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DIHYDROISOCOUMARINS FROM FUNGI: ISOLATION, STRUCTURE ELUCIDATION, CIRCULAR DICHROISM AND BIOLOGICAL ACTIVITY*

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Key Word Index—*Pezicula livida*; *Plectophomella*; *Cryptosporiopsis*; fungal metabolites; melleins; circular dichroism; X-ray; absolute configuration; Baker's yeast.

Abstract—Five known and three new dihydroisocoumarins were isolated from different fungi. The new isocoumarins are 5-chloro-6-hydroxymellein, 5-chloro-4,6-dihydroxymellein and 5,6-dihydroxymellein. The absolute configuration of these secondary metabolites was confirmed by CD measurements and in two cases by X-ray structure analysis. © 1997 Published by Elsevier Science Ltd. All rights reserved

INTRODUCTION

As part of a program directed towards the isolation of biologically active metabolites from fungi [1], we screened different endophytic and soil fungi for the production of pentaketide derived dihydroisocoumarins. This work is in close connection with ongoing investigations to establish a chemotaxonomic basis for classifying endophytic fungi [2, 3]. The classification relies on the distribution of these and related ketide metabolites among the fungal taxa. It is noteworthy that some of the mellein derivatives are produced in substantial amounts facilitating their use for chemotaxonomic purposes. A further matter of prime interest was the absolute configuration of the isolated dihydroisocoumarins, since in some cases the enantiomeric form of the commonly occurring (*R*)-enantiomer was isolated [4, 5].

For that purpose five natural products (**1c**, **1e**, **2**–**4**), and one synthetic derivative **8**, were selected for CD measurements to elucidate their absolute configurations. An additional confirmation by X-ray analysis was obtained for the chlorine containing compound **2**. The biological activity arouses new interest following the isolation of dihydroisocoumarins as pheromones from ants [6]. A comparison of the biological activity of the metabolites using a number of test systems is included.

RESULTS

(−)-Mellein (**1a**) is a widely distributed dihydroisocoumarine derivative in fungi [7–18]. It was isolated by our group from culture broths of the endopytic fungi *Pezicula livida*, *Plectophomella* sp., *Cryptosporiopsis malicorticis* and *Cryptosporiopsis* sp.; 5-methylmellein (**1b**) [11, 19–21] was isolated from the soil isolate *Idriella bambusae* and from the endophyte *Phomopsis* sp.; 6-hydroxymellein (**1c**) [22–25] from the endophytes *Plectophomella* sp. and *Cryptosporiopsis* sp.; 5-chloro-6-methoxymellein (**1d**) [7, 26] and 6-methoxymellein (**1e**) from the soil isolate *Coniothyrium* sp. (for reviews on natural dihydroisocoumarins see [27–30]).

In addition to the known melleins **1a**–**1e**, we isolated the two unknown natural dihydroisocoumarins **2** and **3** from *Plectophomella* sp. The endophytic fungus *Plectophomella* sp. (Coelomycetes, Fungi imperfectii) was isolated from an unidentified tree in Lower Saxony by Prof. H. Butin (BBA, Braunschweig). Compound **2** has been suggested as an intermediate in the biosynthesis [31] of pentanoid secondary metabolites and is already known as a synthetic product [32]. A third unknown compound **4** was isolated from *Cryptosporiopsis* sp., which had been isolated as an endophyte from a spruce tree growing in a forest in

* Part 8 in the series 'Biologically Active Metabolites from Fungi'. For part 7 see ref. [17].

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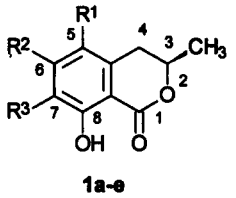
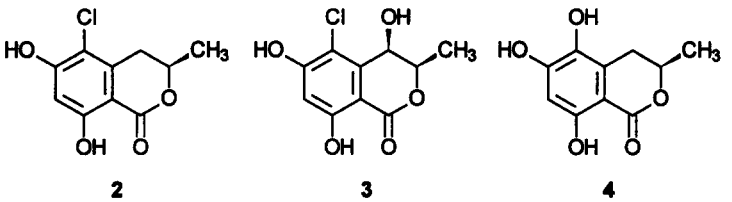


	R1	R2	R3
1a	H	H	H
1b	CH_3	H	H
1c	H	OH	H
1d	H	OCH_3	Cl
1e	H	OCH_3	H

1a-e



2 **3** **4**

Lower Saxony, Germany. *Cryptosporiopsis* is the anamorphic genus of the teleomorphic genus, *Pezicula*, (Ascomycetes, Helotiales). All compounds were separated and purified from fungal culture broth by a combination of column and layer chromatography followed by crystallization (see Experimental).

Structure determination

The structure elucidation relied on a combination of spectroscopic methods, especially NMR and in the cases of **1b** and **2** on X-ray structure analysis. The absolute configuration of **2** was confirmed by CD measurement (see below). The isocoumarins isolated can be regarded as closely related derivatives of mellein (8-hydroxydihydroisocoumarin **1a**) differing only in the substitution pattern and showing characteristic similarities in the IR, UV and NMR spectra. All compounds have absorption bands at 1640–1660 cm^{-1} and 3450–3460 cm^{-1} in the IR spectra typical for aromatic chelated carbonyl and hydroxyl groups, respectively. The UV spectra depended on the substitution pattern of the aromatic ring, but the di- and tri-hydroxylated compounds **2–4** showed a typical absorption at *ca* λ_{max} = 315–330 nm. The chelation of the phenolic hydroxy group was confirmed by lowfield signals in the ^1H NMR spectra at *ca* δ = 11.5 and by signals at about δ = 170 in the ^{13}C NMR spectra, indicating carboxyl groups. Further typical ^1H NMR signals could be detected as spin systems of a methine proton at δ = 4.5–4.8 with a methyl group at 1.3–1.5 ppm and the methylene protons at C-4 (except for **3**) at about δ = 2.8 and 3.3. The nature of the substituents in **1b–4** were elucidated by a combination of the number of carbon atoms (from the ^{13}C NMR spectra, 11 for the methyl or methoxy derivatives) and the molecular mass. The location at the aromatic ring was confirmed by the simple spin systems in the ^1H NMR spectra; the assignment for **1b** was confirmed by the X-ray structure of single crystals of 5-methylmellein shown in Fig. 1.

The new fungal metabolites **2** and **3** have very similar structures. The mass spectrum (ΔM 16) showed one additional oxygen atom for **3** which could be

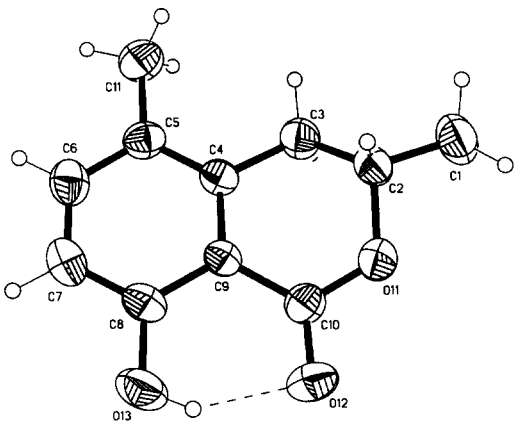


Fig. 1. The molecule of 5-methylmellein (**1b**) in the crystal.

located at C-4 in the ^1H NMR spectrum. According to a comparison of the literature for the related 4-hydroxymelleins [12], the downfield signal for H-4 at δ = 4.83 showed a coupling constant of 1.8 Hz indicating a *cis*-relationship with the neighbouring methyl group. In addition, the chemical shift of δ = 1.52 for the methyl protons are in good agreement with the literature value (δ = 1.63) given for the *cis*-configuration [33]. The coupling constant $J_{3,4}$ and the chemical shift of the methyl group are also in the same order of magnitude as determined for the corresponding 4-chloro-or 4-bromomellein derivatives [18]. The presence of a chlorine substituent for both compounds **2** and **3** is shown by the characteristic molecular ion pattern in the mass spectra and the additional non-chelated hydroxy groups at C-6 by broad signals at δ = 10.15 (**2**) and 10.13 ppm (**3**) in the ^1H NMR spectra. The substitution pattern with *meta*-position for the oxygen atoms is in agreement with biosynthetic considerations [proton signals for 7-H at δ = 6.49 (**2**) and 6.47 (**3**) in the ^1H NMR spectra] and was confirmed by an X-ray structure analysis of single crystals of compound **2** shown in Fig. 2, confirming the structures of 5-chloro-6-hydroxymellein (**2**) and 5-chloro-4,6-dihydroxymellein (**3**). Compound **2** is related to the 3,4-dehydrogenated compound 5-chloro-6,8-dihydroxy-3-methylisocou-

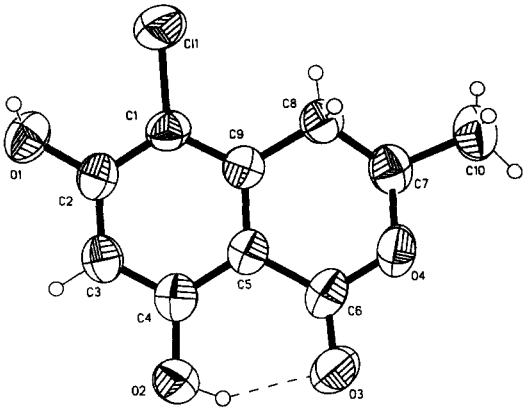


Fig. 2. The molecule of 5-chloro-6-hydroxymellein (**2**) in the crystal.

marin isolated from the fungus *Periconia macrospinosa* [31–35].

The third new mellein has the same substitution pattern as **2**. A molecular formula of $C_{10}H_{10}O_5$ can be deduced by a combination of the data from NMR and the mass spectra (M = 210) and elemental analysis. The chlorine atom in **2** is replaced by a hydroxy group and the new compound can be assigned as 5,6-dihydroxymellein (**4**).

Determination of the absolute configuration of dihydroisocoumarins 2–4

Dihydroisocoumarins possess a benzoic ester chromophore, whose chiroptical properties have been systematically investigated [36]. It was found that the sign of the Cotton effect of $n\rightarrow\pi^*$ origin could be quite safely used for establishing the absolute conformation of the heteroring of this chromophore system, because the sign of this type of Cotton effect is independent of the substitution pattern of the aromatic ring system [37, 38]. The CD measurements of mellein derivatives (**1c**, **2**–**4**), and the synthetic product **8** (see below) were carried out in the UV absorption region in solvents of different polarity. The CD data are shown in Fig. 3 and are given in the Experimental.

In order to assign the Cotton effect of $n\rightarrow\pi^*$ origin, (*R*)-(−)-6-hydroxymellein (**1c**) has been selected as the most suitable reference compound. Its CD spectra were studied in the range from 220 to 320 nm and compared to those of (−)-**2**-(−)-**4** and (+)-**8**.

In the CD spectra of **1c** four Cotton effects were observed in ethanol. The band of negative sign around 270 nm could be unequivocally assigned to the $n\rightarrow\pi^*$ transition of the carbonyl group on the basis of its characteristic hypsochrome shift in *n*-hexane. Accord-

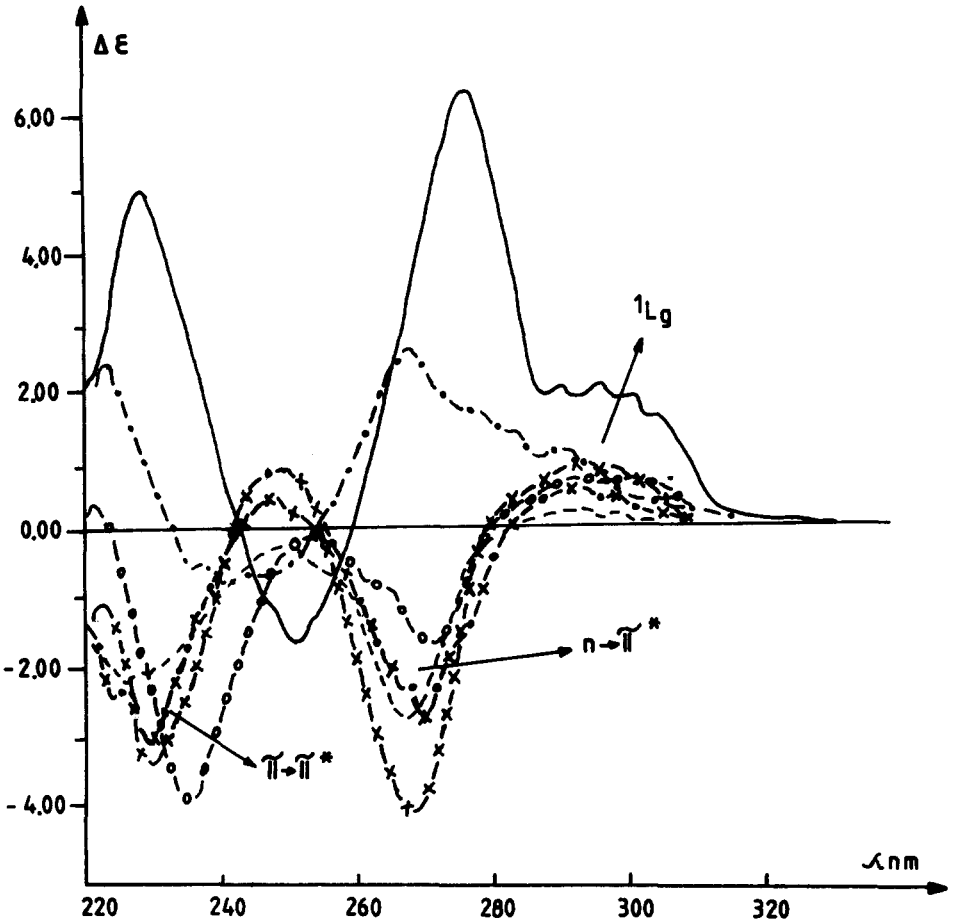
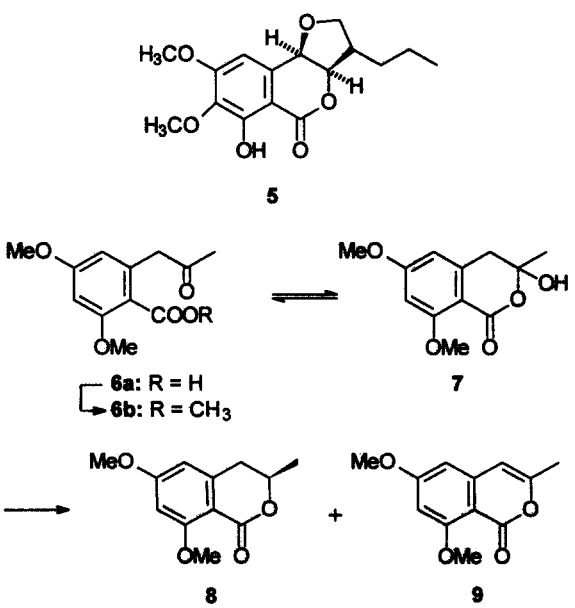


Fig. 3. CD curves of compounds (−)-**1c**, (−)-**2c** and (+)-**8** in ethanol (-●-∗-/○○/-) and in *n*-hexane (-∗-/--/-●-●-), respectively.

Fig. 4. Standard projection from the aromatic into the heterocyclic ring of (3*R*)-(−)-**1c**, (−)-**2**, (+)-**3** and (−)-**4** (half-chair and boat conformation, left and right, respectively).

ing to the helicity rule of the chiral benzoic ester chromophore based on the sign of the $n\rightarrow\pi^*$ band of conformationally fixed dihydrocoumarin derivatives [36], the heterocyclic ring of (−)-**1c** must adopt a half-chair or a chair conformation as illustrated in Fig. 4, in which the methyl group at C-3 is oriented equatorially. Therefore the 3*R* absolute configuration could also be deduced from its CD data. The high similarity of the CD spectra of (*R*)-(−)-**1c** to that of (−)-**2** both in ethanol and in *n*-hexane has clearly shown that they are homochiral compounds of *R* absolute configuration. It is noteworthy that much larger absolute values of the $n\rightarrow\pi^*$ band of **1c** and **2** were found in *n*-hexane than in ethanol. This clearly demonstrated the solvent-dependence of the conformation of the heteroring. According to a theoretical calculation of the correlation between the deviation from the coplanarity of an C = C-C = O chromophore [39], one can assume that the half-chair population of **1c** and **2** is higher in *n*-hexane than in ethanol due to the characteristically different polarity and solvatation of these solvents.

Although the CD spectra of (+)-**3** and (−)-**4** could not be measured in *n*-hexane due to their poor solubility, we can conclude their homochirality by simple comparison of their CD data in ethanol with those of (*R*)-(−)-**2**. Since a negative sign was found for the $n\rightarrow\pi^*$ Cotton effect of (+)-**3** [265 nm (5.40)], its heteroring must also adopt the same conformations as (−)-**2**, depicted in Fig. 4, if the attached methyl group is oriented equatorially. Taking into account its *cis*-relationship with the neighbouring hydroxyl group, the absolute configuration of (+)-**3** is therefore 3*R*, 4*S*. It is interesting to note that the homochiral analogue, monocerin (**5**), also possesses a positive optical rotation of $[\alpha]_D = +53°$. The comparison of the CD data of (+)-**8** with those of (−)-**2**–(−)-**4** has clearly shown that they are not homochiral compounds and (+)-**8** possesses (3*S*)-absolute configuration.



5

6a: R = H
6b: R = CH_3

7

8 **9**

Enantioselective synthesis

For the determination of the absolute configuration of the new isocoumarins (−)-**2**–(−)-**4** using CD measurements (see above) the availability of both dihydroisocoumarin enantiomers was desirable. Therefore, a number of different reagents was tested in the reduction of the easily available prochiral ketoacid **6a** and the ketoester **6b**. The ketoacid was prepared according to a procedure of Staunton *et al.* [40]. In solution ($CDCl_3$) the open chain ketoacid **6a** is in equilibrium with the cyclic hemiacetalic form **7** (81:19 by ^1H NMR). Treatment of the tautomeric mixture **6a**/**7** in methanol with diazomethane afforded the corresponding ester **6b** in 93% yield.

Both the acid **6a** and the ester **6b** were used in a variety of reduction experiments for conversion to the isocoumarin **8**. The results are summarized in Table 1. Treatment of the acid **6a** with an excess of sodium borohydride followed by acidic workup afforded the racemic dihydroisocoumarin *rac*-**8** in addition to the isocoumarin **9**. Next, we tried a number of enantioselective reduction reagents. No reaction was observed with Midlands Alpine-borane [41], while the more reactive diisopinocamphenyl-borane (Ipc_2BCl) [42] reduced the acid **6a** and the ester **6b** to the dihydroisocoumarin (*R*)-**8** (41 and 62% enantiomeric excess (ee), respectively). Very high ee values are reported using enzymes in conjunction with NAD or NADP. In our case reactions with horse liver alcohol dehydrogenase (HLADH) [43] or an alcohol dehydrogenase from the thermostable bacterium *Thermoanaerobium brokii* (TBADH) [44] were not successful. The dimethylether **6a** was also not reduced by the *Coniothyrium* strain which produced the 6-methoxymellein **1e**. However, very good enantioselectivities were finally achieved using baker's yeast (*Saccharomyces cerevisiae*). This organism was extensively used in the reduction of a variety of carbonyl com-

Table 1. Reduction of **6a**/**6b** with different reducing agents

Compound	Reagent	Yield (%)	$[\alpha]_D$	Config.	ee
6a	$NaBH_4$	90	—	*rac*-8	—
6a	IPC_2BCl	45	−60	(*R*)-8	41
6b	IPC_2BCl	27	−93	(*R*)-8	62
6b	Baker's yeast	25	+154	(*S*)-8	>99

Table 2. Fungicidal, antibacterial and algicidal activity of melleins (**1a–4** and **8**) in agar diffusion tests

		Radius of the zone of inhibition zone (mm)						
Compound	mg ml^{-1}	B.m.	E.c.	U.v.	M.m.	E.r.	F.o.	C.f.
1a	18	2	2	8	0	6	3	14
1b	24.5	0	0	3	0	0	—	5
1c	5	0	0	2	0	0	0	6
1e	10	0	0	5	4	2	3	4
2	10	0	0	2	2	0	0	2
4	10	0	1	2	0	0	—	4
rac-**8**	10	0	0	3	0	1	—	15
(*S*)-**8**	10	0	0	2	2	0	—	8
(*R*)-**8**	10	0	0	2	2	2	—	14

Concentration: 50 μl of the given mg ml^{-1} solution; test organisms: B.m. = *Bacillus megaterium*, E.c. = *Escherichia coli*, U.v. = *Ustilago violacea*, M.m. = *Mycotypha microspora*, E.r. = *Eurotium repens*, F.o. = *Fusarium oxysporum*, C.f. = *Chlorella fusca*, — = not tested.

pounds [45]. Good enantioselectivities are often obtained and the (*S*)-alcohol is normally produced in excess, although the reductases present in this whole cell system show different substrate selectivities. Fortunately, ester **6b** was converted to enantiomerically pure (>99% ee) dihydroisocoumarin (*S*)-**8**, albeit with long reaction times (42 d) and at low conversion (25%) (see Table 1). As shown by the CD measurements (see above) the absolute configuration of this synthetic derivative was opposite to that of the natural products **2–4**.

Biological activity

The mellein derivatives **1a–4** generally showed only weak bioactivity against a variety of test organisms as shown in Table 2. It was interesting to compare the biological activities of the synthetic dihydroisocoumarins **8** of opposite configuration [*rac*-**8**; (*R*)-**8** (62% ee), (*S*)-**8**]. In accordance with the data on the natural products no activity against Gram-positive (*Bacillus megaterium*) or Gram-negative bacteria (*Escherichia coli*) and only weak activity against fungi were observed. However, the growth of *Chlorella fusca* and alfalfa (*Medicago sativa*, not shown) were inhibited and there was no difference between the racemic or optically active materials.

EXPERIMENTAL

For general remarks and instrumentation see ref. [46]. CD spectra were recorded on a Jobin-yvon dichrograph-VI at 20°C at concentrations of 12 mmol l^{-1} in cells of 0.050 × 20 cm. The ee values were determined by ^{1}H NMR using optically active shift reagents and the absolute configuration was checked by optical rotation.

Culture was in liquid medium unless otherwise noted. MPY-medium: 20 g l malt extract^{-1}, 2.5 g l yeast extract^{-1}, 2.5 g l peptone^{-1}. CS-medium: 1 g l yeast extract^{-1}, 4 g l glucose^{-1}, 1 g l standard-I-nutrition bouillon (Merck, 7882). SM-medium: see ref. [47]. S-medium: 50 g l glucose^{-1}, 20 g l yeast extract^{-1}. Biomalt medium: 50 g l biomalt^{-1} (Vitaborn, Hameln, Germany). For semi-solid media, 3 g l agar^{-1} were added.

Crystal structure determination of **1b**. Further details of the crystal structure determination are available on request from the Fachinformationszentrum Karlsruhe, Gesellschaft für wissenschaftlich-technische Information mbH, D-76344 Eggenstein-Leopoldshafen, on quoting the depository number CSD-404000 (**1b**) and CSD 404001 (**2**), the names of the authors and the journal citation. $C_{11}H_{12}O_3$, M_r = 192.2, monoclinic, space group P 2_1, a = 710.0(1), b = 1616.2(4), c = 837.3(2) pm, β = 93.98(1)°, V = 958.5 10^6 pm^3, Z = 4, D_r = 1.332 g cm^{-3}, F(000) = 408, T = 296(1)K. Siemens R3m diffractometer, graphite monochromator, λ(MoKα) = 71.073 pm, μ = 0.10 mm^{-1}, colourless crystal, size 0.18 × 0.25 × 0.34 mm, $\omega - 2\theta$ scan, 4439 intensities collected $3 < 2\theta < 55°$, $0 < h < 9$, $0 < k < 21$, $-10 < l < 10$ and Friedel equivalents, 3 standards every 400 reflections showed 7% decrease, intensities corrected accordingly, Lp correction, 3983 unique intensities (R_{int} = 0.026), 2298 with $F > 4\sigma(F)$. Structure solved by direct methods [48], full-matrix least-squares refinement based on F^2 and 260 parameters [49], all but H atoms refined anisotropically, H atoms located from difference Fourier maps and refined with riding model on idealized positions, 2 independent and geometrically equal molecules per asymmetric unit, refinement converged at R1(F) = 0.057, wR2(F^2, all data) = 0.141, S = 1.027, max(Δ/σ) < 0.001, min/max height in final ΔF map −0.14/0.17 e/Å^3. The absolute structure could not be determined reliably. Figure 1 shows the molecular structure.

Crystal structure determination of **2**. $C_{10}H_9ClO_4$, M_r = 228.6, orthorhombic, space group P $2_12_12_1$,

a = 670.4(4), b = 836.4(2), c = 3579.5(10) pm, V = 2007.1 10^6 pm^3, Z = 8, D_r = 1.513 g cm^{-3}, F(000) = 944, T = 296(1)K. Diffractometer and data collection as for **1b**, μ = 0.37 mm^{-1}, colourless crystal, size 0.22 × 0.30 × 0.41 mm, 4055 intensities collected $3 < 2\theta < 50°$, $0 < h < 7$, $0 < k < 9$, $0 < l < 42$ and Friedel equivalents, 3 standards every 400 reflections showed only random deviations, Lp correction, 3478 unique intensities (R_{int} = 0.046), 2424 with $F > 4\sigma(F)$. Structure solution and refinement as for **1b**, 278 parameters, 2 independent molecules per asymmetric unit, refinement converged at R1(F) = 0.056, wR2(F^2, all data) = 0.199, S = 1.122, max(Δ/σ) < 0.001, min/max height in final ΔF map −0.27/0.31 e/Å^3. The absolute structure was determined refining the η parameter. Figure 2 shows the molecular structure.

(R) - (−) - 5 - *Chloro* - 3,4 - *dihydro* - 6,8 - *dihydroxy* - 3-*methyl*-1*H*-2-*benzopyran*-1-*one* (**2**). The culture filtrate from *Plectophomella* sp. (80 l, SM-medium, addition of Co(II)-salts) was extracted × 3 with EtOAc and the combined extracts were concd *in vacuo* to afford 7.0 g of extract. Crude isocoumarin **2** (330 mg) was isolated from the residue by chromatography on Sephadex LH 20 (Pharmacia) with MeOH as eluent followed by chromatography on silica gel 60 (Merck) with dichloromethane–MeOH (1–10% MeOH). Purification was performed by preparative TLC (Macherey & Nagel, 1 mm, 1% MeOH–dichloromethane), followed by recrystallisation from dichloromethane–MeOH to yield 120 mg of colourless needles; mp 178°; $[\alpha]_D^{25}$ −69 (MeOH; *c* 1.08); IR (KBr): ν_{max} cm^{-1} 3463, 3339, 3210 (OH), 1657 (C = O), 1624 (Ar), 1385, 1256, 1129; UV (MeOH): λ_{max} (log ε) nm 287 (3.72), 296 (4.09), 301 (4.16), 309 (4.16), 315 (4.18); CD (EtOH): λ_{max} ($\Delta\varepsilon$) nm 312 (+0.44), 271 (−1.82), 237 (−4.46); ^{1}H NMR (300 MHz, [D]$_6$acetone): δ 1.52 (*d*, $J_{3,3\text{-Me}}$ = 6.3 Hz, 3 H, 3-CH$_3$), 2.75–2.85 (*dd*, $J_{3,4a}$ = 11.7 Hz, $J_{4a,4b}$ = 17 Hz, 1H, H-4a), 3.24–3.31 (*dd*, $J_{4a,4b}$ = 17 Hz, $J_{3,4b}$ = 3.3 Hz, 1H, H-4b), 4.71–4.82 (*m*, $J_{3,4b}$ = 3.3 Hz, 1H, H-3), 6.49 (*s*, 1H, H-7), 10.15 (*s*, 1H, 6-OH), 11.39 (*s*, 1H, 8-OH). ^{13}C NMR (75 MHz, [D]$_6$acetone): δ 18.95 (*q*, 3-CH$_3$), 30.96 (*t*, C-4), 73.95 (*d*, C-3), 100.48 (*s*, C-8a), 101.04 (*d*, C-7), 109.3 (*s*, C-5), 138.0 (*s*, C-4a), 159.24 (*s*, C-6). 161.6 (*s*, C-8), 168.56 (*s*, C-1); MS (70 eV); *m/z* (%): = 228 (100) [M]$^+$, 210 (48) [M$^+$–H$_2$O], 199 (28) [M$^+$ − CHO], 184 (92) [M$^+$ − CO$_2$], 156 (13), 147 (20), 69 (40). HRMS for $C_{10}H_9ClO_4$ requires 228.019; found 228.018 ± 3 ppm.

(3R, 4S) - (+) - 5 - *Chloro* - 3,4 - *dihydro* - 4,6,8 - *trihydroxy*-3-*methyl*-1*H*-2-*benzopyran*-1-*one* (**3**). The fraction next in polarity (60 mg) obtained by column chromatography on silica gel (see above, **2**) was further separated by TLC (Macherey & Nagel, 1 mm, 5% MeOH–dichloromethane) to afford 10 mg of compound **3**. Recrystallization from dichloromethane–MeOH afforded 7 mg of pure **3**; mp 172.5°; $[\alpha]_D^{20}$ = −76.92 (CHCl$_3$, *c* 0.26); IR (KBr): ν_{max} = 3567 cm^{-1}, 3463, 3407, 2917, 1653 (C = O), 1389, 1379, 1291, 1252, 1192, 1136, 1126, 1042; UV (MeOH): λ_{max} (log ε) nm 320 (4.09); CD (EtOH): λ_{max} ($\Delta\varepsilon$) nm 314 (+1.28), 265 (−5.40), 240 (−3.68); ^{1}H NMR (300 MHz, CD$_3$OD: δ 1.52 (*d*, $J_{Me,3}$ = 6.6 Hz, 3H, 3-CH$_3$), 4.59 (*dq*, $J_{Me,3}$ = 6.6 Hz, 1H, 3-H), 4.83 (*d*, $J_{3,4}$ = 1.8 Hz, 1H, 4-H), 6.47 (*s*, 1H, 7-H), additional signals in [D$_6$]acetone: 4.91 (broad *s*, 1H, 4-OH), 10.13 (broad *s*, 1H, 6-OH), 11.42 (*s*, 1H, 8-OH); ^{13}C NMR (75 MHz), CD$_3$OD): δ 16.5 (*q*, 3-CH$_3$), 65.0 (*d*, C-4), 79.4 (*d*, C-3), 101.8 (*s*, C-8a), 104.4 (*d*, C-7), 112.6 (*s*, C-5), 141.2 (*s*, C-4a), 162.0 (*s*, C-6), 163.6 (*s*, C-8), 170.8 (*s*, C-1); MS (EI); *m/z* (%): = 246 (13) [M$^+$(^{37}Cl)], 244 (43) [M$^+$(^{35}Cl)], 215 (10), 200 (36) [M$^+$ − CO$_2$], 172 (75), 171 (100), 69 (15). HRMS for $C_{10}H_9ClO_5$ requires 244.013; found 244.013 ± 3 ppm.

(R) - (−) - 3,4 - *Dihydro* - 3 - *methyl* - 5,6,8 - *trihydroxy*-1*H*-2-*benzopyran*-1-*one* (**4**). The culture filtrate (10 l) of *Cryptosporiopsis* sp. (S-medium, $MnCl_2$-addition) was extracted × 3 with EtOhAc. The combined extracts were concentrated *in vacuo*. The residue was separated by layer chromatography on silica gel ((Macherey & Nagel), 1 mm, 5% MeOH–dichloromethane). From the polar fraction 30 mg of trihydroxydihydroisocoumarin **4** were isolated. From *Fusarium* sp. (semisolid agar (2 l) 300 mg of **4** were obtained by a similar procedure. Mp 185°; $[\alpha]_D^{20}$ = −61.7 (CHCl$_3$, *c* 0.06); IR (KBr): ν_{max} = 3453–3424 cm^{-1} (OH), 3094, 2980 (CH), 1660, 1626 (C = O), 1596, 1527, 1513, 1486, 1473, 1456, 1391, 1330, 1313, 1293, 1240, 1216,1185, 1147, 1116; UV (MeOH): λ_{max} (log ε) nm 208 (4.18), 234 (4.05), 272 (3.93), 328 (3.72); CD (EtOH): λ_{max} ($\Delta\varepsilon$) nm 331 (+0.46), 273 (−2.71), 242 (−4.46); ^{1}H NMR ([D]$_6$DMSO): δ 1.26 (*d*, *J* = 6.24 Hz, 3H, 3-Me), 2.40–2.44 (*dd*, $J_{3,4a}$ = 11.5 Hz, $J_{4a,4b}$ = 16.8 Hz, 1H, H-4a), 2.91–2.96 (AB, $J_{4a,4b}$ = 16.8 Hz, $J_{3,4b}$ = 3.3 Hz, 1H, H-4b), 4.46–4.51 (*m*, $J_{3\text{-Me}}$ = 6.24 Hz, $J_{3,4}$ = 3.45 Hz, 1H, H-3), 6.12 (*s*, 1H, H-7), 9.32 (*br*, 2H, 5,6-OH), 10.62 (*s*, 1H, 8-OH); ^{13}C NMR ([D]$_6$DMSO): δ 19.85 (*q*, 3-CH$_3$), 27.7 (*t*, C-4), 74.5 (*d*, C-3), 98.0 (*s*, C-4a), 100.12 (*d*, C-7), 124.52 (*s*, C-8a), 133.63 (*s*, C-5), 153.58 (*s*, C-6), 155.98 (*s*, C-8), 169.2 (*s*, C-1); MS (70 eV); *m/z* (%): = 210 (100) [M]$^+$, 192 [M$^+$ − H$_2$O], 181 (25), 166 (20), 164 (18), 138 (16). Found: C, 57.01; H, 4.71. $C_{10}H_{10}O_5$ requires C 57.13, H 4.80%.

(−) - 6 - *Methoxy* - 3 - *methyl* - 8 - *hydroxy* - 1*H* - *benzopyran*-1-*one* [(−)-6-*Methoxymellein*] (**1e**). m p = 75°; CD (EtOH): λ_{max} ($\Delta\varepsilon$) nm 301 (+0.64), 270 (−2.65), 248 (+0.49), 233 (−3.62).

Methyl 2,4-*dimethoxy*-6-(2-*oxopropyl*)-*benzoate* (**6b**). A solution of the ketoacid **6a** [40] (2.38 g, 10 mmol) in MeOH (3 ml) is treated in small portions with a 0.1 M solution of diazomethane in Et$_2$O until a faint yellow colour persists. The solvent is removed at reduced pressure and the residue is purified by LC on silica gel (diethyl ether) to afford **6b** (2.35 g, 93%), mp 67°. IR (KBr): ν_{max} = 2950 cm^{-1} (CH$_3$), 1712 (C = O), 1606, 1590, 1471 (Ar); UV (MeOH): λ_{max} (log ε) nm 210 (4.42), 252 (3.80), 286 (3.55); ^{1}H NMR (CDCl$_3$): δ 2.15 (*s*, 3H, CH$_3$), 3.68 (*s*, 2H, CH$_2$), 3.81 (*s*, 3H, OCH$_3$), 3.82 (*s*, 3H, OCH$_3$), 3.84 (*s*, 3H, OCH$_3$),

6.31 (*d*, J_{meta} = 2.2 Hz, 1H, Ar-H), 6.41 (*d*, J_{meta} = 2.2 Hz, 1H, Ar-H); ^{13}C NMR ($CDCl_3$): δ 29.27 (*q*, CH_3), 49.13 (*t*, CH_2), 52.07 (*q*), 55.45 (*q*), 56.04 (*q*), 97.75 (*d*, C-5), 107.46 (*d*, C-3), 116.0 (*s*, C-6), 135.20 (*s*), 159.17 (*s*), 161.89 (*s*), 168.0 (*s*, C = O), 205.43 (*s*, C = O). MS (20°); *m/z* (%): 252 (28) $[M]^+$, 221 (30) $[M^+–OCH_3]$, 178 (100), 77 (30). Found: C, 61.96; H, 6.54. $C_{13}H_{16}O_5$ requires: C, 61.91; H 6.40%.

Reduction of ketoacid **6a** *and ketoester* **6b**: (*a*) *Reduction of* **6a** *with sodium borohydride.* A soln of ketoacid **6a** (129 mg, 0.5 mmol) in MeOH (5 ml) is treated in small portions at 0° with $NaBH_4$ (94.5 mg, 2.5 mmol). The mixture is then stirred for 10 hr at 20°. Aq. NH_4Cl-soln (2 ml) and then 2 N HCl (2 ml) are added (pH *ca* 1) and the mixture is extracted × 3 with Et_2O (15 ml) to afford rac-**8** [88 mg, 73% after LC on silica gel (Et_2O), mp 105° (ref. [50] mp 102–103.5°)] and **9** [15 mg, 13%, mp 153° (ref. [51] mp 154–156°)].

(*b*) *Reduction of* **6b** *with diisopinocamphenyl borane* (*Ipc_2BCl*). A solution of ester **6b** (254 mg, 1 mmol) in dry THF (5 ml) is added with a syringe under N_2 at 0° to a soln of Ipc_2BCl (705 mg, 2.2 mmol) in THF (10 ml). After stirring for 5 d at 20° the reaction is stopped by addition of NaOH (5 ml) and H_2O_2 (30%, 6 ml). The mixt. is adjusted to pH 1 by addition of 2 N HCl, extracted × 3 with Et_2O (25 ml). The combined organic phases are dried ($MgSO_4$), filtered, evapd to dryness at reduced pressure, and separated by LC on silica gel (Et_2O) to afford enantiomerically enriched (*R*)-**8** (59 mg, 27%, mp 112°, $[\alpha]_D^{25}$ = 94 (MeOH, *c* 1.1; 61% ee; 62% ee by NMR). The reduction of the acid **6a** by the same procedure afforded 41% ee.

(*c*) *Reduction of* **6b** *with baker's yeast.* Baker's yeast (50 g) is suspended in 0.1 M Tris buffer (1 l, pH 6.8, glucose 1%) in a 2 l Erlenmeyer flask. After 1 d of incubation at 30° ester **6b** (254 mg, 1 mmol) is added and the flask is shaken for 42 d (TLC control). 10 g of glucose as a 1% soln are added every 4th day. The yeast is then removed by centrifugation, the clear fermentation broth is adjusted by addition of 2 N HCl to pH 1, and is extracted × 4 with Et_2O (200 ml). The combined organic phases are dried (Na_2SO_4), filtered, evapd to dryness at reduced pressure, and the residue is purified by LC on silica gel (Et_2O) to afford in addition to acid **6a** (100 mg, 40%) the enantiomerically pure (*S*)-**8** (55 mg, 25%, mp 127°, $[\alpha]_D^{25}$ = +154 (methanol, *c* = 0.31, > 99% ee; > 99% ee by NMR). CD (*n*-hexane): λ_{max} ($\Delta\varepsilon$) nm 265 (+2.78), 242 (−0.68), 223 (+2.69); (EtOH): λ_{max} ($\Delta\varepsilon$) = 295 nm (+1.92), 270 (+6.22), 250 (−1.66), 229 (+35.10).

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