The chemistry of cyclohexenediones produced by the blackleg fungus

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Abstract: Several oxidation products of metabolites produced by avirulent isolates of *Phoma lingam*, the blackleg fungus, are reported; in addition, the profile of metabolites common to *P. lingam* and *P. wasabiae* is clarified.

Key words: cyclohexenedione, *Phoma lingam, P. wasabiae*.

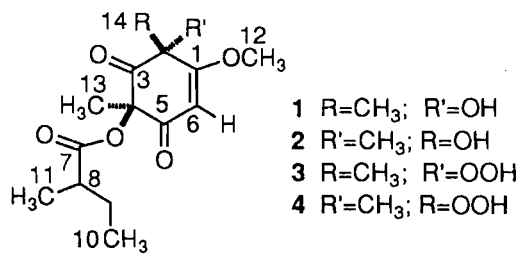
Résumé : On a étudié plusieurs produits d'oxydation tirés des métabolites produits par des isolats avirulents du *Phoman lingam*, un champignon; de plus, on a clarifié le profil des métabolites communs au *P. lingam* et au *P. wasabiae*.

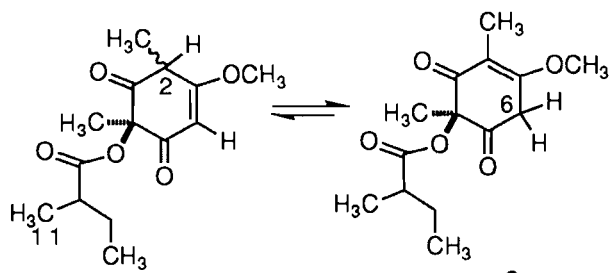
Mots clés : cyclohexènedione, *Phoma lingam, P. wasabiae*.

[Traduit par la rédaction]

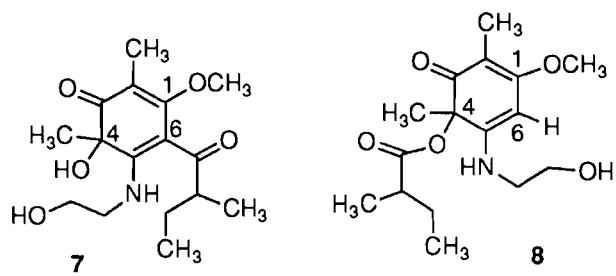
Recent work on the so-called weakly virulent, avirulent, or nonaggressive isolates of the blackleg fungus (*Leptosphaeria maculans* (Desm.) Ces. et de Not., asexual stage *Phoma lingam* (Tode ex Fr.) Desm.) led to the isolation of several metabolites having a cyclohexenone ring as a common structural feature (1–3). Most importantly, this work revealed an unknown relationship between *P. lingam* and *P. wasabiae*. This unforeseen relationship[2] was also supported by analysis of particular DNA sequences of isolates of both species (3). As a consequence of that work, it was proposed that the avirulent isolates of *P. lingam* be formally reclassified (4).

As part of our continuing studies of the chemistry of the blackleg fungus,[3] it was important to determine the phytotoxicity of the cyclohexenones **1**, **2**, **5–8** produced by avirulent isolates. During the purification of these metabolites, unexpected products resulting from the slow oxidation of **5a/5b** and **6** were identified. Here is reported the transformation of phomaligadiones **5** and **6** and phomaligin A (**7**) to phomaligols **1** and **2**, and the intermediates of these oxidative transformations, as well as the products of reaction of phomaligadiones **5** and **6** with diazomethane. These results allow an important clarification regarding the structures of metabolites common to *P. wasabiae* and *P. lingam*. In addition, the implications of these findings on the biogenesis of the cyclohexenone-containing metabolites is discussed and the structures of metabolites common to *P. wasabiae* and *P. lingam* are noted.





5a/5b **6**



7 **8**

Results and discussion

Phomaligols A (**1**) and A_1 (**2**), phomaligadiones A (**5a/5b**) and B (**6**), and phomaligin A (**7**) were obtained from culture extracts of *P. lingam*, as previously described (1, 3). Phomaligadiones constituted an inseparable yellowish mixture (**5a**:**5b**:**6**, ca. 3:1:2), homogeneous by TLC and HPLC, which

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[2] There are more than 2000 species in the genus *Phoma*.

[3] For a recent review of blackleg chemistry see ref. 4; for a recent review of blackleg biology see ref. 5.

Table 1. NMR data[a] for hydroperoxides **3** and **4** in $CDCl_3$.

Position no.	3			4		
	δ_H	δ_C	HMBC	δ_H	δ_C	HMBC
1	—	172.4		—	171.7	
2	—	84.6		—	85.0	
3	—	202.8		—	206.0	
4	—	81.0		—	83.3	
5	—	192.0		—	190.8	
6	5.67, s	102.0	C-1, C-4, C-5	5.71, s	102.6	C-1, C-4, C-5
7	—	176.2		—	175.6	
8	2.46, ddq (6.8, 7.0, 7.0)	39.90	C-7, C-9, C-10, C-11	2.48, ddq (6.8, 7.0, 7.0)	39.95	C-7, C-9, C-10, C-11
9	1.68, m 1.47, m	26.6	C-7, C-8, C-10, C-11	1.71, m 1.48, m	26.6	C-7, C-8, C-10, C-11
10	0.94, t (7.5)	11.4	C-7, C-8, C-9, C-11	0.94, t (7.5)	11.4	C-7, C-8, C-9, C-11
11	1.13, d	16.2	C-7, C-8, C-9, C-10	1.17, d	16.4	C-7, C-8, C-9, C-10
12	3.89, s	56.9	C-1	3.90, s	57.1	C-1
13	1.60, s	21.7[b]	C-3, C-4, C-5	1.61, s	21.7	C-3, C-4, C-5
14	1.59, s	21.9[b]	C-1, C-2, C-3	1.57, s	23.1	C-1, C-2, C-3
OH	8.60, br s	—		8.70, br s	—	

[a]Data recorded at 500 (^{1}H NMR) and 125.8 (^{13}C NMR) MHz, respectively. Values in parentheses refer to J_{HH} in Hz.
[b]May be interchanged.

decomposed to a rather complex reddish mixture on standing. To determine the products resulting from decomposition of phomaligadiones, a solution of the mixture was left standing for 4 weeks at room temperature. Preparative TLC (CH_2Cl_2–MeOH, 97:3) of this mixture recovered 40% of phomaligadiones and four additional compounds. Analysis of the ^{1}H and ^{13}C NMR spectra of each compound revealed the structures of these components: phomaligols A (**1**) **and** $\mathbf{A_1}$ (**2**), and the corresponding hydroperoxides **3** and **4**. The structures of phomaligols A (**1**) and A_1 (**2**) were readily assigned by comparison of the ^{1}H and ^{13}C NMR spectra, and the optical rotation of each compound with data obtained previously (1). The structures of the hydroperoxides **3** and **4** were assigned from analysis of the spectroscopic data (NMR in Table 1) and reduction of the hydroperoxide groups to the corresponding alcohols, as described below.

The ^{1}H NMR spectra of compounds **3** and **4** were similar (within 0.05 ppm) to those of the corresponding alcohols **1** and **2** (1), except for H-6 at 5.67 ppm in **3** (5.53 ppm in **1**) vs. 5.71 ppm in **4** (5.53 ppm in **2**), and the D_2O exchangeable signals at 8.60 ppm in **3** (2.62 ppm in **1**) vs. 8.70 ppm in **4** (3.44 ppm in **2**). The molecular formula ($C_{14}H_{20}O_7$, obtained by HRCIMS) of each new compound, together with ^{1}H and ^{13}C NMR data, indicated that they might be hydroperoxides. Structures **3** and **4** were elucidated by analysis of the HMBC (6) and HMQC (7) spectra of each compound and by reduction of the hydroperoxide groups to the corresponding alcohols. Analysis of the HMBC and HMQC spectra of **3** and **4** indicated key correlations (Table 1) similar to those previously observed for **1** and **2** (1). For example, in compound **3** the methyl groups at δ_H 1.60 (H_3-13) and 1.59 (H_3-14) showed correlations with the carbonyl carbon at δ_C 202.8 (C-3); the methyl group at δ_H 1.59 displayed additional correlations with carbons at δ_C 84.6 (C-2) and δ_C 172.4 (C-1). The latter carbon (C-1) showed further correlations with an OMe group at δ_H 3.89 (H_3-12) and a methine proton at δ_H 5.67. The methine proton (H-6) was attached to a carbon at δ_C 102.0 (C-6) and displayed further correlations with carbons at δ_C 192.0 (C-5) and 81.0 (C-4) ppm. The methyl group at δ_H 1.60 (H_3-13) displayed an additional long-range correlation with the latter carbon (C-4). Similar correlations indicated in Table 1 were observed in compound **4**.

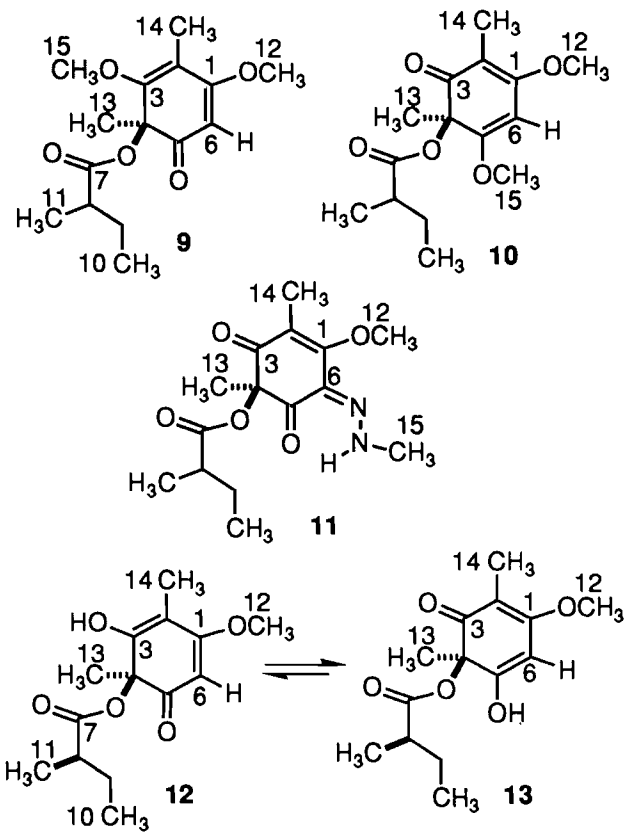
Finally the relative configurations of the C-2 and C-4 stereogenic centers of compounds **3** and **4** were assigned after reduction of the respective hydroperoxide groups with $P(OMe)_3$ and comparison of the spectroscopic data and optical rotation of each reaction product with those of **1** and **2**, respectively. Based on these results the relative configuration of compound **3** is *trans*, like that of **1**, and the relative configuration of compound **4** is *cis*, as is that of **2**. Similarly to phomaligadiones, phomaligin A (**7**) oxidized on standing. In attempting to purify a sample of **7**, which had been previously purified and stored at 0°C for several months, only 30% was recovered; alcohols **1** and **2**, (10%) and **8** (30%) were isolated, along with several minor unidentified products. A rearrangement of **7** to **8**, followed by oxidation and hydrolysis of the $NHCH_2CH_2OH$ group, would explain the spontaneous transformation of **7** to **1** and **2**.

Following the characterization of the oxidation products of phomaligadiones **5** and **6**, their reaction with diazomethane was examined. The reaction products were identified as the methyl ethers **9** and **10** and the adduct **11**. Methyl ethers **9** and **10** were initially characterized by ^{1}H NMR spectroscopy as a mixture (**9**/**10**, 3:1). The ^{1}H NMR spectrum of this mixture showed the expected resonances for two isomeric methyl ethers; however, the position of the newly introduced (O)Me

Table 2. NMR data[a] for compounds **9** and **11** in $CDCl_3$.

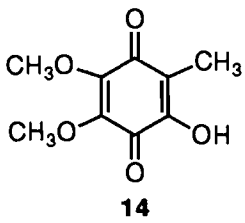
Position no.	9			11		
	δ_H	δ_C	HMBC	δ_H	δ_C	HMBC
1	—	172.7	—	166.1		
2	—	112.8	—	116.8		
3	—	163.3	—	194.0		
4	—	79.2	—	82.6		
5	—	195.4	—	191.5		
6	5.42, s	96.1	C-1, C-4, C-5	—	123.5	
7	—	175.6	—	175.8		
8	2.45, ddq (6.8, 6.8, 7.0)	40.1	C-7, C-9, C-10, C-11	2.50, ddq (6.8, 6.8, 7.0)	40.0	C-7, C-9, C-10, C-11
9	1.72, m 1.47, m	26.6	C-7, C-8, C-10, C-11	1.70, m 1.49, m	26.7	C-7, C-8, C-10, C-11
10	0.96, t (7.5)	11.3	C-7, C-8, C-9, C-11	0.96, t (7.5)	11.4	C-7, C-8, C-9, C-11
11	1.14, d (7.0)	16.3	C-7, C-8, C-9, C-10	1.16, d (7.)	16.4	C-7, C-8, C-9, C-10
12	3.77, s	56.2	C-1	3.90, s	61.9	C-1
13	1.84, s	24.0	C-3, C-4, C-5	1.50, s	23.7	C-3, C-4, C-5
14	1.59, s	9.5	C-1, C-2, C-3	1.90, s	9.0	C-1, C-2, C-3
15	3.74, s	61.2	C-3	3.41, d (4.1)	40.1	
Others	—	—		12.6, br s	—	

[a]Data recorded at 500 (^{1}H NMR) and 125.8 (^{13}C NMR) MHz, respectively. Values in parenthesis refer to J_{HH} in Hz.



group in each isomer could not be determined unambiguously (i.e., C-3 or C-5). Further separation of the mixture of **9** and **10** yielded sufficient amounts of **9** to allow the unambiguous assignment of this structure. Firstly, the ^{1}H NMR spectrum of **9** (Table 2) displayed the resonances seen for the major product present in the initial mixture of **9** and **10**, that is, a *sec*-butyl group (9 hydrogens) in addition to two Me groups (δ_H 1.84 and 1.59), two Me(O) groups (δ_H 3.74 and 3.77), and a vinylic proton at δ_H 5.42. The proton decoupled ^{13}C NMR spectrum (Table 2) of compound **9** showed the expected 15 resonances, which corroborated the presence of 2 × Me, 2 × Me(O), and a *sec*-butyl group, as indicated in the ^{1}H NMR spectrum. In addition, signals attributable to four carbonyl groups or equivalents (δ_C 195.4, 175.6, 172.7, and 163.3), two sp^2 carbons (δ_C 112.8 and 96.1), and one oxygenated quaternary carbon (δ_C 79.2) confirmed that **9** was a methylated derivative of **5/6**. The EIMS of compound **9** revealed the molecular ion expected for $C_{15}H_{22}O_5$ at *m/z* 282. Finally, the structure of compound **9** was unambiguously assigned on the basis of its HMBC spectral data (Table 2). The location of the Me(O) at C-3 and not at C-5 was deduced from the long-range correlations of C- 3 (δ_C 163.3) with the methyl groups at δ_H 3.74 (H_3-15), δ_H 1.84 (H_3-13), and δ_H 1.59 (H_3-14), as well as the correlation of the lowest field carbonyl carbon (δ_C 195.4, C-5) with the methyl group at δ_H 1.84 (H_3-13). Although insufficient sample was recovered to obtain complete spectroscopic data for **10**, the ^{1}H NMR data attributable to the minor isomer of the methylation product mixture indicated the proposed structure.

The major product of the reaction of phomaligadiones **5/6** with diazomethane was a bright yellow oil (**11**). The HREIMS of product **11** suggested the molecular formula $C_{15}H_{22}N_2O_5$, consistent with the formation of an adduct of **5/6** ($C_{14}H_{20}O_5$) with CH_2N_2. Analysis of the ^1H NMR spectrum of **11** showed the expected resonances for the cyclohexenedione substituents also present in the starting material (**5/6**): one *sec*-butyl group (9 hydrogens), two Me groups (δ_H 1.90 and 1.50), and one Me(O) group (δ_H 3.90). Two new signals at δ_H 3.41 (d, 3H) and 12.6 (bs, 1H, D_2O exchangeable) were attributed to the addition of CH_2N_2 to cyclohexenediones **5/6**, resulting in a =N-NH-Me substituent. The locations of the 2 × Me, MeO, *sec*-butyl-C(=O)O, and 2 × C=O groups on the cyclohexenedione were determined to be identical to those observed in **5/6**, by analysis of the proton decoupled ^{13}C NMR and HMBC spectral data (Table 2). Consequently, the *N*-methylhydrazone (=N-NH-Me) could only be located at C-6 (δ_C 123.5). In this way the structure of the major product was established as adduct **11**.[4]



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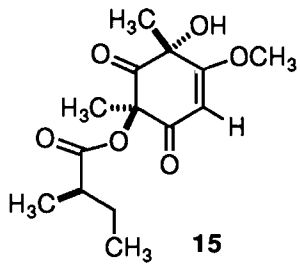

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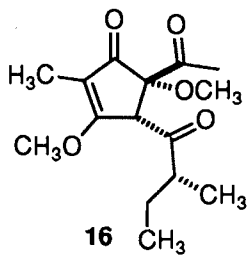
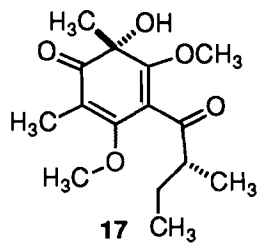


16 CH_3



17 CH_3

The products of the reaction of phomaligadiones **5/6** with diazomethane allowed further conclusions with respect to metabolites produced by *P. wasabiae*. It was reported that a compound named wasabidienone A, on treatment with diazomethane, yielded two Me ethers (1:1) having structures **9** and **10** (8). Later on, analysis of the p-nitrobenzoate derivative of wasabidienone A by X-ray crystallography allowed the assignment of its stereogenic centers (9). Based on these results (8, 9) the tautomeric forms **12** and **13** were assigned to wasabidienone A. Furthermore, it was reported that wasabidienone A on standing on a TLC plate decomposed to a brownish material, which was identified as a mixture of compounds **14** (purple) and **15** (colorless) (10). The structure of compound **15**, including absolute configuration, was determined by X-ray crystallography (10). The purple compound **14** had been previously reported as a metabolite of *P. wasabiae* (11[5] but, interestingly, **15** was never reported to be present in cultures of *P. wasabiae*. The NMR, IR, and optical rotation data reported for compound **15** are in close agreement with data obtained for phomaligol A (**1**) (1). In conclusion, wasabidienone A and phomaligadiones appear to be the same compound, existing, at least in $CDCl_3$, as a tautomeric mixture, described more accurately as **5** and **6**, since the resonances corresponding to C-2 in the former, and C-6 in the latter, are clearly due to sp^3 carbons (1), and not sp^2 carbons as in **12** and **13**. The data obtained for methyl ether derivatives **9** and **10** are in complete agreement with data reported previously (8); however, Soga et al. did not report adduct **11** (8). Because the conditions used to prepare these methyl ethers were not specified in that report (8), it is not possible to comment on those results. Our previous work (3) indicated that some of the compounds isolated from cultures of *P. lingam* were also present in cultures of *P. wasabiae*. Now it is clear that the profile of metabolites common to *P. wasabiae* and *P. lingam* is much broader; not only phomaligols (**1**, **2**) and phomaligadiones (**5**, **6**) are present in liquid cultures of both species, but also wasabidienones B_0 (**16**) and B_1 (**17**) (12) were present in similar liquid cultures.

Biogenetically, metabolites **1**, **2**, **5–8** may derive from a pentaketide that is further methylated and oxidized; however, the results reported here indicate that metabolites **1** and **2** can also result from non-enzymatic oxidation of **5–7**.[6] Furthermore, this work has uncovered a relationship for metabolites **1**, **2**, **7**, and **8**; that is, **8** could result from a non-enzymatic rearrangement of **7**, and could then be further oxidized to **1** and **2** (**7** → **8** → **1** + **2**). In fact, it is likely that the reddish pigments characteristic in liquid cultures of avirulent blackleg isolates (13) are most likely oxidation products of the metabolites **1–8** and (or) related ones such as **14**. The phytotoxicity of these metabolites to canola susceptible to blackleg was evaluated; phomaligols **1** and **2** did not cause obvious lesions even at relatively high concentrations (10^{-3} M), whereas **7** and **8** caused only slight lesions at similar concentrations.

Experimental

General

All chemicals were purchased from Aldrich Chemical Company, Inc., Madison, Wis. All solvents were HPLC grade and used as such. Preparative TLC: Merck Kieselgel 60 F_{254}, 20 × 20 cm × 0.25 mm; analytical TLC (Merck Kieselgel 60 F_{254}, aluminum sheets) 5 × 2 cm × 0.2 mm; compounds were visualized by exposure to UV and by dipping the plates in a 5% aqueous (w/v) phosphomolybdic acid solution containing a trace of ceric sulfate and 4% (v/v) H_2SO_4, followed by heating at 200°C. Flash column chromatography: Merck silica gel, grade 60, mesh size 230–400, 60 Å. NMR spectra were recorded on a Bruker AMX 500 or AM 300 spectrometer; δ

[4] No literature precedent was found for this mode of reaction of diazomethane (T. H. Black, Aldrichimica Acta, **16**, 3 (1983)). It may be a simple protonation of diazomethane, followed by enolate attack on the electrophilic terminal nitrogen.

[5] In the course of this work a reddish polar material was isolated, but due to further decomposition, its structure could not be assigned.

[6] The only indication that enzymatic oxidation might occur arises from detection of **1** and **2** in 2–3-day-old cultures, while **5/6** accumulated in 7–9-day-old cultures.

values were referenced to $CHCl_3$ (7.24 ppm) for ^1H (500 MHz), and referenced to $CDCl_3$ (77.0 ppm) for ^{13}C (125.8 MHz). Mass spectra (MS) were obtained on a VG 70-250 SEQ hybrid mass spectrometer or on a Finnigan Mat model 4500 mass spectrometer (high resolution (HR), electron impact (EI), fast atom bombardment (FAB), or chemical ionization (CI) with ammonia as carrier gas), employing a solid probe in both cases.

Air oxidation of phomaligadiones A (5a/5b) and B (6)

A solution of phomaligadiones A (**5a/5b**) and B (**6**) (28 mg in $CHCl_3$–MeOH, 1:1, 1 mL) was left standing for 4 weeks at room temperature. Preparative TLC (CH_2Cl_2–MeOH, 97:3, multiple development) of that mixture gave phomaligols **1** (2 mg; R_f 0.42, hexane–EtOAc, 1:1) and **2**(1 mg; R_f 0.28, hexane –EtOAc, 1:1), hydroperoxides **3** (3 mg; R_f 0.46, hexane–EtOAc, 1:1) and **4** (1 mg; R_f 0.34, hexane–EtOAc, 1:1), and a mixture of phomaligadiones **5a/5b** and **6** (12 mg; R_f 0.60, hexane–EtOAc, 1:1).

Reaction of phomaligadiones A (5a/5b) and B (6) with diazomethane

Diazomethane in ether was added (excess) to a stirred solution of phomaligadiones A (**5a/5b**) and B (**6**) (8 mg in CH_2Cl_2, 1 mL) at room temperature. After 30 min the solvents were removed with a stream of nitrogen and the reaction products were separated by preparative TLC (CH_2Cl_2–MeOH, 97:3, developed twice) to give a mixture of compounds **9** and **10** in one band (2 mg, 3:1) and adduct **11** (3.2 mg). Compounds **9** and **11** were further purified separately by preparative TLC (hexane–EtOAc, 7:3, multiple elution) to yield pure **9** (1.7 mg; R_f 0.75, hexane–EtOAc, 1:1), **10** (0.5 mg; R_f 0.5, hexane–EtOAc, 1:1), and **11** (2.1 mg; R_f 0.75, hexane–EtOAc, 1:1).

Reduction of hydroperoxides 3 and 4

$P(OMe)_3$ (2 mg in CH_2Cl_2, 1 mL) was added to a solution of the hydroperoxide **3** (2.4 mg in CH_2Cl_2, 1 mL) at room temperature. The solvent was immediately evaporated and the reaction product was purified by preparative TLC (hexane–EtOAc, 1:1, multiple elution) to give a pure compound (1.5 mg), identical to phomaligol A (**1**) in all respects, including optical rotation. Similar reaction with hydroperoxide **4** yielded a pure compound (1.1 mg), identical to phomaligol A_1 (**2**).

Phomaligol A hydroperoxide (3): colorless oil; HRCIMS *m/z* (relative intensity) measured: 301.1282 (301.1287 calcd. for $C_{14}H_{21}O_7$); FABMS *m/z* (relative intensity): 301 ($[M+1]^+$ 100; ^1H and ^{13}C NMR, see Table 1.

Phomaligol A_1 hydroperoxide (4): colorless oil; HRCIMS *m/z* (relative intensity) measured: 301.1283 (301.1287 calcd. for $C_{14}H_{21}O_7$); FABMS *m/z* (relative intensity): 301 ($[M+1]^+$ 100; ^1H and ^{13}C NMR, see Table 1.

Compound 9: light yellow film; ^1H NMR ($CDCl_3$) δ:5.44(s), 3.88(s), 3.72(s), 2.45 (ddq, J = 6.8, 7.0, 7.0 Hz), 1.84(s) 1.72(m), 1.59(s), 1.47(m), 1.13(d, J = 7.0 Hz), 0.90 (t, J = 7.5).

Compound 10: light yellow oil; EIMS *m/z* (relative intensity): 282 ($[M]^+$, 9%), 198 (20%), 155 (30%); ^1H and ^{13}C NMR, see Table 2.

Adduct 11: yellow oil; HREIMS *m/z* (relative intensity) measured: 310.1529 (310.1526 calcd. for $C_{15}H_{22}N_2O_5$); EIMS *m/z* (relative intensity): 310 ($[M]^+$, 15%), 209 (40%), 154 (28%); ^1H and ^{13}C NMR, see Table 2.

Acknowledgments

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References

1. M.S.C. Pedras, V.M. Morales, and J.L. Taylor. Tetrahedron, **49**, 8317 (1993).
2. M.S.C. Pedras, V.M. Morales, and J.L. Taylor. Phytochemistry, **36**, 1315 (1994).
3. M.S.C. Pedras, J.L. Taylor, and V.M. Morales. Phytochemistry, **37**, 1215 (1995).
4. M.S.C. Pedras. Rev. Latinoam. de Quim. In press.
5. R.K. Gugel and G.A. Petrie. Can. J. Plant Pathol. **14**, 36 (1992).
6. A. Bax and M.F. Summers. J. Am. Chem. Soc. **108**, 2093, (1986).
7. A. Bax and S. Subramanian. J. Magn. Reson. **67**, 565 (1986).
8. O. Soga, H. Iwamoto, S. Date, T. Watanabe, K. Tanaka, K. Hate, A. Takuwa, and M. Nakayama. Chem. Lett. 339 (1984).
9. O. Soga, H. Iwamoto, A. Takuwa, H. Nozaki, J. Kuramoto, and M. Nakayama. Agric. Biol. Chem. **51**, 283 (1987).
10. O. Soga, H. Iwamoto, K. Hata, R. Maeba, A. Takuwa, T. Fujiwara, Y. Hsu, and M. Nakayama. Agric. Biol. Chem. **52**, 865 (1988).
11. O. Soga. Z. Naturforsch. B: Anorg. Chem. Org. Chem. **35B**, 1497 (1980).
12. O. Soga, H. Iwamoto, A. Takuwa, T. Takata, Y. Tsugiyama, K. Hamada, T. Fujiwara, and M. Nakayama. Chem. Lett. 1535 (1988).
13. R.L. Gabrielson. Seed Sci. Technol. **11**, 749 (1983).