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SCREENING OF THE GENUS *CERCOSPORA* FOR SECONDARY METABOLITES*

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Key Word Index—*Cercospora*; Deuteromycetes; cercosporin; cercosporin esters; 3-methoxy-2,5,7-trihydroxy-1,4-naphthaquinone; *cis*-4,6-dihydroxymellein.

Abstract—Screening of 61 species of *Cercospora* grown on a potato-agar medium showed the presence of the phytotoxin cercosporin in 24 of them, and of dothistromin in 8. Some strains of *C. beticola* produce a yellow phytotoxin (CBT). The new metabolites cercosporin esters, ligustrone A, B, C, taiwapyrone, 3-methoxy-2,5,7-trihydroxy-1,4-naphthaquinone, *cis*-4,6-dihydroxymellein and (−)-11-acetyldehydrocurvularin were isolated besides the known cynodontin, (−)-dehydrocurvularin, (+)-mellein and *cis*-3*S*,4*S*-4-hydroxymellein.

INTRODUCTION

Among plant pathogens, the genus *Cercospora* (Deuteromycetes) [1] is well known, as it includes several species responsible for leaf spot diseases of many plants, including some of economic importance, such as sugar beet [2] and soya beans [3]. The possible phytotoxic role played by secondary metabolites of *Cercospora* has been emphasized by Schlösser [2]. However, the first report of the production of a phytotoxin by *C. kikuchii* is due to Kuyama and Tamura [4], who isolated cercosporin. The structure of cercosporin (**1**) was elucidated a few years later [5], and its unusual stereochemical features clarified [6, 7]. Reports of the isolation of cercosporin from other *C.* species, namely *C. beticola* [8], *C. hayii* [9], *C. personata* [10] and *C. ricinella* [11] have also appeared. Recently, the biosynthesis of cercosporin has been studied [12], and its photodynamic and antibacterial activity demonstrated [13].

Due to the interest of cercosporin as a phytotoxin, to the possible synergism with other metabolites, and in the hope of finding new biologically active substances, we have undertaken the screening of a large number of *Cercospora* species for secondary metabolites.

RESULTS AND DISCUSSION

The results reported in Table 1 show that 23 of the 61 species of *Cercospora* examined produce cercosporin (**1**), which could so far be considered as a metabolite typical of the genus.

Besides cercosporin, *Cercospora setariae* produces four acetate and benzoate side chain esters (**2**–**5**). Their structure and configuration was easily established from NMR and MS, and by hydrolysis to cercosporin with sodium methoxide in methanol. In particular the monoacetate monobenzoate (**5**) was obtained as a crystalline compound suitable for X-ray analysis, which is now in progress [14].

All the other metabolites which have been isolated are of polyketide origin. *C. ligustrina* produces the new chromones ligustrone A, B and C (**6**–**8**), whereas *C. taiwanensis* yields mellein (**9**), *cis*-3*S*-4*S*-4-hydroxymellein (**10**), and taiwapyrone (**11**). The elucidation of these structures has been reported elsewhere [15, 16]. From *C. cari* cynodontin (1,4,5,8-tetrahydroxy-2-methylanthraquinone) was obtained, a known metabolite of some *Helminthosporium* sp. and of other fungi [17]. The major metabolites of seven species are the two epimers dothistromin (**12**) and 2-epidothistromin (**13**) [18]. It is noteworthy that dothistromin has been recently isolated from the fungus *Dothistroma pini*, and recognized as the phytotoxin responsible for the 'pine blight' disease [19]. A culture of *C. smilacis* showed also the presence of other anthraquinones, averufin and averythrin, which both are produced by *Dothistroma pini*.

A new metabolite (**14**) was obtained from *C. melonis*. The UV, NMR and MS data indicated that it was a trihydroxymethoxy-1,4-naphthaquinone. The presence in the NMR spectrum of signals from two *meta* aromatic hydrogens, which were shifted to lower field by acetylation and were not decoupled by irradiation of the methoxy protons and of only one chelated OH, restricted the choice to the two structures, 3-methoxy-2,5,7-trihydroxy-1,4-naphthaquinone (**14**) and 2-methoxy-3,5,7-trihydroxy-1,4-naphthaquinone (**18**). In order to distinguish between these two possibilities, the latter compound was prepared by selective demethylation of 2,3-dimethoxy-5,7-dihydroxy-1,4-naphthaquinone (**17**) with HCl in ethanol, [20], a reaction based on the different hydrolysis rates of the two methoxyls, the OH at C-3 being more acidic than that at C-2 in the parent tetrahydroxyquinone. As the product (**18**) appeared different from the metabolite

* Part 4 of a series on metabolites of *Cercospora*: for preceding papers see refs. [5], [15] and [16].

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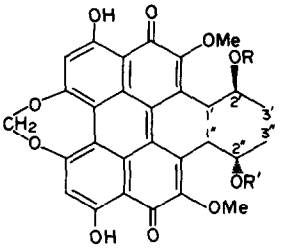
Table 1. Species of *Cercospora* examined for phytotoxins

	Species	Source*	Metabolites	
			Cercosporin (1)†	Others
1	*Cercospora aleuritidis*	CBS 281.62‡	−	
2	*Cercospora althaeina*	CBS 248.67	−	
3	*Cercospora ampelopsidis*	CBS 249.67	−	
4	*Cercospora angolensis*	CBS 149.53	−	
5	*Cercospora apii*	CBS 119.25	−	
6	*Cercospora ariminiensis*	CBS 137.56	+ +	
7	*Cercospora armoraciae*	CBS 250.67	−	
8	*Cercospora astragali*	CBS 537.71	−	
9	*Cercospora bellynchii*	CBS 150.49	−	
10	*Cercospora bertoreae*	CBS 538.71	+ +	CBT‖
11	*Cercospora beticola*	CBS 152.52	+ +	
12	*Cercospora beticola*	CBS 121.31	+ +	CBT
13	*Cercospora beticola*	IPV-F586‡	+ +	CBT
14	*Cercospora beticola*	IPV-F587‡	+ +	CBT
15	*Cercospora beticola*	IPV-F588‡	+ +	CBT
16	*Cercospora beticola*	IPV-F573§	−	CBT
17	*Cercospora bizzozeriana*	CBS 540.71	+	
18	*Cercospora bolleana*	CBS 541.71	−	
19	*Cercospora calotropidis*	CBS 129.30	−	
20	*Cercospora canescens*	CBS 153.55	+	
21	*Cercospora cantuariensis*	CBS 112.24	−	
22	*Cercospora cari*	CBS 148.52	−	cynodontin
23	*Cercospora carotae*	CBS 101.65	+ +	
24	*Cercospora chenopodii*	CBS 126.29	+	
25	*Cercospora cistinearum*	CBS 257.67	+ +	
26	*Cercospora cladosporioides*	CBS 159.48	+	
27	*Cercospora diazu*	CBS 138.28	+ +	
28	*Cercospora dulcamarae*	CBS 544.71	+	
29	*Cercospora erysimi*	CBS 545.71	+ +	
30	*Cercospora exosporioides*	CBS 751.70	−	
31	*Cercospora ferruginea*	CBS 546.71	−	dothistromin (12)
32	*Cercospora festucae*	CBS 143.51	−	
33	*Cercospora fusca*	CBS 106.14	−	dothistromin (12)
34	*Cercospora italica*	CBS 130.32	−	
35	*Cercospora kaki*	CBS 128.39	−	
36	*Cercospora kikuchii*	CBS 128.27	+ +	
37	*Cercospora kikuchii*	CBS 135.28	+ +	
38	*Cercospora ligustrina*	CBS 148.59	−	ligustrone A(6), B(7), C(8)
39	*Cercospora magnoliae*	CBS 541.63	−	
40	*Cercospora malvacearum*	CBS 126.26	+	
41	*Cercospora malvicola*	CBS 548.71	+	
42	*Cercospora medicaginis*	CBS 108.22	+ +	
43	*Cercospora melonis*	CBS 161.60	−	(14)
44	*Cercospora mercurialis*	CBS 551.71	−	
45	*Cercospora microsora*	CBS 552.71	−	dothistromin(12)
46	*Cercospora musae*	CBS 143.36	−	
47	*Cercospora nicotianae*	CBS 131.32	+	
48	*Cercospora olivascens*	CBS 253.67	−	
49	*Cercospora oryzae*	CBS 145.37	+	
50	*Cercospora personata*	CBS 220.31	+ +	
51	*Cercospora plantaginis*	CBS 252.67	+	
52	*Cercospora plumbaginea*	CBS 553.71	—	
53	*Cercospora psoraleae-bituminosae*	CBS 554.71	−	
54	*Cercospora rautensis*	CBS 555.71	−	
55	*Cercospora rhapisicola*	CBS 282.66	−	
56	*Cercospora rosicola*	CBS 138.35	−	dothistromin(12)¶
57	*Cercospora rubi*	CBS 256.35	−	dothistromin(12)
58	*Cercospora salina*	CBS 141.60	−	
59	*Cercospora scirpicola*	CBS 104.40	−	(19), (20), (22)
60	*Cercospora setariae*	CBS 494.71	+	(2), (3), (4), (5)
61	*Cercospora smilacis*	CBS 556.71	−	dothistromin(12,13), averufin and averythrin
62	*Cercospora taiwanensis*	CBS 139.35	−	mellein(9), 4-hydroxymellein(10), taiwapyrone(11)
63	*Cercospora unamunoi*	CBS 156.62	+	
64	*Cercospora vaginae*	CBS 140.34	−	¶
65	*Cercospora violae*	CBS 151.49	+	
66	*Cercospora zebrina*	CBS 129.39	−	
67	*Cercospora zonata*	CBS 557.71	−	

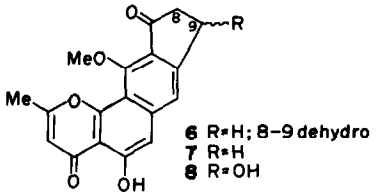
* CBS = Centraal Bureau voor Schimmelcultures, Baarn, Netherlands. IPV = Istituto di Patologia Vegetale, Università di Milano, Italy. † +, + + shows qualitatively the production of cercosporin. ‡ From prof. V. D'Ambra. Università di Padova, Italy. § From prof. E. Schlösser, Institut für Pflanzenkrankheiten der Universität, Bonn, Germany. ‖ CBT = *Cercospora beticola* toxin. ¶ Structural work on other metabolites is in progress.

of *C. melonis* on TLC, the structure (**14**) was attributed to this latter.

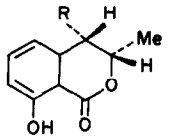


1 R=H ; R'=H
2 R=COMe ; R'=COMe
3 R=COMe ; R'=H
4 R=COPh ; R'=COPh
5 R=COPh ; R'=COMe

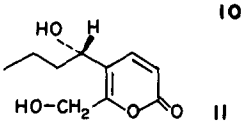
The macrolide dehydrocurvularin (**19**), a known metabolite of *Curvularia* sp. [21] was isolated from *C. scirpicola*, together with a new compound (**20**). The structure of **19** was easily established by comparison of spectral data and optical rotation with those of the literature



6 R=H; 8–9 dehydro
7 R=H
8 R=OH



9 R=H
10 R=OH



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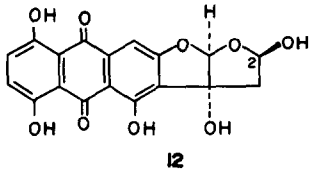

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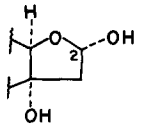

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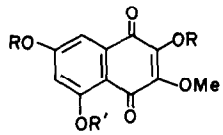
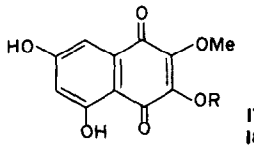


14 R=H; R'=H
15 R=Ac; R'=Ac
16 R=Me; R'=H

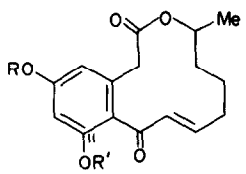
[22]. Spectral data for **20** and conversion of both **19** and **20** to the same diacetate (**21**) indicated that **20** must be a monoacetate of **19**. The presence of a MeCO group and the lack of a chelated OH in the NMR spectrum of **20** led



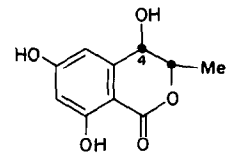
17 R=Me
18 R=H

us to attribute to this compound the structure of 11-*O*-acetyldehydrocurvularin (**20**). A small amount of another metabolite was also obtained, the NMR and MS of which, compared with those of **9** and **10**, indicated that it is the new *cis*-4,6-dihydroxymellein (**22**).

The extracts from a few strains of *C. beticola* and *C. bertoreae* contain a yellow substance, that is named CBT (*Cercospora beticola* toxin) in Table 1. The presence of this metabolite was previously reported by Schlösser, who named and partially characterized it, and studied its phytotoxic and antibiotic activity [23]. We have found CBT in the mycelia of some strains of *C. beticola* isolated from infected sugarbeets, and also in that of the original strain of Dr. Schlösser, kindly provided by him.



19 R=H; R'=H
20 R=H; R'=Ac
21 R=Ac; R'=Ac



22

Although the substance is rather unstable, we have been able to obtain it in a reasonably pure state. Therefore the allegation of Balis and Payne [8] that it is a mixture of cercosporin and of fatty acids, is wrong. Structural work on CBT is in progress in our laboratories. The substance is also of interest, as it appears together with cercosporin in a few active strains, and preliminary experiments could suggest a possible synergism in the phytotoxic activity of both compounds. Further investigations on this particular subject are also in progress.

EXPERIMENTAL

Mp's are uncorrected UV spectra were measured in 95% EtOH. NMR spectra were recorded at 100 MHz, chemical shifts are in pp, (δ), from TMS as internal standard. Column chromatography and TLC were performed with Si gel. Unless otherwise indicated the purity of the products was checked by TLC, NMR and MS and deemed sufficient for the purposes of structural elucidation.

Materials and methods. The 61 species of *Cercospora* which have been examined are reported in Table 1. Most of them were obtained from the Centraal Bureau voor Schimmelcultures, Baarn, Holland. A survey of the different factors (culture medium, temperature, pH, light, addition of specific substances, etc.) influencing the growth led us to choose the following standard conditions for the cultures to be screened. The strains were cultivated on potato-agar medium, obtained by boiling 200 g potatoes for 30 min, filtering, adding 20 g glucose and 13 g agar, diluting to 1 l. and adjusting the pH to 6.5–6.8, in Roux flasks at 22–24°, containing 100 ml each of the culture medium. After a growth period of 15–20 days, the content of each flask was extracted twice with 100 ml EtOAc, and the extract dried on Na_2SO_4 and evaporated *in vacuo* at 40°. The metabolites were isolated by PLC or column chromatography on Si gel. It was observed that all strains showed better production on media containing agar than in liquid media. For some species (6, 21, 24, 27, 40, 42, 65) the addition of 2% of yeast and for others (11, 12, 13, 14, 15, 16, 36, 42, 47, 49) of a further 10% of glucose to the standard medium increased the production of metabolites, whereas addition of biotin, thiamine or β-alanine [9] had no effect. Ferrous salts apparently induced a deeper pigmentation, but no systematic investigation was carried out.

Cercospora setariae. Extraction with EtOAc of 35 Roux flasks gave after evaporation of the solvent, 0.5 g of crude products; by PLC with C_6H_6–Et_2O–formic acid (50:50:1) 5 metabolites were obtained and identified as follows. *Cercosporin* (**1**, 20 mg).

2',2''-diacetylcercosporin (**2**). 45 mg of a red powder, mp 80–82°. (Found: C, 63.17; H, 4.50. $C_{33}H_{30}O_{12}$ requires: C, 64.07; H, 4.89%); MS 618; UV λ_{max}(nm): 290, 297*sh*, 485, 600 and 640 (ε 32700, 32400, 21800, 8600, 12000); IR ν_{max}^{Nujol} cm^{-1}: 1740 (Ac), 1620 (conj. CO); CD (in EtOH, *c* 2.1 × 10^{-2} g/100 ml): 246, 297, 320, 352 and 410 nm (Δε + 23.5, −43.5, −7.95, +2.94, −7.35); NMR ($CDCl_3$): δ 0.55 (*d*, 2 Me; *J* = 6 Hz), 1.70 (2 Ac), 3.73 and 3.0 (*m*, 2 —$\underline{CH_2}$—CH—O), 4.28 (2 OMe), 4.68 (*m*, 2—CH_2—$\underline{CH}$—O), 5.8 (*s*, O—$\underline{CH_2}$—O), 7.04 (*s*, 2 arom. H), 14.76 (2 chel. OH).

2'-Acetylcercosporin (**3**). 15 mg yield with mp 133–134°; MS 576; UV λ_{max} (nm): 253, 291, 299*sh*, 480, 590*sh* and 640 (ε 15500, 23000, 23300, 14100, 5000, 6200); IR ν_{max}^{Nujol} cm^{-1}. 3450 (OH), 1740 (Ac), 1620 (conj. CO); CD (in EtOH, *c* 0.9 × 10^{-2} g/100 ml): 240, 297, 352 and 410 nm (Δε + 23.6, −42.2, +1.92, −8); NMR ($CDCl_3$); δ 0.56 and 0.64 (*d*, 2 Me), 1.66 (Ac), 2.80 − 3.80 (*m*, —CH_2—$\underline{CH}$—O and 2 —$\underline{CH_2}$—CH—O), 4.24 and 4.22 (OMe), 4.64 (*m*, —CH_2—$\underline{CH}$—OAc), 5.76 (*s*, O—$\underline{CH_2}$—O), 7.04 (*s*, 2 arom. H), 14.82 and 14.76 (2 chel. OH).

2',2''-Dibenzoylcercosporin(**4**). 5 mg of a red solid, mp 120–123 : (Found: C, 68.76; H, 4.60. $C_{43}H_{34}O_{12}$ requires: C, 69.6: H. 4.62%); MS 742; UV λ_{max} (nm): 224, 270, 475 and 565 (ε 36700, 16300, 11800, 4300); IR ν_{max}^{neat} cm^{-1}: 1715 (OCOPh), 1620 (conj. CO); CD (in EtOH, *c* 1.23 × 10^{-2} g/100 ml): 240, 297, 318 and 410 nm (Δε + 21, −24.2, −6.95, −3.62); NMR ($CDCl_3$): 0.83 (*d*, 2 Me), 3.40–3.86 (*m*, 2 —$\underline{CH_2}$—CH—O), 4.32 (2 OMe), 5.0–5.2 (*m*, 2 —CH_2—$\underline{CH}$—OCOPh), 5.62 (*s*, O—$\underline{CH_2}$—O), 6.80 (*s*, 2 arom. H), 7.0–7.40 (10 arom.), 14.70 (2 chel. OH).

2'-Acetyl-2''-benzoylcercosporin (**5**). 25 mg, red crystals mp 153–155°; (Found: C, 67.00; H, 4.74. $C_{38}H_{32}O_{12}$ requires: C, 67.05; H, 4.75%); MS 680; UV λ_{max} (nm). 223, 273, 380*sh*, 480, 570 and 620 (ε 41500, 24400, 4550, 18700, 5850, 4000); IR ν_{max}^{Nujol} cm^{-1}: 1740 (Ac), 1720 (OCOPh), 1620 (conj. CO); CD (in EtOH, *c* 3.21 × 10^{-2} g/100 ml): 240, 298, 320, 360 and 410 nm (Δε + 16.2, −20, −4.86, +0.42, −3.82); NMR ($CDCl_3$). δ 0.53 and 0.82 (*d*, Me), 1.61 (Ac), 2.92–3.88 (*m*, 2 —$\underline{CH_2}$—CH—O), 4.28 and 4.30 (OMe), 4.65 (*m*, CH_2—$\underline{CH}$—OAc), 5.07 (*m*, CH_2—$\underline{CH}$—OCOPh), 5.70 (*d*, O—$\underline{CH_2}$—O; *J* 7 Hz), 6.85 and 7.06 (*s*, 2 aromatic protons), 7.16–7.50 (C_6H_5), 14.66 and 14.80 (2 chel. OH).

Hydrolysis of the esters (**2–5**). To 5 mg (**2–5** dissolved in MeOH, were added 10 mg MeONa, at room temp. for 1 day; all the products after acidification, gave (**1**), identified by TLC comparison.

Cynodontin. A strain of *Cercospora cari* (5 flasks), gave, after PLC a few mg of cynodontin; it was identified by TLC and mp comparison with an authentic sample and by MS of the tetraacetate (Py and Ac_2O), *m/e*: 454, 412, 370, 328, 286, 257, 229.

Cercospora melonis. *Isolation and identification of metabolite* **14**. A strain of *C. melonis* was grown on potato-agar in the usual manner for 1 month. Mycelia were crushed and extracted twice with EtOAc. The less polar compound **14** was separated from the crude mixture by PLC in C_6H_6–Et_2O–formic acid (50/50/1).

2,5,7-*Trihydroxy*-3-*methoxy*-1,4-*naphthaquinone* (**14**). Mp 255° (dec.); (Found: C, 55.16; H, 3.73. $C_{11}H_8O_6$ requires: C, 55.94; H, 3.41%); MS *m/e* (rel. int): 236 (100), 207 (8), 190 (20), 179 (10), 165 (42), 137 (27), 121 (34); UV λ_{max} (nm): 225*sh*, 269.5, 320, 384 and 470*sh* (ε 12600, 15300, 6550, 3140, 1200), basic EtOH 227, 291, 374 and 580 (ε 23200, 24400, 5600, 1750); IR ν_{max}^{KBr} (cm^{-1}: 3360 (OH), 1650 (conj. CO) and 1615, NMR (DMSO): δ 3.88 (OMe), 6.94 and 6.50 (*d*, 2 arom. *m*-H; *J* 2.5 Hz), 12.2 (chel OH).

2,5,7-*Triacetoxy*-3-*methoxy*-1,4-*naphthaquinone* (**15**). 20 mg **14** in 0.2 ml of dry C_5H_5N and 0.4 ml Ac_2O were left overnight at room temp. Dilution with H_2O, extraction with Et_2O gave **15** as a yellow solid mp 145–147° (Et_2O); IR ν_{max}^{KBr} cm^{-1}: 1780 (Ac), 1680 (conj. CO), 1630 and 1600, NMR ($CDCl_3$): δ 2.43, 2.38 and 2.33 (Ac), 4.15 (OMe), 7.80 and 7.21 (*d*, 2 arom. *m*-H).

5-*Hydroxy*-2,3,7-*trimethoxy*-1,4-*naphthaquinone* (**16**). 10 mg **14**, dissolved in MeOH, were treated with an ethereal soln of CH_2N_2; evap gave orange crystals of **16**, mp 90–92°; MS 264; UV λ_{max} (nm): 266, 312, 390 and 430*sh* (ε 20000; 10700, 3000, 2940), NMR (acetone–d_6): δ 4.08, 4.07 and 3.95 (OMe), 7.03 and 6.66 (*d*, 2 arom. *m*-H), 12.13 (OH). Compound **17**; NMR (acetone–d_6): δ 4.70 and 4.50 (OMe), 7.03 and 6.56 (*d*, arom. *m*-H), 12.10 (OH). Compound **18**; NMR (acetone–d_6): δ 4.0 (OMe), 7.08 and 6.55 (*d*, arom. *m*-H), 11.64 (OH). The compound **18** is different from **14** as shown by the NMR spectrum of the mixture and by TLC in three solvents.

Cercospora scirpicola. *Identification of metabolites.* A strain of *C. scirpicola* was grown as usual for 3 weeks; the crude extract was separated by PLC in C_6H_6–Et_2O–formic acid (50:50:1) as eluent; 3 main products were obtained: the less polar one was *dehydrocurvularin* (**19**), mp 224°, identified by MS and NMR spectra. The more polar compound was 11-*O-acetyldehydrocurvularin* (**20**), mp 105–110°; $[\alpha]_D^{20}$ −31.7° (in MeOH, *c* 0.4); UV λ_{max} (nm): 222, 232*sh*, 275 and 308 (ε 10200, 8800, 5800, 4900); NMR (acetone–d_6/DMSO–d_6). δ 1.10 (*d*, Me; *J* 6 Hz), 1.2 2.1 (6 aliphatic protons), 2.09 (Ac), 3.74 (*q*, Ar—$\underline{CH_2}$—CO), 4.94 (*m*, O—$\underline{CH}$—Me), 6.26–6.48 (2 aromatic and 2 vinylic protons). 5 mg of **20** were acetylated with Py and Ac_2O at room temp. for 12 hr., to give the diacetate (**21**) identical to the product obtained by acetylation of **19**. The third compound was 4,6-*dihydroxymellein* (**22**), white solid, mp 183–185°; (Found: *m/e* 210.0566 ± 0.004. $C_{10}H_{10}O_5$ requires M, 210.0528), MS *m/e*. 210, 192, 177, 166, 150, 137, 121; UV λ_{max} (nm): 223*sh*, 267 and 302 (ε 12600, 10700, 5800); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1670 (CO), NMR (acetone–d_6): δ 1.45 (*d*, Me), 4.56 (H-4), 4.67 (H-3; $J_{3,4}$ = 2, $J_{3,Me}$ 6 Hz), 6.33 and 6.49 (*d*, 2 arom. *m*-H; *J* 2.5 Hz), 11.26 (chel. OH).

Cercospora beticola *toxin.* (CBT) A strain of *C. beticola* (IPV-F 573), was grown as usual. Mycelia were extracted twice with EtOAc. Extracts were adsorbed on the top of a chromatographic column and eluted with a mixture of $CHCl_3$ and MeOH. CBT was obtained with $CHCl_3$–MeOH 9:1. Yellow solid, mp > 300° (dec.); $[\alpha]_D^{20}$ + 326° (in MeOH, *c* 0.16); UV λ_{max} (nm) 344, 435 and 455*sh*; IR ν_{max} cm^{-1}: 3450 (broad OH), 1730 (CO), 1610 (conj. CO), 1450; a hydrogenation product (Pd 10% on $BaSO_4$) had M^+ 640.

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