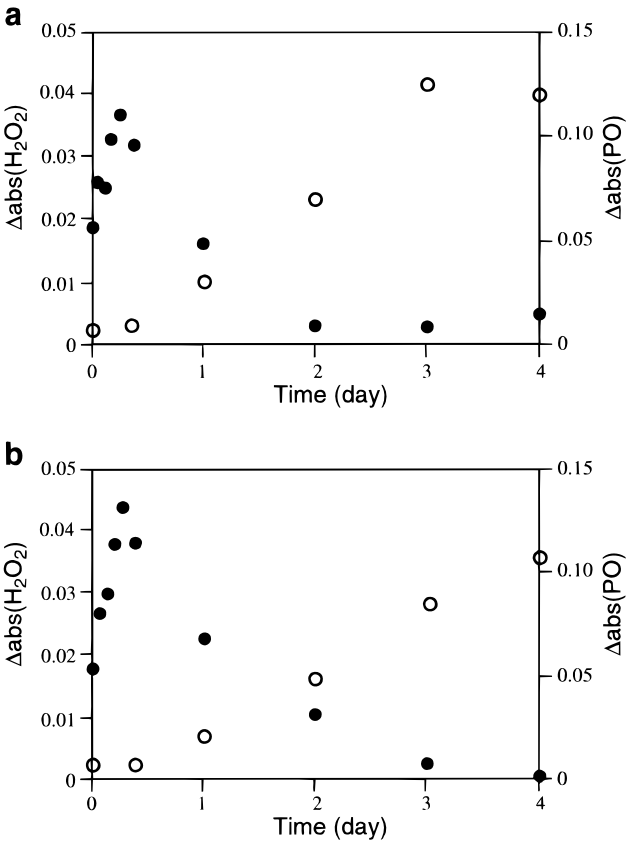


Figure 2. Formation of hydrogen peroxide (H_2O_2, ●) and peroxidase (PO, ○) in UV-nonirradiated tissue (**a**) and in UV-irradiated tissue (**b**). The sample solutions were prepared from cut, cubed lily scales after peeling. The reaction with ABTS was initiated by adding 0.1 mM CPO (H_2O_2 measurement) or H_2O_2 (PO measurement) and monitored by the absorption at 414 nm.

by chloroperoxidase (CPO, eq 1). This enzyme requires

$$2AH + 2Cl^- + H_2O_2 \xrightarrow{CPO} 2A\text{-}Cl + 2H_2O \quad (1)$$
(AH = substrate)

hydrogen peroxide as the first substrate,[19] and generation of hydrogen peroxide in damaged or infected plant tissues has been reported.[20] Therefore, the formation of hydrogen peroxide in the lily bulb scales was first examined using 2,2′-azido-di-(ammonium 3-ethylbenzthiazoline-6-sulfonate) (ABTS) as an indicator in the presence of commercial CPO (obtained from *Caldariomyces fumago*, Sigma Chemical Co., St. Louis, MO). This indicator gives a stable radical cation with a maximum absorption at 414 nm on enzymatic oxidation with hydrogen peroxide.[21] To intact or UV-irradiated unpeeled lily bulb scales was added a mixture of CPO and ABTS. The color of the tissues, however, remained unchanged. On the other hand, when peeled and UV-irradiated or peeled nonirradiated scales were treated with the reagents, both the scales turned reddish after 5 to 20 min. These results indicated that peeling or injury resulted in the production and release of hydrogen peroxide. Using small, cubed blocks of peeled lily bulb scales (about 5 × 5 × 5 mm), the formation of hydrogen peroxide was followed colorimetrically (Figure 2). UV-irradiated or nonirradiated blocks were incubated for indicated periods and then immersed in acetate buffer (pH 4.5) with stirring. The upper layer

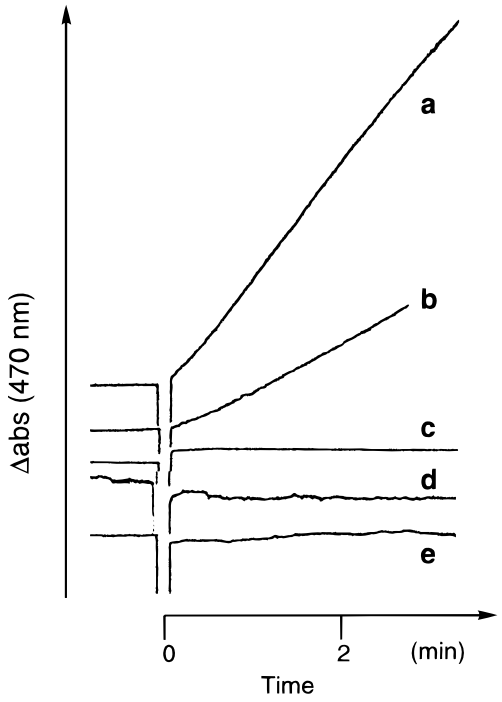


Figure 3. Peroxidase activity of lily bulb scales under different conditions. The activity of the crude enzyme solution was estimated by PO-catalyzed production of tetraguaiacoquinone (maximum absorption, 470 nm) from guiacol (10 mM) and hydrogen peroxide (0.1 mM) in 0.1 M acetate buffer (pH 4.5, total reaction volume 2.00 mL). The reaction was initiated by the addition of 20 μL of hydrogen peroxide (10 mM) and monitored by increase of absorption at 470 nm. Each sample contains enzyme solution from: (**a**) browned part of UV-irradiated tissue (20 μL); (**b**) necrotic part of diseased tissue (20 μL); (**c**) necrotic part of diseased tissue (20 μL) and 1 mM KCN (20 μL); (**d**) healthy tissue (20 μL); and (**e**) fungal culture of *Fusarium oxysporum* (40 μL).

was centrifuged, and the amounts of hydrogen peroxide in each supernatant were estimated by measuring the absorption at 414 nm after addition of ABTS and CPO. Both samples reached their respective maximal concentrations after 6 h of incubation and then declined gradually. Thus, the early formation of hydrogen peroxide in the treated tissues was confirmed.

Peroxidase activity in diseased plant tissues has been often reported.[22] Prior to the examination of CPO, a special type of peroxidase (PO), PO activity in treated or in untreated lily tissues was examined using guaiacol as a substrate. PO-catalyzed oxidation of guaiacol with hydrogen peroxide yields tetraguaiacoquinone with a maximum absorption at 470 nm.[23] Crude enzyme preparation from the necrotic or UV-irradiated scales showed the PO activity, but those from the intact ones showed no PO activity (Figure 3). Furthermore, addition of potassium cyanide to the crude enzyme preparation from the necrotic scales inhibited the enzyme activity as in a usual PO.[21b,24] These results and the absence of PO activity in a preparation from the pathogenic fungus *F. oxysporum* f. sp. *lilii* suggested the induction of PO in the plant tissue under stress conditions.

CPO activity in the diseased plant tissue was next examined as follows. Chlorination of monochlorodimedone (MCD) in the presence of hydrogen peroxide and CPO results in the decrease of UV absorption at 278

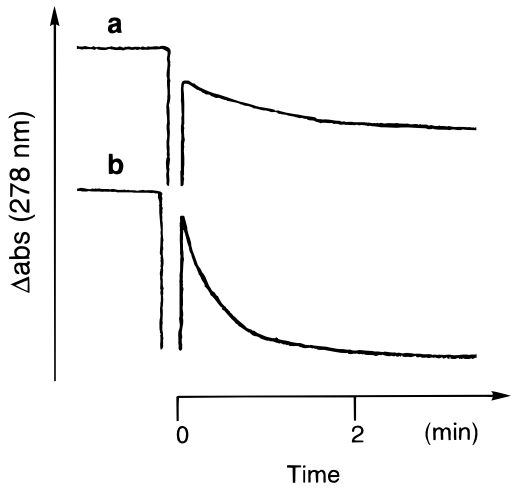
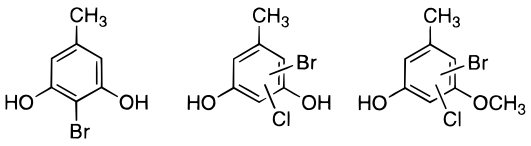


Figure 4. CPO activity of necrotic tissue of diseased bulb scales (**a**) and of CPO from *C. fumago* (**b**). The activity was determined by using the reaction MCD (1 mM, 100 μL) to DCD with KCl solution (0.1 mM, 20 μL) in 0.1 M acetate buffer (pH 3.0, total volume 2.00 mL). The reaction was initiated by adding 10 μL of hydrogen peroxide (10 mM) and monitored by decrease of absorption at 278 nm.

nm due to dichlorodimedone (DCD) formation.[25] Addition of the above substrates to the crude enzyme preparation from the infected necrotic tissue showed a decrease of the absorption (Figure 4), suggesting the presence of CPO.

The data available strongly suggested that chlorinated orcinol derivatives (**2**−**8**) are derived from orcinol (**9**) through biochlorination catalyzed by CPO, followed by biomethylation in the diseased lily bulbs. In the present study, the CPO activity in the crude enzyme preparation was not strong enough to induce *in vitro* chlorination of orcinol (**9**). Enzymatic chlorination of orcinol (**9**), however, was confirmed using commercial CPO. In an experiment using a mixture of CPO (0.1 μunit), orcinol (**9**, 0.2 mM), and KCl (0.1 M) in acetate buffer (pH 3.0), the formation of two monochlorinated orcinols **7** and **8** was proved by GC−MS. Furthermore, more oxidizable bromide ions were incorporated to orcinol (**9**) in the plant tissue without external addition of CPO. When a potassium bromide solution was poured into hollows of UV-irradiated or nonirradiated bulb scales and incubated for 4 days at 25 °C, bromine-containing compounds were detected only in the sample of UV-irradiated tissue. The structures were assigned by GC−MS as **13**, **14**, **15a**, and **15b**, if an orcinol skeleton is assumed.



13 **14** **15a 15b**

As mentioned previously, the chlorinated antifungal orcinol derivatives localized in the necrotic lesions of the diseased lily bulbs with high concentrations. Such high concentrations, however, were not reproduced in UV-irradiated or in inoculated lily bulb scales. This discrepancy could be attributed to a restricted supply of chloride ions in the treated scales. Generally, chlorine is a minor element in higher plants.[26] The content of chloride ions in a lily bulb was estimated as approximately 200 ppm by a silver nitrate titration method. Therefore, the restricted supply of chloride ions would be a limiting factor for the production of chlorinated orcinols. Formation of these organochlorine compounds in the bulbs would depend on many factors: production of hydrogen peroxide, induction of CPO, ample supply of chloride ions from the soil surrounding the lily plant, methylation of phenolic hydroxyl groups of induced chlorinated compounds, and microbial interaction to initiate a series of reactions in the host plant. It was found that the organochlorine compounds in lily bulbs from different fields showed considerable variations in their concentration levels. This would be explained by the above-mentioned variable factors.

It is noteworthy that the lily bulbs produce two different types of antifungal compounds: chlorinated orcinol derivatives (**2**−**9**) and yurinelide (**1**). Under natural conditions, the former compounds were the major active principles, while the latter is induced as the major phytoalexin in UV-irradiated or in inoculated lily bulbs. Induction of two different types of compounds in diseased lily bulbs is indicative of diverse plant defense mechanisms. The natural "organochlorine pesticides" **2**−**8** from lily bulbs are unstable, in contrast to synthetic organochlorine pesticides, which persist for a long time and have resulted in many environmental problems.[27]

Experimental Section

General Experimental Procedures. The ^{1}H (90, 400 MHz) and ^{13}C (22.5 MHz) NMR spectra were obtained on a JEOL JNM-EX90 or a JNM-EX400 NMR spectrometer using $CDCl_3$ unless otherwise stated. Chemical shifts are given in parts per million (ppm) relative to the TMS or by reference to the solvent signal. IR spectra were recorded on a JASCO IR-700 spectrometer. UV spectra were obtained using a JASCO Ubest-30, a Hitachi 200-10, or a Shimadzu UV-240 spectrophotometer. LREIMS and HREIMS were obtained using a JEOL JMS-DX303 or JMS-DX300 spectrometer. Melting points were determined by a Yanagimoto MP micro-melting point apparatus and are uncorrected. HPLC separations were performed on a JASCO 800S instrument equipped with a JASCO UVIDEC-100V detector and a Shimadzu Chromatopac C−R6A integrator, using an analytical μ-Bondapak C_{18} or Radial-Pak cartridge Resolve C_{18} column (8 mm × 10 cm, Waters Associates). GC analysis was performed on a Shimadzu GC system model GC-14APF with Thermon 600T capillary column (25 m) using a FID detector. The GC−MS analysis was performed on JEOL JMS-DX300 with Thermon 600T capillary column (25 m) or 3% OV-17 (1 m). Reagents, an enzyme, and solvents were obtained from Nacalai Tesque (Kyoto, Japan), Aldrich Chemical Co. (Milwaukee, WI), Sigma Chemical Co. (St. Louis, MO), and Wako Pure Chemical Industries (Osaka, Japan). All chemicals of reagent grade were used without further purification unless otherwise noted.

Plant Material. *L. maximowiczii* cv. Hakugin was cultivated in Biei, Hokkaido, Japan, and harvested in October 1990. This cultivar is grown widely in Hokkaido, and the type species is maintained at Hokkaido

Prefectural Plant Genetic Resources Center, Takikawa, Hokkaido, Japan.

Bioassay Procedure. For each 2D TLC bioassay,[6] a developed Si gel sheet (i, Et_2O; ii, CH_2Cl_2−MeOH, 49:1) was sprayed with a dense conidial suspension of *B. leersiae* in a potato−glucose medium, and incubated in a moist box at 25 °C for 2 days. Fungitoxic spots appeared white against a dark gray background. The antifungal activity of each chromatographic fraction was monitored by 1D TLC using solvent system i or ii. For semiquantitative bioassays, fresh conidia of *B. leersiae* were collected and added to a solution consisting of H_2O (100 mL), 1/15 M KH_2PO_4 (100 mL), and potato dextrose broth (2 mL). Under stirring with a magnetic stirrer, 1 mL of the conidial suspension was transferred into each vial (10 mL). Each sample solution in Me_2CO (10 μL) was added to the vial, and it was capped and kept at 20 °C for 24 h. Conidial germination was examined under a microscope.

Extraction and Isolation. Diseased edible lily bulb scales (4.9 kg, fresh wt) of *L. maximowiczii* cv. Hakugin collected from an infested field in the Kamikawa area of Hokkaido, Japan, were homogenized in EtOAc using a Polytron homogenizer, allowed to stand for 3 days, and centrifuged. The combined supernatant was evaporated under reduced pressure to give a crude extract (22 g). To remove tarry materials, the EtOAc-soluble portion of the extract was passed through a Si gel column using EtOAc containing 0.005% BHT as eluent. The eluate from the column was separated, in two portions, by column chromatography on Sephadex LH-20 (200 g, MeOH containing 0.005% BHT), and the fractions were combined into three fractions (F-1, F-2, and F-3) guided by the TLC bioassay. F-1 was inactive, and F-3 contained the previously reported yurinelide (**1**).[5] F-2 (4.3 g) was separated into four fractions (F-2−1 to F-2−4) by column chromatography on Sephadex LH-20 (200 g, CH_2Cl_2−MeOH, 4:1, 0.005% BHT). F-2−1 (2.5 g) was separated to give two active fractions (F-2−1−1 and F-2−1−2). F-2−1−1 (360 mg) decomposed during further separation. F-2−1−2 (460 mg) was separated by sequential column chromatography on Si gel (i, 65 g, hexane−CH_2Cl_2, 2:1; ii, 20 g, hexane−Et_2O, 9:1) to yield **2** (34 mg) and an active fraction (94 mg), which gave **3** (7 mg) and **4** (33 mg) on preparative HPLC (Radial-Pak cartridge Resolve C_{18}, MeOH−H_2O, 4:1). F-2−2 (220 mg) was separated by column chromatography on Si gel (25 g, hexane−EtOAc, 19:1 to 2:5) to give two active fractions (F-2−2−1 and F-2−2−2). F-2−2−1 (11 mg) gave **5** (8 mg) on preparative HPLC (Radial-Pak cartridge Resolve C_{18}, MeOH−H_2O, 11:9). F-2−2−2 (8 mg) on preparative HPLC (Radial-Pak cartridge Resolve C_{18}, H_2O−MeOH, 3:2) gave **6** (4 mg). F-2−3 (140 mg) gave **7** (23 mg) by column chromatography on Si gel (20 g, CH_2Cl_2). F-2−4 (300 mg) was separated to give two active fractions (F-2−4−1, 19 mg; F-2−4−2, 230 mg). F-2−4−1 and F-2−4−2 gave **8** (11 mg) and **9** (16 mg), respectively, on preparative HPLC on a Radial-Pak cartridge Resolve C_{18} using H_2O−MeOH (3:2) and H_2O−MeOH (7:3).

2,4,6-Trichloro-3-hydroxy-5-methoxytoluene (2): colorless crystals; mp 119−121 °C; UV (CH_3CN) λ_{max} (log ϵ) 284 (3.28), 292 (3.34) nm; IR ($CHCl_3$) ν_{max} 3518, 2938, 1562, 1458, 1396, 1350, 1284, 1094, 1035, 949 cm^{-1}; ^1H NMR ($CDCl_3$, 90 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 22.5 MHz), see Table 2; EIMS *m/z* 244 $[M]^+$ (38), 242 $[M]^+$ (97), 240 $[M]^+$ (100), 229 (16), 227 (45), 225 (47), 201 (17), 199 (46), 197 (48), 164 (19), 162 (28), 135 (23), 133 (34), 127 (12); HREIMS *m/z* 239.9500 (calcd for $C_8H_7Cl_3O_2$, 239.9483).

2,6-Dichloro-3,5-dimethoxytoluene (3): colorless crystals; mp 134−135 °C; UV (CH_3CN) λ_{max} (log ϵ) 291 (3.54) nm; IR ($CHCl_3$) ν_{max} 3014, 2962, 2938, 2838, 1578, 1456, 1430, 1339, 1207, 1110, 1081, 940, 804 cm^{-1}; ^1H NMR ($CDCl_3$, 90 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 22.5 MHz), see Table 2; NOE correlation ($CDCl_3$, 400 MHz) δ 3.91 (s, 6H)/6.46 (s, 1H); EIMS *m/z* 224 $[M]^+$ (14), 222 $[M]^+$ (70), 220 $[M]^+$ (100), 207 (6), 205 (8), 181 (6), 179 (31), 177 (47), 164 (5), 162 (7), 144 (6), 142 (17), 127 (10); HREIMS *m/z* 220.0049 (calcd for $C_9H_{10}Cl_2O_2$, 220.0058).

2,4-Dichloro-6-hydroxy-3,5-dimethoxytoluene (4): colorless crystals; mp 86−87 °C; UV (CH_3CN) λ_{max} (log ϵ) 291 (3.51) nm; IR ($CHCl_3$) ν_{max} 3526, 3012, 2936, 2836, 1577, 1461, 1407, 1349, 1296, 1191, 1088, 1047, 971, 937, 872 cm^{-1}; ^1H NMR ($CDCl_3$, 90 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 22.5 MHz), see Table 2; HMBC correlations ($CDCl_3$, 400 MHz) CH_3-1/C-1, C-2, C-6: OCH_3-3 or -5/C-3 or C-5; EIMS *m/z* 240 $[M]^+$ (11), 238 $[M]^+$ (87), 236 $[M]^+$ (87), 225 (12), 223 (67), 221 (100), 195 (12), 193 (19), 180 (13), 178 (20); HREIMS *m/z* 236.0009 (calcd for $C_9H_{10}Cl_2O_3$, 236.0007).

2,4-Dichloro-3,6-dihydroxy-5-methoxytoluene (5) or 2,4-dichloro-5,6-dihydroxy-3-methoxytoluene (5′): colorless crystals; mp 146−149 °C; UV (CH_3CN) λ_{max} (log ϵ) 297 (3.70) nm; IR ($CHCl_3$) ν_{max} 3530, 3010, 2938, 1459, 1420, 1370, 1312, 1262, 1083, 1038, 941, 891 cm^{-1}; ^1H NMR ($CDCl_3$, 90 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 22.5 MHz), see Table 2; EIMS *m/z* 226 $[M]^+$ (9), 224 $[M]^+$ (51), 222 $[M]^+$ (77), 211 (12), 209 (67), 207 (100), 183 (3), 181 (19), 179 (30).

4-Chloro-2,5-dihydroxy-3-methoxytoluene (6): colorless crystals; mp 103−104 °C; UV (CH_3CN) λ_{max} (log ϵ) 293 (3.36) nm; IR ($CHCl_3$) ν_{max} 3542, 3016, 2940, 2842, 1594, 1475, 1427, 1363, 1280, 1161, 1067, 1001, 926, 865 cm^{-1}; ^1H NMR ($CDCl_3$, 90 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 22.5 MHz), see Table 2; HMBC correlations ($CDCl_3$, 400 MHz) CH_3-1/C-1, C-2, C-6: OCH_3-3/C-3: H-6/C-2, C-4, C-5; EIMS *m/z* 190 $[M]^+$ (33), 188 $[M]^+$ (90), 175 (36), 173 (100), 147 (15), 145 (42).

4-Chloro-3,5-dihydroxytoluene (7):[11] colorless crystals; mp 134−136 °C; UV (CH_3CN) λ_{max} (log ϵ) 274 (2.99) nm; IR ($CHCl_3$) ν_{max} 3544, 3254, 3022, 2920, 1591, 1488, 1460, 1360, 1324, 1262, 1169, 1058, 984, 827 cm^{-1}; ^1H NMR ($CDCl_3$, 90 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 22.5 MHz), see Table 2; EIMS *m/z* 160 $[M]^+$ (38), 158 $[M]^+$ (100), 157 (39), 140 (11), 123 (75), 95 (7); HREIMS *m/z* 158.0159 (calcd for $C_7H_7ClO_2$, 158.0134).

2-Chloro-3,5-dihydroxytoluene (8):[16] colorless crystals; mp 139−141 °C; UV (CH_3CN) λ_{max} (log ϵ) 282 (2.78) nm; IR ($CHCl_3$) ν_{max} 3594, 3528, 3314, 1601, 1479, 1350, 1259, 1156, 1048, 978, 842 cm^{-1}; ^1H NMR ($CDCl_3$, 90 MHz), see Table 1; ^{13}C NMR [$(CD_3)_2CO$, 22.5 MHz], see Table 2; EIMS *m/z* 160 $[M]^+$ (37), 158 $[M]^+$ (100), 140 (6), 123 (88), 105 (8), 95 (12).

Orcinol (9): colorless crystals; mp 82−84 °C; UV (CH_3CN) λ_{max} (log ϵ) 275 (3.20), 281 (3.20) nm; IR ($CHCl_3$) ν_{max} 3592, 3320, 1600, 1479, 1336, 1299, 1147,

1032, 969, 832 cm^{-1}; ^1H NMR ($CDCl_3$, 90 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 22.5 MHz), see Table 2; EIMS *m/z* 124 $[M]^+$ (91), 123 (52), 107 (14), 95 (42).

Synthesis of 2,4,6-Trichloro-3,5-dimethoxytoluene. To a solution of orcinol monohydrate (**9**, 101 mg, 0.71 mmol) in HOAc (1 mL) was added sulfuryl chloride (240 μL) gradually at room temperature. The mixture was kept for 2 h with stirring. After removal of the solvent, the residue was recrystallized from hexane to give 2,4,6-trichloro-3,5-dihydroxytoluene (**10**, 63 mg, 39%): ^1H NMR ($CDCl_3$, 90 MHz) δ 2.46 (s, 3H), 5.91 (s, 2H); ^{13}C NMR ($CDCl_3$, 22.5 MHz) δ 17.8, 106.0, 113.0, 133.2, 147.1; EIMS *m/z* 232 $[M]^+$ (7), 230 $[M]^+$ (37), 228 $[M]^+$ (97), 226 $[M]^+$ (100), 195 (12), 193 (61), 191 (89). To a solution of **10** (32 mg, 0.14 mmol) in ether was added an ethereal solution of CH_2N_2 at room temperature. The mixture was kept for 6 h with stirring. The reaction mixture was evaporated to give almost pure 2,4,6-trichloro-3,5-dimethoxytoluene (31 mg, 86%): IR ($CHCl_3$) ν_{max} 3012, 2936, 2854, 1452, 1396, 1379, 1335, 1194, 1096, 1047, 958, 935 cm^{-1}; ^1H NMR ($CDCl_3$, 90 MHz) δ 2.48 (s, 3H), 3.88 (s, 6H); ^{13}C NMR ($CDCl_3$, 22.5 MHz) δ 18.0, 60.6, 122.0, 125.7, 134.6, 151.7. To a solution of **2** (4 mg, 17 μmol) in ether was added an ethereal solution of CH_2N_2 at room temperature. The mixture was kept for 1 h with stirring. The reaction mixture was evaporated to give almost pure 2,4,6-trichloro-3,5-dimethoxytoluene (4 mg, 94%), which was identical with the synthetic sample.

Synthesis of 4. To a solution of orcinol monohydrate (146 mg, 1.03 mmol) in ether was added gradually an ethereal solution of CH_2N_2 at room temperature. The mixture was kept for 5 days with stirring. After removal of the solvent, the residue was submitted to Si gel chromatography (CH_2Cl_2) to give 3,5-dimethoxytoluene (34 mg, 22%): ^1H NMR (90 MHz) δ 2.31 (s, 3H), 3.78 (s, 6H), 6.33 (m, 3H). To a solution of *m*-chloroperbenzoic acid (39 mg) in CH_2Cl_2 (1 mL) was added gradually a solution of 3,5-dimethoxytoluene (21 mg, 0.14 mmol) in CH_2Cl_2 (1 mL) and stirred for 5 h at room temperature. The reaction mixture was taken up in Et_2O, and the Et_2O layer was washed with saturated $NaHCO_3$ aqueous solution and H_2O and then dried over Na_2SO_4. The residue obtained after removal of the solvent was submitted to Si gel chromatography [hexane−CH_2Cl_2 (4:1)] to give 2-hydroxy-3,5-dimethoxytoluene (4 mg, 17%):[13] ^1H NMR ($CDCl_3$, 90 MHz) δ 2.24 (s, 3H), 3.75 (s, 3H), 3.85 (s, 3H), 5.27 (s, 1H), 6.29 (d, J = 2.7 Hz, 1H), 6.36 (d, J = 2.7 Hz, 1H). To a solution of 2-hydroxy-3,5-dimethoxytoluene (4 mg, 24 μmol) in HOAc (400 μL) was added sulfuryl chloride (5 μL) at room temperature.[11,14] The mixture was kept for 2 h with stirring. The residue obtained after removal of the solvent was submitted to Si gel chromatography [hexane−Et_2O (2:1)] to give **4** (0.2 mg, 4%).

Methylation of 4 and 5. To a solution of **4** (3 mg, 13 μmol) in ether was added gradually an ethereal solution of CH_2N_2 at room temperature. The mixture was kept for 16 h with stirring. The reaction mixture was evaporated to give almost pure 2,4-dichloro-3,5,6-trimethoxytoluene (2 mg, 63%): IR ($CHCl_3$) ν_{max} 2974, 1719, 1598, 1458, 1383, 1342, 1290, 1108, 1075, 1014, 954 cm^{-1}; ^1H NMR ($CDCl_3$, 90 MHz) δ 2.30 (s, 3H), 3.81 (s, 3H), 3.86 (s, 3H), 3.89 (s, 3H). To a solution of **5** (2 mg, 9 μmol) in ether was added gradually an ethereal solution of CH_2N_2 at room temperature. The mixture was kept for 9 h with stirring. The reaction mixture was evaporated to give almost pure 2,4-dichloro-3,5,6-trimethoxytoluene (2 mg, 90%), which was identical with that derived from **4**.

Sample Preparations for GC Analysis. Intact tissue: The intact lily bulb scales (120 g) were homogenized in EtOAc using a Polytron homogenizer, left for 1 day, and centrifuged, and the resulting EtOAc layer was evaporated. The residue was taken up in ether, and the extract was filtered. After removal of the solvent, the residue was submitted to successive chromatography on SepPak Si gel cartridge (MeOH), Sephasorb column (MeOH), and SepPak Si gel cartridge (benzene−diethyl ether, 1:4) to give a sample (1 mg).

Injured tissue: The intact lily bulb scales (690 g) were washed with H_2O and then carefully slashed with a razorblade. The injured scales were incubated for a specified time (14 h and 1, 2, 4, and 7 days) at 25 °C in moist plastic cases covered loosely with a special polyethylene film (Aisaika film, Nissho Co., Osaka, Japan). The sample scales were homogenized and treated as described above to give samples of 3 mg (14 h), 1 mg (1 day), 4 mg (2 days), 9 mg (4 days), and 9 mg (7 days).

UV-irradiated tissue: The intact lily bulb scales (650 g) were washed with H_2O and irradiated for 20 min with a 15-W germicidal lamp. They were mixed to ensure even irradiation and irradiated twice more in a similar manner. The irradiated scales were incubated for a specified time (14 h and 1, 2, 4, and 7 days) at 25 °C in moist plastic cases and treated as above to give samples of 5 mg (14 h), 1 mg (1 day), 7 mg (2 days), 10 mg (4 days), and 1 mg (7 days).

***F. oxysporum*-inoculated tissue:** The fungus *F. oxysporum* f. sp. *lilii* was cultivated on potato dextrose agar (PDA). The culture in three Petri dishes was homogenized briefly to give a suspension. The intact lily bulb scales (660 g) were washed with H_2O, carefully injured with a razorblade, and inoculated with the above suspension. The inoculated scales were incubated for a specified time (14 h and 1, 2, 4, and 7 days) at 25 °C in moist plastic cases, and treated as above to give GC samples of 5 mg (14 h), 2 mg (1 day), 2 mg (2 days), 4 mg (4 days), and 7 mg (7 days).

Culture extract of *F. oxysporum* f. sp. *lilii*: The PDA culture of *F. oxysporum*. sp. *lilii* (one Petri dish) was homogenized in EtOAc and treated in a manner similar to the second scenario to give 0.8 mg of a reference sample.

GC−MS Analysis of a Sample from the Scales (UV-irradiated and Incubated for 7 Days). The analysis was carried out by using a Thermon 600T column (25 m) with a gas flow rate of 40 mL/min. The column temperature was programmed from 180 to 230 °C at 2 °C/min. Molecular ion peaks (*m/z*) observed in the sample: **2**, 244 (30), 242 (90), 240 (100); **4**, 240 (10), 238(46), 236 (65), 223 (65), 221 (100); **8**, 160 (36), 158 (100); **9**, 124 (100), 123 (44); **10**, 230 (32), 228 (95), 226 (100), 195 (11), 193 (60), 191 (93); **11**, 196 (3), 194 (22), 192 (33), 157 (27), 149 (100); **12**, 210 (12), 208 (66), 206 (100).

Detection of Hydrogen Peroxide. The intact lily bulb scales (200 g) were washed with H_2O, peeled carefully, and left for 1 day at 25 °C in moist plastic cases. The scales were cut into small cubes (about 5 × 5 × 5 mm). After incubation for a specified time (0, 1.5, 3, 4.5, 6, and 9 h and 1, 2, 3, 4, 6, and 7 days), they were immersed in acetate buffer (0.1 M, pH 4.5, 1 g/1.5 mL). The aqueous phase was centrifuged to give supernatants as sample solutions of the hydrogen peroxide test. To the sample solution (1979 μL) in the cubic UV cell (1 cm) was added 10 mM ABTS aqueous (20 μL) solution. The reaction was initiated by adding 0.1 mM CPO (obtained from *Caldariomyces fumago*, Sigma Chemical Co.) and monitored by the absorption at 414 nm. In case of UV-irradiated tissue, the scales were irradiated with a 15-W germicidal lamp for every 5 min three times with careful mixing after aging for 1 day. The UV-irradiated tissue was treated similarly. These experiments were performed three times.

Enzyme Activities. The PO activity was estimated by PO-catalyzed production of tetraguaiacoquinone (maximum absorption, 470 nm) from guiacol and hydrogen peroxide. The scales (healthy tissue, necrotic tissue of diseased bulbs, browned part of UV-irradiated tissue, white part of tissue immediately after UV irradiation, or fungal culture of *F. oxysporum* f. sp. *lilii*) were homogenized in 20 mM phosphate buffer (pH 7.4). The homogenates were centrifuged to give supernatants, which were used as crude enzyme solutions. The crude enzyme solution (10−40 μL) and guaiacol as 40% ethanol solution (100 mM, 200 μL) were added to 0.1 M acetate buffer (pH 4.5, total reaction volume 1.98 mL) and incubated at room temperature for several minutes in a cubic UV cell (1 cm). The reaction was initiated by the addition of 20 μL of hydrogen peroxide (10 mM) and monitored by increase of absorption at 470 nm.

The CPO activity was determined by using the reaction MCD to DCD. Decrease of MCD absorbance at 278 nm was monitored. Each scale (necrotic tissue of a diseased bulbs, browning part of UV-irradiated tissue) was homogenized in 20 mM phosphate buffer (pH 7.4) and centrifuged to give supernatants as crude enzyme solutions. A mixture of the crude enzyme solution (10−80 μL), MCD (1 mM, 100 μL), and KCl solution (0.1 mM, 20 μL) was incubated in 0.1 M acetate buffer (pH 3.0, total reaction volume 1.99 mL) at room temperature for several minutes in a cubic UV cell (1 cm). The reaction was initiated by addition of 10 μL of hydrogen peroxide (10 mM) and monitored by decrease of absorption at 278 nm.

Enzymatic Reaction of Orcinol (9). A mixture of the enzyme (CPO, 0.1 mM, 5 μL), orcinol (**9**, 10 mM, 100 μL), hydrogen peroxide (10 mM, 100, 300, or 500 μL), and KCl solution (1M, 500 μL) was added to 0.1 mM acetate buffer (pH 3.0, total reaction volume 5.0 mL) and incubated at room temperature for 30 min. The reaction mixture was extracted with ether (5 mL) twice, and the combined extract was washed with H_2O (5 mL). The extract was dried over Na_2SO_4, and then submitted to GC analysis after evaporation.

Incorporation Studies of Bromide Ions. Intact tissue: The intact lily bulb scales (39 g) were washed with H_2O and left for 1 day at 25 °C in moist plastic cases. A KBr aqueous solution (1.0 M) was poured into the concave surface of the bulb scales and incubated for 4 days at 25 °C. After draining of the aqueous phase, the scales were homogenized in EtOAc, left for 3 days, centrifuged, and the obtained supernatant was evaporated. The residue dissolved in MeOH was next subjected to chromatography on a Sephasorb column (MeOH) to remove the less polar nonaromatic fractions. The more polar fractions were evaporated, and the residue (20 mg) was passed through a Adsorbex SI (100 mg) cartridge. The cartridge was then eluted with ether. The eluate was further separated on a small Si gel column using benzene−ether (1:4) to give a sample (2 mg) with R_f higher than 0.2 on a Si gel TLC sheet (diethyl ether).

UV-irradiated tissue: The intact lily bulb scales (35 g) were washed with H_2O and left for 1 day at 25 °C in moist plastic cases. After were both sides of each surface UV-irradiated for 12 min, the KBr solution (1.0 M) was fed as above and incubated for 2 days at 25 °C. The scales were homogenized in EtOAc and treated similarly to give 2 mg of GC sample.

GC−MS analysis: The analysis was carried out by using 3% OV-17 column (1 m) with a gas flow rate of 40 mL/min. The column temperature was programmed from 100 to 280 °C at 4 °C/min. Bromine-containing peaks were observed only in the UV-irradiated tissue: **13**, 6.5 min, *m*/*z* 202, 204 $[M]^+$; **14**, 10.0 min, *m*/*z* 236, 238, 240 $[M]^+$; **15a**, 13.0 min, *m*/*z* 250, 252, 254 $[M]^+$; **15b**, 13.5 min, *m*/*z* 250, 252, 254 $[M]^+$.

Chloride Ion Concentration in the Lily Bulb Scales. Intact bulb scales (24.9 g) were homogenized in deionized H_2O (210 mL) using a Polytron homogenizer and centrifuged (10 000 rpm). The supernatant was filled up to 250 mL, and a part of it was titrated with 0.0251 M $AgNO_3$ solution using K_2CrO_4 solution as an indicator. The sample solution (100 mL) consumed 2.25 mL of the $AgNO_3$ solution. Therefore, the chloride ion level in the bulb scales was estimated at 201 ppm.

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