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DETOXIFICATION OF THE POTATO PHYTOALEXIN RISHITIN BY *GIBBERELLA PULICARIS*

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Key Word Index—*Gibberella pulicaris*; Fusarium sambucinum; Solanum tuberosum; Solanaceae; potato; phytoalexin metabolism; rishitin; sesquiterpene.

Abstract—The ability of strains of *Gibberella pulicaris* to cause dry rot of potato tubers is related to their tolerance of the potato phytoalexin rishitin. All highly virulent strains studied to date have proven tolerant of and able to metabolize rishitin. Here we report that a rishitin-tolerant strain metabolizes the potato phytoalexin rishitin to 13-hydroxyrishitin and 11,12-epoxyrishitin. The identity of the latter compound was confirmed by chemical epoxidation of rishitin. 11,12-Epoxyrishitin has no effect on the growth of a rishitin-sensitive strain and is further metabolized by this strain. This is the first report of a nontoxic fungal metabolite of rishitin.

INTRODUCTION

Gibberella pulicaris (Fr.) Sacc. (anamorph. *Fusarium sambucinum* Fuckel) is a major cause of dry rot in stored potatoes (*Solanum tuberosum* L.) worldwide [1]. The ability of strains of *G. pulicaris* to cause dry rot is correlated with their tolerance of potato sesquiterpene phytoalexins incorporated within their growth media. All highly virulent field-strains are tolerant of both sesquiterpenes, rishitin (**1**) and lubimin; whereas, strains with a low level of phytoalexin tolerance are low in virulence on potato tuber [2, 3]. Although many sensitive strains metabolize rishitin, the most tolerant ones are characterized by a higher rate of rishitin metabolism. Some rishitin-tolerant strains are sensitive to lubimin and vice versa, indicating that different mechanisms are responsible for metabolism of the two phytoalexins. Furthermore, classical genetic analysis indicates that high virulence is associated with a genetic locus, designated *Rim1*, that controls rishitin metabolism [4]. We previously demonstrated that metabolism of lubimin by *G. pulicaris* includes a complex pattern of detoxification reactions including dehydrogenation, cyclization and epoxidation [5]. Herein we report the occurrence of 13-hydroxyrishitin (**2**) and 11,12-epoxyrishitin (**3**), rishitin metabolites produced by *G. pulicaris*, and we show that 11,12-epoxyrishitin lacks fungitoxicity. A preliminary report of a portion of this investigation has been published in a recent review [6].

RESULTS AND DISCUSSION

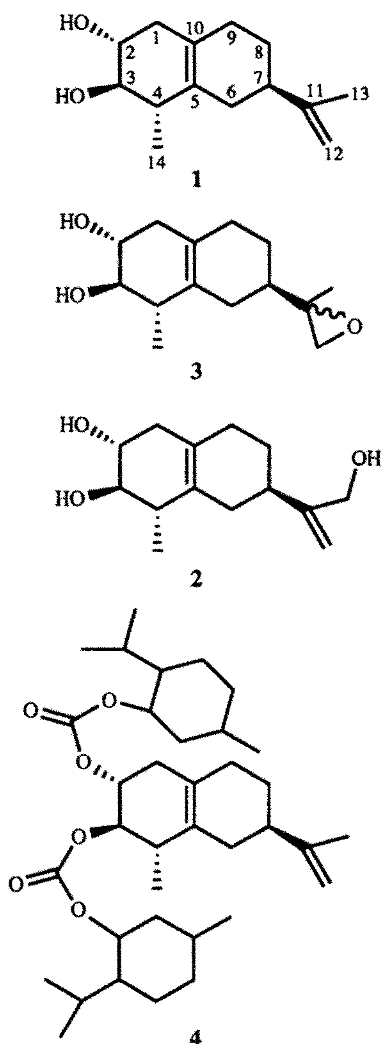
Identification of fungal metabolites

Preliminary experiments in which rishitin was metabolized by a virulent strain of *G. pulicaris* revealed no obvious accumulation of metabolite(s) in the growth media. TLC of the product mixture revealed a number of faint spots, one of which was related to rishitin, based on mass spectral analysis. The rishitin metabolite migrated on TLC just below rishitin [Et_2O–MeOH (19:1); rishitin $R_f = 0.5$; metabolite $R_f = 0.40$] and it afforded a mass spectral molecular ion that was consistent with rishitin containing an extra oxygen.

As described below, this metabolite was subsequently identified as 11,12-epoxyrishitin. In order to characterize it, an incubation of *G. pulicaris*, strain 1845-17-3, was scaled-up to 80 mg rishitin. More than 75% of added rishitin was metabolized, and *ca* 1 mg of 11,12-epoxyrishitin was recovered after open-column chromatographic clean-up followed by HPLC. When the above procedure was repeated several times on a smaller scale, 11,12-epoxyrishitin was consistently observed by HPLC or GC-MS. These data indicate that this biotransformation of rishitin is reproducible, although the yield is always low. While some losses may have occurred during work-up, the low yield of 11,12-epoxyrishitin indicates that its formation may be the rate-limiting step in a sequence of further transformations. There is also the possibility that there is more than one pathway of rishitin metabolism.

To illustrate the latter possibility, *ca* 1 mg of 13-hydroxyrishitin was isolated from the incubation medium in one experiment. However, this compound was not detected in additional incubation experiments. It is not known if 13-hydroxyrishitin was lost during work-up or if it is simply a variable metabolite. No other rishitin-like compound could be found by GC-MS surveys of fractions.

Since the quantity of 11,12-epoxyrishitin was insufficient for full characterization by spectral methods, we



attempted to synthesize it from rishitin using the epoxidizing reagent, 3-chloroperoxybenzoic acid. Unfortunately, under the conditions employed, the oxidizing reagent preferentially epoxidized the cyclohexene 5,10-double bond before attacking the side-chain isopropenyl 11,12-double bond. In this way, both 5,10-epoxyrishitin and 5,10-11,12-diepoxyrishitin were readily obtained. (−)-Menthoxycarbonyl (MC) blocking groups on the 2,3-hydroxyl groups of rishitin (**4**) supplied sufficient steric hindrance to the 5,10-double bond to permit partial epoxidation of only the 11,12-double bond. Thus, 11,12-epoxyrishitin was obtained after removal of the MC blocking groups. In theory, the synthetic compound was comprised of two diastereomeric epoxides, and indeed, capillary GC showed a very slight peak separation of compounds with nearly identical EIMS profiles.

NMR spectral methods were useful in characterizing the structure of the metabolites as well as the synthetic samples. The precursor rishitin, as well as synthetic 11,12-epoxyrishitin, was fully characterized by ^{1}H NMR, ^{13}C NMR, distortionless enhancement by polarization transfer (DEPT) and carbon/hydrogen correlative spectroscopy. The combined NMR methods permitted a complete assignment of all proton (Table 1) and carbon absorption values (Table 2). The metabolite, 13-hydroxyrishitin, and synthetic 5,10-epoxyrishitin and 5,10-11,12-diepoxyrishitin were examined by ^{1}H NMR (Table 1), ^{13}C NMR (Table 2) and DEPT. Because of the lack of sufficient authentic 11,12-epoxyrishitin produced by fungal metabolism, only ^{1}H NMR and EIMS data were obtained for comparison with the spectra of the synthetic sample. Although the main features in the ^{1}H NMR of metabolic and synthetic 11,12-epoxyrishitin were identical, there were notable differences due to the presence of two epoxide diastereomers in the latter. The most notable difference was in the absorbance of the C-12 AB-protons. The compound obtained from metabolism gave only doublets centred at δ2.58 and 2.54 (AB quartet); whereas the synthetic sample gave these absorbances plus doublets centred at δ2.63 and 2.55 due to the other epoxide diastereomer (Table 1). In addition, in the ^{1}H NMR spectrum of the synthetic sample, the methyl absorbance at C-14 was split into an apparent triplet and the C-3 apparent triplet was narrowly split into a doublet of triplets; whereas, the metabolic sample gave only a doublet for the absorbance at C-14 centred at 1.12 and an apparent triplet for C-3. There were also slight differences in the splitting of the C-2 proton multiplet between the two spectra. That the synthetic 11,12-epoxyrishitin was a pair of diastereomers was also demonstrated by the splitting of the proton absorbance at C-13 into an apparent doublet (this absorption appeared as a singlet at δ1.28 in the spectrum of the metabolic sample, but it was partially obscured due to an impurity). Also, it is noted that the ^{13}C NMR spectrum for synthetic 11,12-epoxyrishitin displayed narrowly split absorbances for most of the carbons (Table 2).

GC-MS furnished further structural confirmation of the metabolites and synthetic products. The metabolic and synthetic 11,12-epoxyrishitin gave virtually identical spectra and shared a number of prominent fragment ions with rishitin as follows: EIMS (GC-MS) 70 eV, *m/z* (rel. int.) 238 $[M]^+$ (2), 223 $[M-Me]^+$ (3), 220 $[M-H_2O]^+$ (12), 207 $[M-O-Me]^+$ (15), 202 $[M-2H_2O]^+$ (13), 194 (12), 189 $[M-H_2O-Me-O]^+$ (33), 180 (21), 171 (21), 161 (53), 145 (87), 143 (92), 131 (70), 119 (65), 117 (63), 105 (97), 91 (100), 79 (57), 55 (38) and 43 (60). The mass spectrum of synthetic 5,10-epoxyrishitin gave a very close match to the 11,12-epoxy metabolite as expected, but with several notable differences: EIMS (GC-MS) 70 eV, *m/z* (rel. int.) 238 $[M]^+$ (1), 220 $[M-H_2O]^+$ (30), 205 $[M-H_2O-Me]^+$ (20), 202 $[M-2H_2O]^+$ (79), 187 $[M-2H_2O-Me]^+$ (35), 173 (28), 159 (48), 145 (48), 135 (48), 133 (51), 131 (48), 121 (63), 107 (79), 93 (100), 91 (78), 79 (78), 67 (99), 55 (94) and 41 (99). 13-Hydroxyrishitin afforded a mass spectrum similar to both of the mono-epoxides, but was distinguished from either by a few characteristic ions as well as some striking differences in ion intensity: EIMS (GC-MS) 70 eV, *m/z* (rel. int.) 220 $[M-H_2O]^+$ (23), 205 $[M-H_2O-Me]^+$ (8), 202 $[M-2H_2O]^+$ (5), 189 $[M-H_2O-Me-O]^+$ (15), 187 $[M-2H_2O-Me]^+$ (16), 161 (36), 159 (39), 145 (60), 143 (55),

Table 1. ^{1}H NMR spectral data of rishitin, 11,12-epoxyrishitin, 5,10-epoxyrishitin, 5,10-11,12-epoxyrishitin and 13-hydroxyrishitin

	Chemical shifts [multiplicity; *J* (Hz)]				
Proton	Rishitin	11,12-Epoxyrishitin	5,10-11,12-Diepoxyrishitin	5,10-Epoxyrishitin	13-Hydroxyrishitin
1AB	2.10*	2.07*	nd†	nd	nd
	2.15*	2.18*	nd	nd	nd
2	3.63 (*m*; 5.4, 10.6, 9.5)	3.64 (*m*)	3.54 (*m*)	3.53 (*m*)	3.65 (*m*)
3	3.21 (*dd*; 9.5, 8.9)	3.19 (app† *t*; 9.1)§	3.26 (*dd*)§	3.24 (*dd*)	3.23 (*dd*)
4	2.07* (*m*)	2.07*	nd	nd	nd
6AB	1.73*	1.87*	nd	nd	nd
	2.22*	2.06*	nd	nd	nd
7	2.23*	2.20*	nd	nd	nd
8AB	1.55 (*m*)	1.77*	nd	nd	nd
	1.66* (*m*)	1.85*	nd	nd	nd
9AB	1.81* (*m*)	1.61*	nd	nd	nd
	1.99*(*m*)	2.08*	nd	nd	nd
12AB	4.62 (*m*; 0.7)	2.58 (*d*; 4.7)	2.62 (*d*; 4.7)	4.64 (*s*)	4.83 (*s*)
	4.72 (*m*; 1.5)	2.54 (*d*; 4.7)	2.55 (*d*; 4.7)	4.76 (*m*; 1.5)	5.06 (*m*; 1.3)
12'AB‡		2.63 (*d*; 4.8)	2.59 (*d*; 4.7)		
		2.55 (*d*; 4.8)	2.51 (*d*; 4.7)		
13	1.72 (*s*)	1.28 (*s*)	1.24 (*s*)	1.69 (*s*)	4.13 (*s*)
13'‡		1.26 (*s*)			
14	1.14 (*d*; 6.7)	1.12 (*d*; 6.9)†	1.21 (*d*; 6.8)§	1.22 (*d*; 6.9)	1.15 (*d*; 6.7)
14'‡		1.14 (*d*; 7.5)†			
OH	3.08 (*br*† *s*)	2.35 (*br d*)	nd	2.38	nd

*Resonances were partly obscured by other signals; proton chemical shifts were determined by ^{13}C NMR/^{1}H NMR correlation spectroscopy.

†Abbreviations: app, apparent; *br*, broad; nd, the overall absorption pattern was similar to those of rishitin, but overlapping absorbances could not be analysed due to lack of ^{13}C NMR/^{1}H NMR correlation spectroscopy.

‡The second set of proton absorbances designated by prime (12', 13' and 14') are caused by a second epoxide diastereomer produced by synthesis; the primed values were slightly less intense absorbances. The absorbances obtained for the metabolic 11,12-epoxyrishitin corresponding to the unprimed first set of values.

§Absorbance(s) narrowly split presumably due to expoxide stereoisomers.

Table 2. ^{13}C NMR spectral data of rishitin, 11,12-epoxyrishitin, 5,10-11,12-diepoxyrishitin, 5,10-epoxyrishitin and 13-hydroxyrishitin

	Chemical shift (multiplicity)				
C	Rishitin	11,12-Epoxyrishitin*†	5,10-11,12-Diepoxyrishitin*†	5,10-Epoxyrishitin*	13-Hydroxyrishitin
1	38.3 (*t*)	38.4, 38.6 (*t*)	37.4, 37.5 (*t*)	37.4 (*t*)	38.2 (*t*)
2	71.5 (*d*)	71.4 (*d*)	70.4 (*d*)	70.5 (*d*)	71.5 (*d*)
3	79.2 (*d*)	79.0, 79.1 (*d*)	74.9 (*d*)	75.1 (*d*)	79.1 (*d*)
4	41.6 (*d*)	41.4, 41.2 (*d*)	41.0, 40.9 (*d*)	41.4 (*d*)	41.7 (*d*)
5	129.0 (*s*)	128.8, 128.9 (*s*)	66.0, 66.4 (*s*)	66.1 (*s*)	128.9 (*s*)
6	31.0 (*t*)	29.7, 30.8 (*t*)	30.5, 30.4 (*t*)	33.2 (*t*)	31.4 (*t*)
7	40.4 (*d*)	39.2, 40.3 (*d*)	35.9, 35.8 (*d*)	37.0 (*d*)	36.1 (*d*)
8	26.5 (*t*)	24.4, 24.6 (*t*)	23.0, 23.5 (*t*)	24.5 (*t*)	26.6 (*t*)
9	29.6 (*t*)	27.4, 27.6 (*t*)	30.1, 30.0 (*t*)	29.7 (*t*)	29.3 (*t*)
10	124.8 (*s*)	125.3, 125.1 (*s*)	62.2, 62.1 (*s*)	62.1 (*s*)	124.9 (*s*)
11	148.9 (*s*)	58.7, 58.4 (*s*)	58.5, 58.7 (*s*)	148.6 (*s*)	152.2 (*s*)
12	108.9 (*t*)	53.0, 53.1 (*t*)	52.8 (*t*)	109.4 (*t*)	108.7 (*t*)
13	21.1 (*q*)	18.8, 18.3 (*q*)	18.8, 18.7 (*q*)	21.3 (*q*)	65.3 (*t*)
14	16.4 (*q*)	16.3, 16.6 (*q*)	12.9, 13.0 (*q*)	12.7 (*q*)	16.3 (*q*)

*Chemically synthesized from rishitin.

†Carbon absorbances were narrowly split indicating differences in the shifts of diastereomers; the absorbance with slightly greater abundance is listed first.

131 (60), 129 (44), 117 (41), 105 (63), 91 (100), 79 (42), 77 (56), 55 (65) and 41 (87). Other than failing to give a molecular ion, the mass spectrum of synthetic 5,10-11,12-diepoxyrishitin gave the expected ions: EIMS (GC-MS) 70 eV, m/z (rel. int.) 221 $[M-2O-H]^+$ (5), 218 $[M-2H_2O]^+$ (3), 203 $[M-2H_2O-Me]^+$ (16), 189 $[M-H_2O-Me-2O]^+$ (11), 179 (21), 175 (19), 161 (31), 149 (34), 145 (31), 135 (40), 133 (36), 121 (52), 109 (65), 91 (58), 79 (59), 55 (65) and 43 (100).

Toxicity and metabolism of 11,12-*epoxyrishitin*

The effects of rishitin and 11,12-epoxyrishitin on a rishitin-sensitive strain of *G. pulicaris* are shown in Table 3. The sensitive strain 1845-11-2 was clearly inhibited by and unable to metabolize rishitin, but was tolerant of and able to further metabolize the 11,12-epoxyrishitin produced by tolerant strain 1845-17-3, as shown by its complete disappearance. Interestingly, strain 1845-11-2 was completely tolerant of synthetic 11,12-epoxyrishitin, but only one diastereomer almost completely disappeared after seven days incubation. The second diastereomer was more resistant to metabolism and it amounted to 21% of that recovered from uninoculated seven day controls. Although the data show that epoxidation detoxified rishitin, it is not clear if this event represents the principal strategy for detoxification. Because of inadequate sample size and its inconsistent formation by the fungus, the fungitoxicity of 13-hydroxyrishitin was not tested. This hydroxylation of rishitin is catalysed by potato tuber tissue itself [7], in which case the oxygen atom is derived from molecular oxygen, suggesting the involvement of a cytochrome P450 monoxygenase [8]. It has been hypothesized that conversion of rishitin to 13-hydroxyrishitin is a detoxification mechanism employed by the plant [8], but we are not aware of any specific study in which this hypothesis has been tested.

EXPERIMENTAL

Culture methods. The strains of *G. pulicaris* used in this study were obtained as previously described [4]. Strains 1845-17-3 and 1845-11-2 were progeny from asci of cross 1845 between a rishitin-tolerant strain and a rishitin-sensitive strain. Strain 1845-17-3 is tolerant of rishitin and is virulent on potato tubers, whereas strain 1845-11-2 is sensitive to rishitin and is not virulent. For long-term storage, strains were maintained in V-8 agar medium (M-20) [9] at 4° and as lyophilized suspensions at the Fusarium Research Center (The Pennsylvania State University, University Park, PA). For each experiment, fresh transfers of each strain were grown on V-8 medium with an alternating 12 hr 25° light/12 hr 20° dark schedule.

Bioassay. For fungitoxicity and metabolism studies of strain 1845-11-2, duplicate 3.5 × 1.0 cm Petri dishes were filled with 1 ml of V-8 medium and 1% DMSO with or without the test compounds. The plates were inoculated at their edge with a plug (3 mm diameter) cut from the growing margin of cultures less than 10 days old and placed with the mycelial surface appressed to the agar. Plates were incubated for 7 days at 25 ± 2° in the dark. Colony radius was measured daily for 7 days, or until fungal growth reached the opposite edge of the plate. Radial growth rates were approximately linear for both strains in the presence or in the absence of test compounds. Determination of remaining test compounds in the agar was by GC as previously described [2]; the GC column was a 0.32 mm × 30 m capillary coated with 0.25 μm SPB-1 from Supelco.

Preparation of rishitin metabolites. Rishitin was prepd from arachidonic acid-treated tubers of potato cultivar Russet Burbank as described previously [2, 10]. Rishitin was incubated with *G. pulicaris* strain 1845-17-3 on agar plates. The rishitin was dissolved in DMSO (10 or 20 mg ml^{-1}) and mixed with V-8 agar medium [9] at a final concn of 200 μg ml. This prepn was poured into 9 × 1.5 cm plastic Petri dishes in 20 ml portions. Each of the agar plates was inoculated with 5 plugs (5 mm diameter) cut from the growing margin of strain 1845-17-3 cultures less than 10 days old and placed with the mycelial surface appressed to the agar. Plates were incubated at 25 ± 2° in the dark for 4 days. After incubation the agar was extracted by adding 300 ml $CHCl_3$–MeOH (1:1), sitting overnight, pouring off the solvent, then repeating this procedure 3 times. Rishitin and its metabolites were recovered from the $CHCl_3$ layer after

Table 3. Effect of rishitin and 11,12-epoxyrishitin on the growth of a sensitive strain of *G. pulicaris*

	(Radial growth rate (mm day^{-1}))*				Recovery (%)†		
			11,12-*Epoxyrishitin*			11,12-*Epoxyrishitin*	
Strain number	Control	Rishitin	Metabolite	Synthetic	Rishitin	Metabolite	Synthetic‡
1845-11-2	3.4 ± 0.2	1.2 ± 0.2	3.2 ± 0	3.4 ± 0.1	58 ± 24	nd	27

*Mean and standard deviation of tests on 1-ml agar plates amended with 200 μg of test compound in DMSO (1% DMSO in agar media) and incubated in the dark at 25° for 7 days. Control plates (DMSO only), 6 tests; rishitin plates, 6 tests; 11,12-epoxyrishitin plates, 2 tests each.

†Analysed by GC as the per cent of test compound based on uninoculated plates containing the test compound (mean of all plates). Recovery from 7-day uninoculated plates was 85% for rishitin and 71% for 11,12-epoxyrishitin. nd, not detected.

‡Of the two isomers present in synthetic 11,12-epoxyrishitin, the diastereomer eluting slightly earlier by GC was present in 4-fold greater amount after incubation with strain 1845-11-2. The per cent recovery is the mean of two; 18 and 35%.

adding 300 ml H_2O to the combined sample. After evapn of the $CHCl_3$, the residue was mixed with 2 g SilicAR CC4 (Mallinckrodt, Paris, KY) in hexane and applied to an open column (2.5 cm i.d.) containing 50 g SilicAR CC4 packed in hexane. Elution was with 0.3 l 70% Et_2O in hexane, 0.4 l Et_2O, and 0.4 l 5% MeOH in Et_2O. Rishitin eluted between 0.26 and 0.38 l, and 11,12-epoxyrishitin between 0.41 and 0.55 l. 11,12-Epoxyrishitin was isolated after HPLC purification of the material obtained from the open column using a 5 μ particle size Zorbax-sil column (9.4 × 250 mm from Dupont) eluting with hexane–Me_2CO (84:16) at a flow rate of 3.3 ml min^{-1} (elution time, 50 min). Detection was by absorbance at 206 nm. In one experiment, 13-hydroxyrishitin was obtained in SilicAR CC4 frs eluting between 0.56 and 0.8 l. About 1 mg 13-hydroxyrishitin was recovered after HPLC with hexane–Me_2CO (75:25) at a flow rate of 3 ml min^{-1} (elution time, 44 min).

Chemical synthesis of rishitin epoxides. The synthesis of 11,12-epoxyrishitin required blocking groups to slow the epoxidation of the 5,10-double bond. Thus, rishitin (15 mg) was reached with 3 mmol (−)-menthyl chloroformate (Aldrich), in 6 ml toluene–pyridine (12:1) for 2 hr at 50°. The product was purified by prep. TLC (2 mm thick plate, Merck) developing with hexane–EtOAc (95:5) and recovering the band at $R_f = 0.52$ (yield, 21 mg). Products were detected by spraying with a 0.1% soln of 8-anilino-1-naphthalenesulphonic acid and viewing by long wavelength UV light. The isolated material, 2,3-bis-MC-rishitin, was oxidized with an equimolar amount of 3-chloroperoxybenzoic acid in 6 ml $CHCl_3$ for 1 hr at 25° after which the $CHCl_3$ layer was washed with aq. 10% $NaHCO_3$. Three 2,3-bis-MC derivatives were isolated from the $CHCl_3$ layer by TLC [hexane–EtOAc (95:5)], rishitin ($R_f = 0.52$), 5,10-epoxyirishitin ($R_f = 0.28$) and 11,12-epoxyrishitin ($R_f = 0.12$). A fourth component, not examined, was presumably the 2,3-bis-MC derivative of 5,10-11,12-diepoxyrishitin ($R_f = 0.03$). The 2,3-bis-MC-rishitin remaining from the first oxidation was subjected to 2 more sequential oxidations and isolations affording a total of 5 mg of the 2,3-bis-MC derivative of 11,12-epoxyrishitin. This derivative was saponified by 0.5 ml of 0.5 N KOH in MeOH for 30 min at 70° and the product was extracted by 2 vols of $CHCl_3$–H_2O (1:1). Clean-up by TLC [Et_2O–MeOH (19:1, paper tank-liner)] furnished 1.4 mg 11,12-epoxyrishitin and 0.4 mg 11-methoxy-12-hydroxyrishitin (data not shown); presumably the latter compound arose from methanolysis of the epoxide.

The other epoxides of rishitin were synthesized by direct epoxidation of rishitin. Eleven mg of rishitin were oxidized with a 2 M excess of 3-chloroperoxybenzoic acid in 10 ml $CHCl_3$ for 2 hr. After reaction the $CHCl_3$ layer was washed with 10% aq. $NaHCO_3$ and H_2O, and the recovered material sepd by TLC (pre-coated silica gel F-254, Merck) using Et_2O–MeOH (19:1, paper tank-liner). 5,10-Epoxyrishitin (41% yield, $R_f = 0.45$) and 5,10-11,12-diepoxyrishitin (25% yield, $R_f = 0.30$) were recovered.

^1H NMR (300 and 400 MHz) and ^{13}C NMR (75 and 100 MHz) spectra were recorded with $CDCl_3$ as int. standard and solvent. GC-MS was completed with a Hewlett-Packard capillary column (HP-5MS, cross-linked 5% phenyl methyl silicone, 0.25 mm × 30 m, film thickness 0.25 μm). The column was temp. programmed from 160 to 260° at 5° min^{-1} with a He flow of 0.9 ml min^{-1}; injector temp. was 260°.

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