Absolute Stereochemistry of Spiciferones and Spicifernin, Bioactive Metabolites of the Fungus *Cochliobolus spicifer*: Evidence for their Unique Biosynthesis

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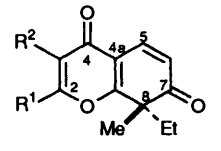
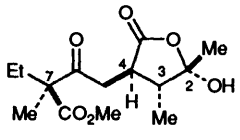
The absolute stereochemistry of spiciferone A and spicifernin, a phytotoxin and a plant-growth promoter, respectively, produced by the phytopathogenic fungus *Cochliobolus spicifer* Nelson, has been determined by the application of Mosher's method after establishment of relative stereochemistry by X-ray analysis. The absolute stereochemistry of spiciferones B and C has been determined by CD spectral comparison. Their absolute stereochemistry was in good agreement with the proposed polyketide biosynthetic pathway in which spiciferones and spicifernin are formed from a common cyclic precursor by a sequence of biological reactions involving a unique retro-aldol condensation.

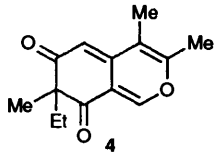
Strain D-5 of *Cochliobolus spicifer* Nelson, which causes leaf spot disease in wheat, simultaneously produces several phytotoxins and a plant-growth promoter. The phytotoxins, *i.e.*, spiciferones A **1**, B **2**, and C **3** and spiciferinone **4**, and the plant-growth promoter, *i.e.*, spicifernin, tautomers **5a** and **5b**,† have been isolated and characterized in our laboratory.[1] Despite different carbon skeletons, they have unique structural features in common: (i) a quaternary carbon bearing an ethyl, a methyl, and a ketonic carbonyl and (ii) vicinal methyls. This commonality strongly suggests that these metabolites have the same origin. Labelled acetic acid and methionine were incorporated



1 $R^1 = R^2 = Me$
2 $R^1 = CH_2OH$, $R^2 = Me$
3 $R^1 = Me$, $R^2 = CH_2OH$



5a



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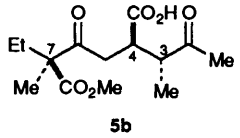


5b

into spiciferone A and spicifernin, and the resulting labelling patterns indicated the operation of the biosynthetic pathway such as in Scheme 1.[2] In this pathway a single hexaketide chain bearing two *C*-methyls from C_1 units is modified into spiciferone A and spicifernin *via* a 10-membered monocyclic intermediate **6** by a sequence of biological reactions including a unique retro-aldol condensation. In this hypothetical pathway the absolute configuration of C-8 in spiciferone A is closely related to that of C-7 in spicifernin through the intermediate **6**. Thus their absolute configurations will afford evidence for this interesting biosynthetic pathway. However, those still remain to be determined. In this paper, we report the absolute stereochemistry of spiciferones and spicifernin and then discuss the validity of our proposed biosynthetic pathway.

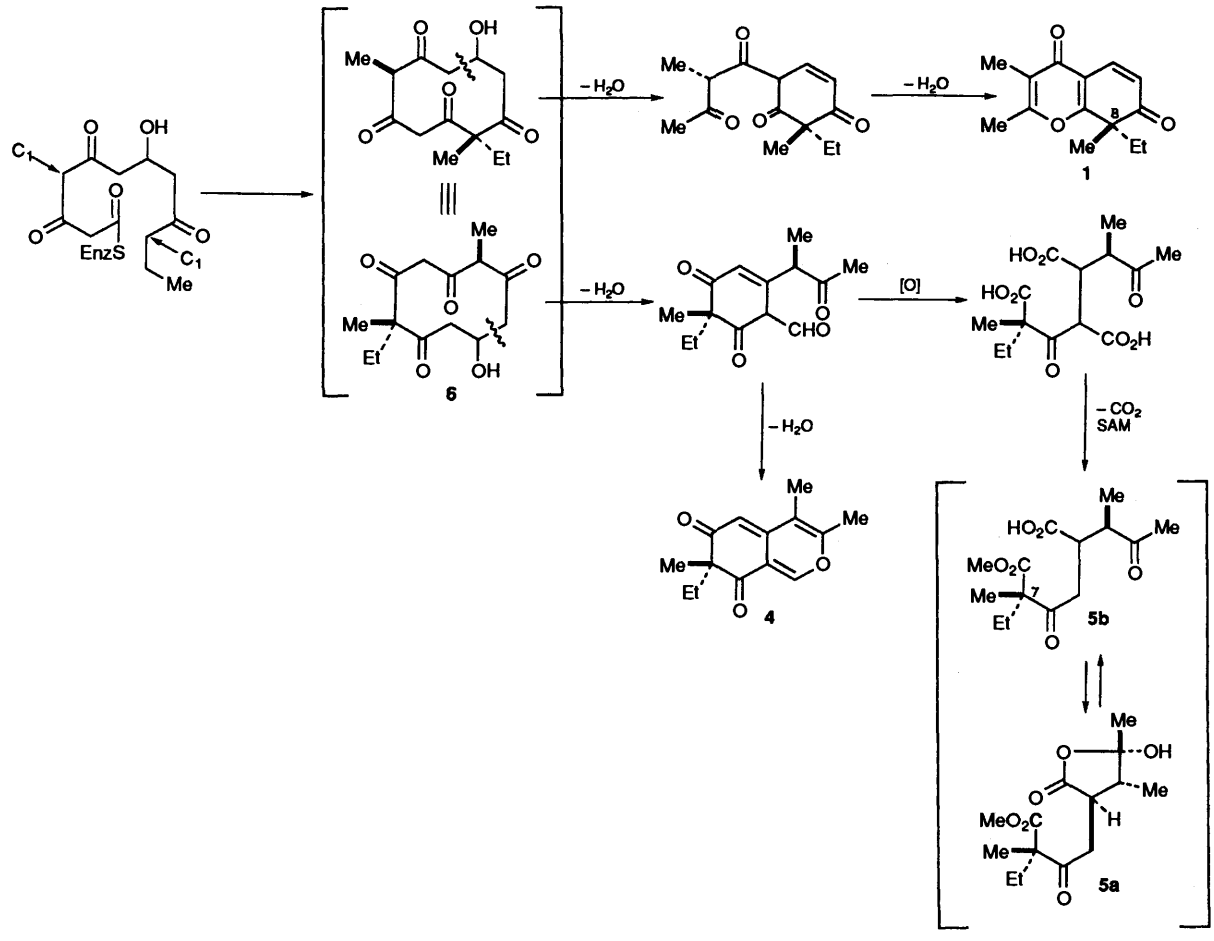
Results and Discussion

The absolute stereochemistry of spiciferone A was determined by application of Mosher's method for its derivative whose relative stereochemistry was established by X-ray analysis. Spiciferone A **1** was converted into alcohols **7a** and **7b** in two steps as shown in Scheme 2 and these two alcohols were separable by HPLC as previously described.[3] The major alcohol **7b**, in which the hydroxy group is oriented in a pseudoaxial position, was found to be fairly nonreactive towards α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPACl) under ordinary conditions. Thus, the minor alcohol **7a** was chosen for X-ray analysis and application of Mosher's method. The alcohol **7a** was recrystallized from EtOAc–hexane to give needles and was subjected to X-ray analysis to establish its relative stereochemistry. The molecular structure of alcohol **7a** is shown in Fig. 1 and atomic coordinates are listed in a supplementary publication.‡ The relative configuration was found to be (7*R**,8*S**). The absolute configuration at C-7 of compound **7a** was established by application of an advanced Mosher's method.[4] Alcohol **7a** was converted into its (*R*)-α-methoxy-α-(trifluoromethyl)phenylacetic acid [(*R*)-MTPA] ester **8a** and (*S*)-MTPA ester **8b**, and their ^1H NMR spectra were measured in $CDCl_3$. The chemical-shift differences ($\Delta\delta = \delta_{8b} - \delta_{8a}$) between the resonances in the ^1H NMR spectra of esters **8a** and **8b** are shown in Fig. 2. The absolute configuration at C-7 in the alcohol **7a** was thus determined to be *R* by Mosher's method. Therefore, the absolute configuration at C-8 of compound **7a** is *S* and consequently that of spiciferone A **1** was established to be *R*.

Next the CD spectra of spiciferones A, B and C, and the alcohol **7a** were measured in order to establish the absolute stereochemistry of spiciferones B and C. Table 1 shows the CD spectral data for these compounds. The spectra of spiciferones revealed CD bands closely similar to each other, whereas the spectrum of alcohol **7a** showed no perceptible CD bands at all. This indicates that the conjugated ketone functionality at C-7

† Spicifernin was found to exist as an equilibrium mixture of tautomers **5a** and **5b** in solution from NMR data.[1b]

‡ For full details of the CCDC deposition scheme, see 'Instructions for Authors,' *J. Chem. Soc., Perkin Trans. 1*, 1994, issue 1.



Scheme 1 Proposed biosynthetic pathway[2] of spiciferone A **1**, spiciferinone **4** and spicifernin **5a/b**. SAM = *S*-adenosylmethionine.

Table 1 CD spectral data for spiciferones A **1**, B **2** and C **3**, and the alcohol **7a**

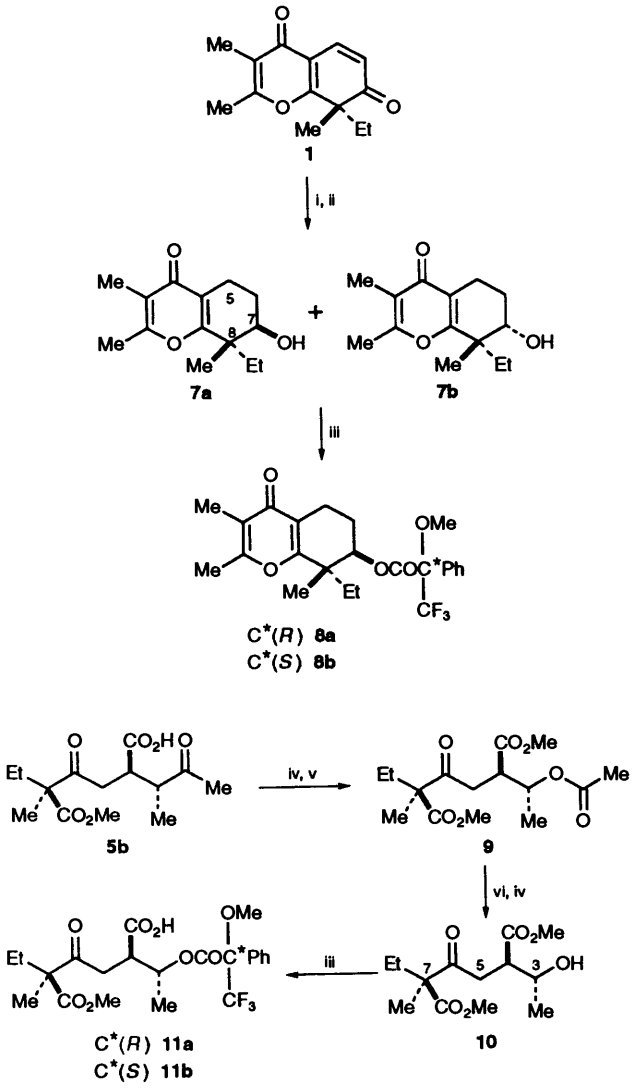
Compound	CD Band λ_{max}(EtOH)/nm ($\Delta\varepsilon$)
Spiciferone A **1**	309 (−2.11) and 363 (+0.48)
Spiciferone B **2**	305 (−1.62) and 360 (+0.35)
Spiciferone C **3**	305 (−1.88) and 363 (+0.38)
Alcohol **7a**	no perceptible CD band

is important for the characteristic CD bands of compounds **1–3** and also that C-8 in spiciferones B and C (compounds **2** and **3**) has the same configuration as in spiciferone A **1**.

The relative stereochemistry of spicifernin **5a/b** was established by X-ray analysis and its absolute stereochemistry was determined by application of Mosher's method for the degraded product. Spicifernin was recrystallized from EtOAc–hexane to yield needles, which were subjected to X-ray analysis. The molecular structure of spicifernin is shown in Fig. 3 and atomic coordinates are listed in the supplementary publication. Thus, the relative stereochemistry of one form of spicifernin, **5a**, was determined to be (2*S**,3*R**,4*R**,7*S**). Interestingly, spicifernin exists only as lactol **5a** in the crystal state, while it shows acidic properties and exists as an equilibrium mixture of lactol **5a** and keto acid **5b** in solution.[1b] Spicifernin was converted, *via* triester **9**, into alcohol **10** in four steps, including Baeyer–Villiger oxidation, as shown in Scheme 2. Alcohol **10** was esterified with (+)- and (−)-MTPACl in pyridine to give (*R*)-MTPA ester **11a** and (*S*)-MTPA ester **11b**, respectively, and their ^{1}H NMR spectra were measured in $CDCl_3$. The chemical-shift differences ($\Delta\delta = \delta_{11b} - \delta_{11a}$) between the resonances in ^{1}H NMR spectra of esters **11a** and **11b** are shown in Fig. 4. The absolute configuration for C-3 in the alcohol **10** was thus determined to be *R* by Mosher's method. The positive $\Delta\delta$-value for methyl protons in the methoxycarbonyl function at C-7 is due to the following reason: this methyl is positioned on the same side of the MTPA plane as is the methyl at C-3 in the most preferable conformation of the MTPA esters **11a** and **11b**. This was confirmed by MM-2 conformational analysis of the corresponding (*S*)-α-methoxy-α-(methyl)phenylacetate† and (*R*)-α-methoxy-α-(methyl)phenylacetate† of alcohol **10** (Fig. 5).[5] Thus, the absolute stereochemistry of one form of spicifernin, **5a**, was established as (2*S*,3*R*,4*R*,7*S*).

In our previous report,[2] we proposed the biosynthetic pathway shown in Scheme 1 for the production of spiciferone A and spicifernin in this fungus. In the proposed pathway they arise from the common monocyclic precursor **6** by retro-aldol condensation, which is a novel reaction in polyketide biosynthesis. If this pathway is operative in this fungus, the absolute configuration of the quaternary carbon in intermediate **6** is deduced to be *S* from the configuration of spiciferone A **1**. Similarly, that carbon is deduced to have the *S* configuration from the stereochemistry of spicifernin. Therefore, our present results concerning the stereochemistry of spiciferone A and spicifernin strongly support the unique biosynthetic pathway we have proposed.

† *i.e.*, The corresponding α-methoxy-α-phenylpropionates.



Scheme 2 Reactions of spiciferone A **1** and spicifernin **5b** to establish their absolute stereochemistry. *Reagents and conditions:* i, H_2, PtO_2, EtOAc; ii, $NaBH_4$, THF, 20 min, 0 °C; iii, (*R*)- or (*S*)-MTPACl, pyridine, $H_2NCH_2CH_2CH_2NMe_2$; iv, CH_2N_2, Et_2O; v, MCPBA, NaH_2PO_4, CH_2Cl_2, 45 °C, 96 h; vi, Na_2CO_3, MeOH, room temperature.

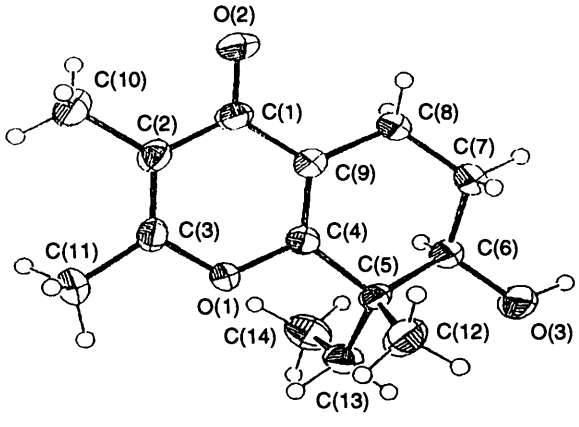


Fig. 1 Perspective view of alcohol **7a** drawn by ORTEP and the numbering system used for the X-ray molecular structure

Experimental

General Procedure.—Optical rotations were measured on a Horiba SEPA-200 high sensitivity polarimeter and $[\alpha]_D$-values are given in 10^{-1} deg cm^2 g^{-1}. CD spectra were recorded on a JASCO J-20C spectropolarimeter. MS were recorded on a JEOL DX-300 spectrometer. NMR spectra were measured on a JNM GX-270 FT NMR spectrometer (^1H, 270 MHz; ^{13}C, 67.8 MHz), for solutions in $CDCl_3$ with Me_4Si as reference. The steric conformations were built up by MM-2 energy minimization of *CSC Chem3D/Plus*™ software (Cambridge Scientific Computing, Inc.) on a Macintosh Centris 650 computer.

X-Ray Crystal Structure Analysis.—Intensity data were

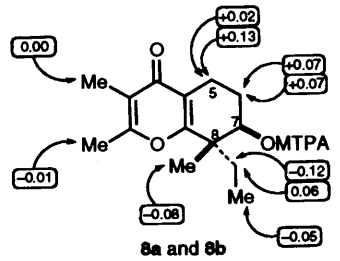


Fig. 2 Determination of the absolute configuration at C-7 in alcohol **7a**: $\Delta\delta$-value was obtained from the ^1H NMR spectra of (*R*)-MTPA ester **8a** and (*S*)-MTPA ester **8b**

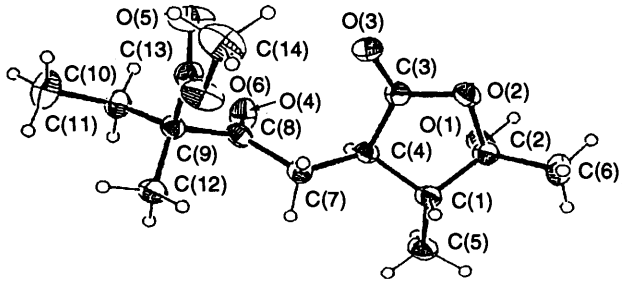


Fig. 3 Perspective view of spicifernin **5a** drawn by ORTEP and the numbering system used for X-ray molecular structure

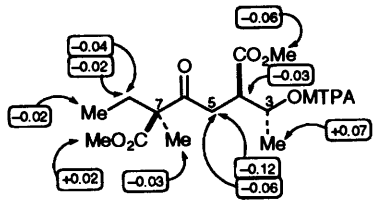


Fig. 4 Determination of the absolute configuration at C-3 in alcohol **10**: $\Delta\delta$-value was obtained from the ^1H NMR spectra of (*R*)-MTPA ester **11a** and (*S*)-MTPA ester **11b**

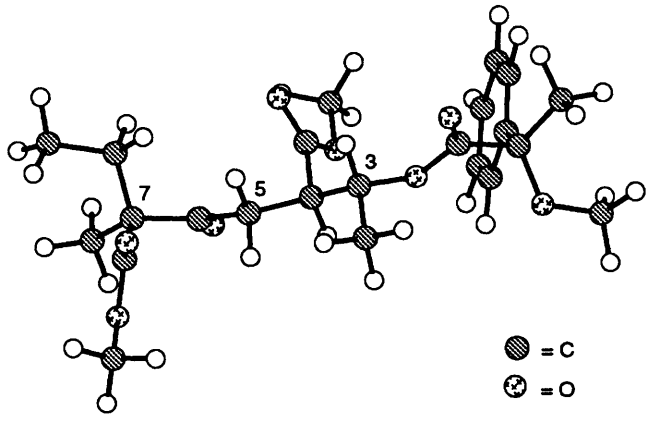


Fig. 5 Stable conformation (37.3 kcal mol^{-1}) of (*S*)-α-methoxy-α-phenylpropionate of alcohol **10** obtained by MM-2 energy minimization (1 cal = 4.184 J)

measured on a Rigaku four-circle diffractometer using Ni-filtered Cu-Kα radiation (λ = 1.5418 Å) and a rotating anode generator. The ω–2θ scan mode was employed, with background measurement at the end of each scan. No significant change was observed in the intensities of the three standard reflections measured every 100 reflections for each compound. Intensity data were corrected only for Lorentz and polarization effects. Both structures were determined by direct methods using SHELX 86[6] and refined by block-diagonal least-squares[7] on F using atomic scattering factors.[8] The hydrogen atoms were located in the difference Fourier maps. The oxygen and carbon atoms were refined anisotropically and the hydrogen atoms isotropically. Molecular structure perspective views (Figs. 1 and 3) were drawn by ORTEP-II.[9]

Crystal Data for Alcohol **7a**.—$C_{14}H_{20}O_3$, M = 236.3, orthorhombic, space group $P2_12_12_1$, a = 12.637(2), b = 7.552(1), c = 13.501(2) Å, V = 1288.5(3) Å^3 (cell parameters by least-squares from the setting angles of 22 reflections), μ(Cu-Kα) = 6.4 cm^{-1}, Z = 4, D_X = 1.22 g cm^{-3}, $F(000)$ = 512, T = 293 K, R = 0.050 and R_W = 0.062 for 1000 unique reflections with $F_o > 2\sigma(F_o)$. A crystal of dimensions 0.05 × 0.1 × 0.6 mm was used. Intensities of 1156 unique reflections were measured to $2\theta_{max}$ = 120° in the range $0 \leq h \leq 14$, $0 \leq k \leq 8$, $0 \leq l \leq 15$, with ω scan width of 1.0° + 0.15° tan θ, a scan speed of 4° min^{-1}, and a background counting time of 6 s. Strongest reflection, 0 2 0, was omitted from the refinement. The weighting scheme used in the final stage of refinement was $w = [\sigma(F_o)^2 + 0.015F_o + 0.0005F_o^2]^{-1}$. The residual electron densities in the final difference Fourier map ranged from −0.18 to 0.17 e Å^{-3}. Atomic coordinates, temperature factors, and bond lengths and angles have been deposited at the Cambridge Crystallographic Data Centre.

Crystal Data for Spicifernin **5a**.—$C_{14}H_{22}O_6$, M = 286.3, orthorhombic, space group $P2_12_12_1$, a = 26.939(5), b = 9.237(2), c = 5.995(1) Å, V = 1491.8(4) Å^3 (cell parameters by least-squares from the setting angles of 21 reflections), μ(Cu-Kα) = 8.7 cm^{-1}, Z = 4, D_X = 1.27 g cm^{-3}, $F(000)$ = 616, T = 293 K, R = 0.041 and R_W = 0.048 for 1202 unique reflections with $F_o > 2\sigma(F_o)$. A crystal of dimensions 0.1 × 0.1 × 0.7 mm was used. Intensities of 1348 unique reflections were measured to $2\theta_{max}$ = 120° in the range $0 \leq h \leq 30$, $0 \leq k \leq 10$, $0 \leq l \leq 6$, with ω scan width of 0.9° + 0.15° tan θ, a scan speed of 3° min^{-1}, and a background counting time of 6 s. The weighting scheme used in the final stage of refinement was $w = [\sigma(F_o)^2 + 0.024F_o]^{-1}$. The residual electron densities in the final difference Fourier map ranged from −0.18 to 0.17 e Å^{-3}. Atomic coordinates, temperature factors, and bond lengths and angles have been deposited at the Cambridge Crystallographic Data Centre.

(R)-*MTPA Ester* **8a**.—To a solution of the alcohol **7a** (2.5 mg, 11 μmol), prepared as described previously,[3] in pyridine (5.0 mm^3) was added (+)-MTPACl (10 mm^3, 53 μmol). After 15 h at room temperature, *N*,*N*-dimethylpropane-1,3-diamine (6.3 mm^3) was added to the reaction mixture. After 10 min, the reaction mixture was diluted with diethyl ether (10 cm^3) and the solution was washed successively with brine, aq. copper(II) sulfate, brine, aq. $NaHCO_3$ and brine. The solution was dried over Na_2SO_4, and filtered and concentrated under reduced pressure. The residue was subjected to TLC [silica gel; acetone–benzene (1:9)] and then to HPLC (COSMOSIL 5C18-AR, 4.6 × 150 mm; 55% aq. MeOH) to give (*R*)-MTPA ester **8a** as an oil (3.1 mg, 65%); δ_H 0.761 (3 H, t, *J* 7.6, 8-CH_2Me), 1.126 (3 H, s, 8-Me), 1.495 (1 H, dq, *J* 14.2 and 7.6, 8-CH_2Me), 1.716 (1 H, dq, *J* 14.2 and 7.6, 8-CH_2Me), 1.795 (1 H, m, 6-H), 1.854 (3 H, s, 3-Me), 1.953 (1 H, m, 6-H), 2.200 (3 H, s, 2-Me)$_3$ 2.385 (1 H, ddd, *J* 6.4, 8.3 and 18.1, 5-H), 2.457 (1 H, ddd, *J* 5.5, 6.5 and 18.1, 5-H), 3.421 (3 H, br s, OMe), 5.229 (1 H, dd, *J* 3.4 and 9.5, 7-H), 7.38 (3 H, m, Ph) and 7.46 (2 H, m, Ph); EI-MS *m*/*z* 452 (M$^+$, 51%), 422 (8), 219 (100), 191 (22) and 189 (87).

(S)-*MTPA Ester* **8b**.—This compound was obtained in 65% yield from alcohol **7a** with (−)-MTPACl by essentially the same procedure as for the preparation of compound **8a**; δ_H 0.710 (3 H, t, *J* 7.3, 8-CH_2Me), 1.048 (3 H, s, 8-Me), 1.375 (1 H, dq, *J* 14.2 and 7.3, 8-CH_2Me), 1.658 (1 H, dq, *J* 14.2 and 7.3, 8-CH_2Me), 1.854 (3 H, s), 1.866 (1 H, m, 6-H), 2.187 (3 H, s), 2.020 (1 H, m, 6-H), 2.401 (1 H, ddd, *J* 17.6, 9.2 and 6.4, 5-H), 2.582 (1 H, ddd, *J* 17.6, 5.9 and 4.8, 5-H), 3.484 (3 H, br s, OMe), 5.253 (1 H, dd, *J* 3.4 and 9.7, 7-H), 7.355 (3 H, m, Ph) and 7.475 (2 H, m, Ph); EI-MS *m*/*z* 452 (M$^+$, 50%), 422 (6), 219 (100), 191 (21) and 189 (89).

Compound **9**.—A solution of methyl spicifernin (Me ester of **5b**, 55 mg, 0.18 mmol), obtained as previously described,[1] *m*-chloroperbenzoic acid (MCPBA) (121 mg, 0.70 mmol) and NaH_2PO_4 (93 mg, 0.78 mmol) in dichloromethane (2.0 cm^3) was stirred for 96 h at 45 °C. The reaction mixture was diluted with dichloromethane (13 cm^3) and the solution was washed successively with aq. sodium thiosulfate, aq. $NaHCO_3$ and brine. After removal of solvent the residue was purified with TLC [silica gel; EtOAc–hexane–AcOH (20:80:1), triple development] to yield compound **9** as an oil (23 mg, 40%); $[\alpha]_D^{24}$ ~0 (*c* 1.0, EtOH); δ_H 0.84 (3 H, t, *J* 7.3, 9-H$_3$), 1.20 (3 H, d, *J* 6.4, 3-Me), 1.33 (3 H, s, 7-Me), 1.86 (1 H, dq, *J* 14.6 and 7.3, 8-H), 2.00 (1 H, dq, *J* 14.6 and 7.3, 8-H), 2.01 (3 H, s, 1-H$_3$), 2.64 (1 H, dd, *J* 3.9 and 18.1, 5-H), 3.00 (1 H, dd, *J* 9.3 and 18.1, 5-H), 3.22 (1 H, ddd, *J* 3.9, 4.9 and 9.3, 4-H), 3.69 (3 H, s, OMe), 3.73 (3 H, s, OMe) and 5.16 (1 H, dq, *J* 4.9 and 6.4, 3-H); δ_C 8.5, 17.1, 18.2, 21.0, 28.0, 35.8, 44.5, 51.9, 52.3, 59.9, 69.6, 169.9, 172.1, 173.3 and 205.6; FAB-MS *m*/*z* 339 (M$^+$ + Na, 6%), 317 (M$^+$ + H, 26), 301 (24), 257 (99), 225 (14), 197 (36), 154 (100), 137 (96), 136 (90), 107 (29), 89 (25) and 59 (12).

Alcohol **10**.—A solution of compound **9** (37 mg, 0.12 mmol) and Na_2CO_3 (48 mg, 0.45 mmol) in methanol (1.8 cm^3) was stirred for 48 h at room temperature. The reaction mixture was filtered through Celite and diluted with water (10 cm^3). The solution was adjusted to pH 2.0 and passed through a Sep Pak C_{18} Cartridge (Waters Associates). The cartridge was washed with dil. HCl (20 cm^3) and was then eluted with methanol (20 cm^3). The methanolic solution was concentrated and treated with ethereal diazomethane until a yellow colour persisted. The residue obtained after removal of solvent was purified with TLC [silica gel; EtOAc–hexane–AcOH (30:70:1), triple development] to yield alcohol **10** as an oil (16 mg, 49%); $[\alpha]_D^{24}$ −6.0 (*c* 1.0, EtOH); δ_H 0.82 (3 H, t, *J* 7.5. 9-H$_3$), 1.19 (3 H, d, *J* 6.4, 3-Me), 1.33 (3 H, s, 7-Me), 1.84 (1 H, dq, *J* 14.5 and 7.5, 8-H), 1.97 (1 H, dq, *J* 14.4 and 7.4, 8-H), 2.80–3.01 (2 H, m, 5-H$_2$), 3.71 (3 H, s, OMe), 3.71 (1 H, m, 4-H), 3.72 (3 H, s, OMe) and 3.95 (1 H, m, 3-H); δ_C 8.5, 18.3, 21.0, 27.9, 37.2, 46.8, 51.8, 52.4, 59.9, 67.6, 173.3, 174.1 and 206.1; FAB-MS *m*/*z* 297 (M$^+$ + Na, 85%), 257 (100), 243 (3), 197 (18), 185 (10), 141 (6), 115 (40) and 93 (18).

(R)-*MTPA Ester* **11a**.—A solution of alcohol **10** (3.0 mg, 11 μmol) and (+)-MTPACl (4.2 mm^3, 22 μmol) in pyridine (37 mm^3) was kept for 50 h at room temperature. *N*,*N*-Dimethylpropane-1,3-diamine (4.0 mm^3) was added to the reaction mixture. After 10 min, the solution was diluted with diethyl ether (2.0 cm^3) and washed successively with brine, aq. copper(II) sulfate, brine, aq. $NaHCO_3$ and brine. After drying over Na_2SO_4, filtration and concentration of the solution, the residue was subjected to TLC [silica gel; hexane–EtOAc–AcOH

(30:70:1)] to give (*R*)-MTPA ester **11a** as an oil (3.4 mg, 63%); δ_H 0.826 (3 H, t, *J* 7.4, 9-H_3), 1.280 (3 H, d, *J* 7.8, 3-Me), 1.294 (3 H, s, 7-Me), 1.825 (1 H, dq, *J* 14.2 and 7.4, 8-H), 1.960 (1 H, dq, *J* 14.2 and 7.4, 8-H), 2.592 (1 H, dd, *J* 3.4 and 18.1, 5-H), 3.025 (1 H, dd, *J* 9.8 and 18.1, 5-H), 3.282 (1 H, ddd, *J* 3.4, 5.8 and 9.8, 4-H), 3.494 (3 H, br s, OMe), 3.660 (6 H, s, 2 × OMe), 5.410 (1 H, dq, *J* 5.8 and 7.8, 3-H) and 7.38–7.50 (5 H, m, Ph); FAB-MS *m/z* 491 (M^+ + H, 6%), 257 (100), 225 (14), 197 (23) and 189 (45).

(S)-*MTPA Ester* **11b**.—This compound was obtained in 98% yield from alcohol **10** with (−)-MTPACl by essentially the same procedure as for the preparation of compound **11a**; δ_H 0.811 (3 H, t, *J* 7.3, 9-H_3), 1.267 (3 H, s, 7-Me), 1.346 (3 H, d, *J* 6.4, 3-Me), 1.781 (1 H, dq, *J* 14.2 and 7.3, 8-H), 1.936 (1 H, dq, *J* 14.2 and 7.3, 8-H), 2.475 (1 H, dd, *J* 18.1 and 3.4, 5-H), 2.961 (1 H, dd, *J* 18.1 and 10.3, 5-H), 3.254 (1 H, ddd, *J* 3.4, 5.4 and 10.3, 4-H), 3.527 (3 H, br s, OMe), 3.602 (3 H, s, OMe), 3.680 (3 H, s, OMe), 5.396 (1 H, dq, *J* 5.4 and 6.4, 3-H) and 7.37–7.50 (5 H, m, Ph); FAB-MS *m/z* 491 (M^+ + H, 14%), 257 (100), 225 (12), 197 (24) and 189 (49).

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