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Synthesis and free radical scavenging activity of a novel metabolite from the fungus *Colletotrichum gloeosporioides*

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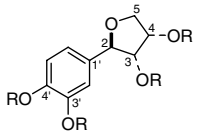
Abstract—A novel metabolite (−)-**1** was isolated as its peracetylated derivative, (−)-2-(3′,4′-diacetoxyphenyl)-3,4-diacetoxytetrahydrofuran (**2**), from a strain of the phytopathogenic fungus *Colletotrichum gloeosporioides* CECT 20122. The synthesis of (−)-**1** was carried out by ring-closing metathesis of diene **6** and stereoselective dihydroxylation of a dihydrofuran derivative **7** as key steps. The tetraol (−)-**1** showed free radical scavenging activity comparable to that of BHT, caffeic acid or protocatechuic acid.

Colletotrichum fungal species include some of the most destructive post-harvest pathogens of a wide range of plants including cereals, legumes, fruits and vegetables.[1,2] As a part of our research programme on the rational control of phytopathogenic fungi,[3] we have focussed on the relationship between the infective capabilities of certain phytopathogenic fungi and the role of their secondary metabolites in the production of active oxygen species.[4–7] These may prove to be a vital resource for medically applicable free radical scavengers in important conditions such as atherosclerosis, stroke, myocardial infarction, trauma, arthritis and cancer.[8–10]

A wide variety of fungi have produced novel antioxidants.[11,12] These include *Penicillium roquefortii*,[13] *Aspergillus candidus*,[14–16] *Mortierella* sp.,[17] *Emericella falconensis*[18] and fungi of the genus *Acremonium*.[19] In this paper, we report the synthesis and radical scavenging activity of the tetrahydroxylated compound (−)-**1**, which was isolated as a peracetylated derivative from the culture broth of a strain of the fungus *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. (teleomorph *Glomerella cingulata*). Radical scavenging activity has been determined using the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·) due to its easy and its widespread use.[20–22]

A liquid culture (65 L) of the fungus *C. gloeosporioides* CECT 20122[23] was filtered and the resulting broth was repeatedly extracted with ethyl acetate. After solvent drying and evaporation, a crude extract (9.3 g) was obtained, which was fractionated by silica gel column chromatography. The ethyl acetate fraction (2.6 g) was acetylated (Ac_2O/py) and further analysed (silica gel, petroleum ether–ethyl acetate gradients) to yield (−)-(2*R*(*S*),3*R*(*S*),4*S*(*R*))-2-(3′,4′-diacetoxyphenyl)-3,4-diacetoxytetrahydrofuran ((−)-**2**) (4 mg), together with acetates of previously known compounds.[24]



1 R = H
2 R = Ac

Compound (−)-**2** was obtained as a yellow oil which gave a negative optical specific rotation ($[\alpha]_D^{25}$ −34 (*c* 2 mg/mL, $CHCl_3$)). EIMS gave a quasi-molecular ion peak at *m*/*z* 381 $[M+1]^+$, while HREIMS gave a fragment ion peak at *m*/*z* 320.0895 [M−AcOH], which is consistent with the formula $C_{16}H_{16}O_7$ (calcd 320.0896). This, together with the analysis of the ^{13}C NMR and

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DEPT spectra, which shows the presence of 4 methyl groups, 1 methylene, 6 methines and 7 quaternary carbons, allows the molecular formula $C_{17}H_{20}O_9$ to be assigned to compound (−)-**2**. The ^{1}H NMR spectrum of compound (−)-**2** showed signals attributed to a 1,3,4-trisubstituted phenyl moiety at δ 7.17 (1H, d, J = 8.4 Hz, H-5′), 7.23-727 (2H, H-2′, H-6′), 4 methyls from acetate groups at δ 2.09 (3H, s, CH_3CO on C-4), 2.11 (3H, s, CH_3CO on C-3), 2.28 (6H, s, $2CH_3CO$ on C-3′,4′), and a 2,3,4 trisubstituted tetrahydrofuran moiety at δ 3.97 (1H, dd, J = 10.3,5.6 Hz, H-5), 4.43 (1H, dd, J = 10.3, 5.6 Hz, H-5′), 4.94 (1H, d, J = 7.0 Hz, H-2), 5.03 (1H, dd, J = 6.8, 5.2 Hz, H-3), 5.41 (1H, ddd, J = 5.3, 5.2,4.0 Hz, H-4). The connectivity among the described fragments was determined by HMBC experiments. The data are consistent with the proposed structure assigned to compound (−)-**2** as (−)-(*2R(S), 3R(S),4S(R)*)-2-(3′,4′-diacetoxyphenyl)-3,4-diac-etoxytetrahydrofuran.

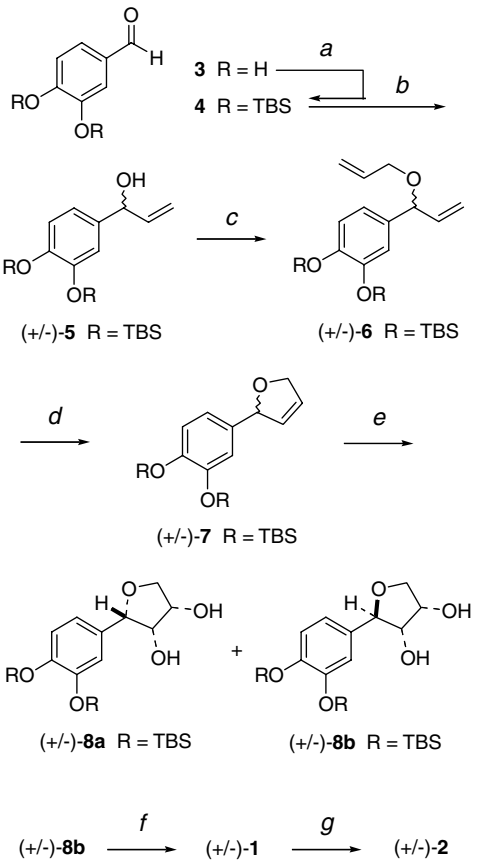
Relative stereochemistry at C-2 for compound **2** was deduced from J coupling analysis but it could not be directly confirmed by NOE analysis. A chemical synthesis of compound (±)-**2** and the related tetraol, (*2R*(*S*),*3R*(*S*),*4S*(*R*))-2-(3′,4′-dihydroxyphenyl)tetrahydrofuran-3,4-diol ((±)-**1**), would support the regio and stereochemical assignations and would produce further material for testing.

The chemical synthesis of the metabolite relied on a stereoselective hydroxylation of a non-conjugated dihydrofuran precursor (±)-**7**, which was prepared via a ring-closing metathesis (Scheme 1).

Diallylether (±)-**6** was prepared by treatment of the protected 3,4-dihydroxybenzaldehyde (**4**)[25] with vinyl bromide and magnesium turnings, in THF,[26] to yield 1-[(3′,4′-bis-(*tert*-butyldimethyl-silyloxy)phenyl)]prop-2-en-1-ol ((±)-**5**) (71%). *O*-Allylation of compound (±)-**5** was efficiently carried out by treatment with $(TMS)_2NLi$ and allyl bromide in THF, which allowed to obtain the allyl ether (±)-**6** in 98% yield.

Ring-closing metathesis on the non-conjugated diene (±)-**6**, mediated by the first generation Grubbs' catalyst,[27] gave a high yield (99%) of the non-conjugated dihydrofuran (±)-**7** after 2 h reaction time. This compound was dihydroxylated with the OsO_4/Me_3NO[28] system to give a 1:8 mixture of syn-diols ((±)-**8a**–(±)-**8b**; 45% yield). The observed product ratio reflected the preference of the reagent for the least hindered face of the olefin in the precursor compound (±)-**7**.

The relative stereochemistry of minor compound (±)-**8a** could be determined by NOE experiments as 2*S*(*R*),3*R*(*S*),4*S*(*R*) (Fig. 1). Compounds (±)-**8a** and (±)-**8b**, syn diols obtained by OsO_4 mediated dihydroxylation, are diastereoisomers which only differ on their configuration at C-2. Therefore, once the relative stereochemistry for (±)-**8a** had been determined as stated above, the relative stereochemistry for its diastereocomplementary diol (±)-**8b** could be assigned as 2*R*(*S*),3*R*(*S*),4*S*(*R*).



Scheme 1. Synthesis of (±)-**1** and (±)-**2**. Reagents and conditions: (a) TBSCl, imidazole, THF, 0 °C (87%); (b) vinyl bromide, magnesium, THF, 25 °C (73%); (c) allyl bromide, $(TMS)_2NLi$, THF, 0 °C, 15′, then 90 °C, 12 h (98%); (d) 10 mol% Grubbs catalyst ($(PCy_3)_2Cl_2RuCH{=}Ph$), CH_2Cl_2, reflux (99%); (e) OsO_4, Me_3NO, *t*-BuOH, H_2O, pyridine, reflux, (45%, 1:8 **8a** vs **8b** diastereoisomeric ratio); (f) iodine, MeOH, reflux (37%); (g) Ac_2O, pyridine, 25 °C (34%).

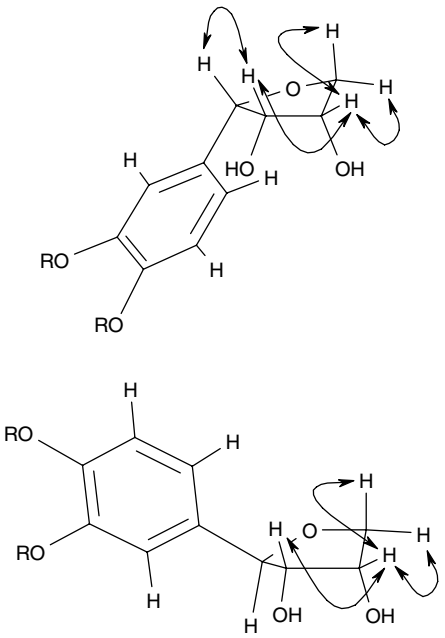


Figure 1. Selected NOE correlations for compounds (±)-**8a** (above) and (±)-**8b** (below); R = TBS.

Deprotection of the major diol (±)-**8b** with I_2/MeOH[29] gave the tetrahydroxylated compound (±)-**1**, which after peracetylation with Ac_2O/py gave compound (±)-**2**. In order to obtain compound (−)-**1** and its peracetate (−)-**2**, major diol (±)-**8b** was treated with (−)-(2*R*)-2-methoxy-2-phenylacetic acid, DCC and a catalytical amount of DMPA in CH_2Cl_2 at room temperature for 1 hour. This produced two diastereoisomeric diesters, which after separate treatment with KOH/MeOH yielded, respectively, the enantiomeric diols (−)-**8b** and (+)-**8b**. Separate treatment of (−)-**8b** and (+)-**8b** with I_2/MeOH gave, respectively, the synthetic tetrols (−)-**1** and (+)-**1**.[30] Treatment of compound (−)-**1** with Ac_2O/py yielded a synthetic product which had identical physical ($[\alpha]_D^{25}$ −34 (*c* 2.2 mg/mL, $CHCl_3$)) and spectral properties to the isolated natural product (−)-**2**.

The radical scavenging activity of the synthetic natural product (−)-**1** was estimated according to the procedure described by Brand-Williams et al.[22] This assay involved measuring the decrease in absorbance at 515 nm that occurred when the DPPH radical was reduced by the tested antioxidant. A 90 μM methanolic solution of DPPH· (Fluka) was employed and compound (−)-**1** was assayed at final concentrations in the range between 30 and 5 μM in methanol. For every evaluated concentration, an aliquot of the DPPH· solution (950 μL) was mixed with an aliquot of the test solution (50 μL) in such a fashion that the desired concentration was achieved and the mixture was incubated in darkness at 25 °C. Simultaneously, a control solution of DPPH· was prepared by mixing an aliquot of the DPPH· stock solution (950 μL) with MeOH (50 μL), which was also incubated in darkness at 25 °C. The absorbance of the mixtures of compound (−)-**1** and DPPH· and their corresponding controls at 515 nm were determined immediately and every 10 min for 120 min. Methanol was used to zero the spectrophotometer. Absorbancies were measured using a Varian model Cary 50 Bio UV–vis spectrophotometer. The measurements were made in triplicate. The inhibition percentage (IP) values referred to the DPPH radical at different times (*t*) were calculated using the following equation: $IP = 100 \times (Abs\ control_t - Abs\ test_t)/Abs\ control_t$. Percentage of remaining DPPH· (calculated as 100-IP) at steady state for every tested concentration of compound (−)-**1** was obtained from a plot of the percentage of remaining DPPH· against time for the above-mentioned concentrations, according to the procedure described by Brand-Williams et al.[22,31]

In order to carry out a comparison of DPPH· scavenging activity of compound (−)-**1**, the amount of compound necessary to decrease the DPPH· concentration by 50% efficient concentration = EC_{50} ((mol/L) (−)-**1**/(mol/L) DPPH·) was calculated from a plot of the steady-state values (120 min) of the percentage of remaining DPPH· versus concentration of (−)-**1**, according to the procedure of Brand-Williams et al. (Table 1).[22]

The synthetic tetraol (−)-**1** showed a radical scavenging activity comparable to that of BHT, caffeic acid or protocatechuic acid, as shown in Table 1. All these compounds can be described as having a slow kinetic behaviour against the DPPH· scavenging, as more than half an hour is needed to reach a steady state in the reaction (no decrease in percentage of remaining DPPH· with time).

Table 1. Comparison of DPPH radical scavenging activity for compound (−)-**1**, BHT, caffeic acid and protocatechuic acid

Compound	EC_{50}[a]	ARP (AE)[b]	Kinetic behaviour[c]
(−)-**1**	0.14	7.14	Slow
BHT[d]	0.23	4.20	Slow
Protocatechuic acid[d]	0.14	7.14	Slow
Caffeic acid[d]	0.11	9.1	Slow

[a] Efficient concentration = EC_{50} ((mol/L) (−)-**1**/(mol/L) DPPH·).
[b] Antiradical power (ARP) = antiradical efficiency (AE) = $1/EC_{50}$.
[c] Time required to reach steady state in the DPPH· scavenging experiment (fast <1 min, intermediate >1 min, <30 min, slow >30 min).
[d] Ref. 22.

In conclusion, we have described the isolation of a novel natural compound (−)-**1** as its tetraacetate (−)-**2**, and established its constitution and relative stereochemistry via spectroscopic studies and stereoselective synthesis. The tetrahydrofuran derivative (−)-**1**, obtained by chemical resolution, proved to be an effective inhibitor of the DPPH radical.

Acknowledgments

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23. Strain originally isolated from *Fragaria* spp. (strawberries) and obtained from a fungal collection depository (Colección Española de Cultivos Tipo).
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30. Compound (−)-**1**. Colourless oil. $[\alpha]_D^{25}$ −38 (*c* 1.85 mg/mL, MeOH). ^{1}H NMR (400 MHz, CD_3OD): δ (ppm) 3.80 (dd, J = 2.4, 8.8 Hz, 1H, H-5b), 3.87 (dd, J = 4.2, 7.6 Hz, 1H, H-3), 4.22 (ddd, J = 2.4, 4.2, 4.6 Hz, 1H, H-4), 4.25 (dd, J = 4.6, 8.8 Hz, 1H, H-5a), 4.51 (d, J = 7.6 Hz, 1H, H-2), 6.70 (2H, H-5′, H-6′), 6.80 (d, J = 2.0 Hz, H-2′); ^{13}C NMR (100 MHz, CD_3OD): δ (ppm) 72.4 (d, C-4), 74.2 (t, C-5), 79.7 (d, C-3), 84.5 (d, C-2), 114.5 (d, C-2′), 116.1 (d, C-6′), 119.0 (d, C-5′), 133.3 (s, C-1′), 146.1 (s, C-3*), 146.3 (s, C-4*) (*interchangeable signals); selected HMBC correlations: C1′ → H-2, H-3, H-5′, H-6′; IR (neat) ν 3334, 1520; EIMS *m*/*z* (rel int.) 212 $[M]^+$ (73), 194 (8), 152 (10), 139 (100); HREIMS *m*/*z* calcd for $C_{10}H_{12}O_5$ = 212.0685, found 212.0679. Compound (+)-**1**. $[\alpha]_D^{25}$ +38 (*c* 1.7 mg/mL, MeOH).
31. Concentrations of compound (−)-**1** were expressed as moles of (−)-**1**/mol of DPPH·. The DPPH· concentration at a given time in the reaction medium was calculated from a calibration curve with the equation: $Abs_{515\ nm} = 25.607 \times Conc_{DPPH\cdot}$ (mg/mL) + 0.0064 (R = 0.9982).