Absolute Configuration of the Fungal Metabolite Spirolaxine

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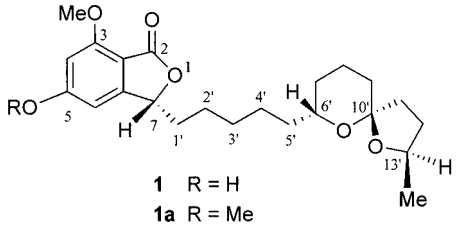
The relative stereochemistry of the four stereocentres of spirolaxine **1**, a bioactive 6,5-spiroacetal phthalide secondary metabolite, was determined through single-crystal X-ray analysis. Its absolute configuration was determined by circular dichroism; the experimental spectrum of spirolaxine is in good agreement with that evaluated by means of DeVoe coupled-oscillator calculations.

Introduction

Spirolaxine **1** is an interesting secondary metabolite that contains a 3-methoxy-5-hydroxy-phthalide nucleus linked to a 6,5-spiroacetal group through a five-membered methylene chain which we previously isolated from cultures of *Sporotrichum laxum* (Basidiomycetes); its structure but not stereochemistry was determined by means of NMR spectroscopy, mass spectrometry and chemical evidence. Together with spirolaxine, MPGA (malt peptone glucose agar) cultures produce the related metabolites, sporotricale and phanerosporic acid.[1]



1 R = H
1a R = Me

Besides spirolaxine **1**, originally discovered as a plant growth inhibitor,[1] two other new 5,5-spiroacetal phthalides, CJ-12,954 and CJ-13,014, have been isolated from cultures of the Basidiomycetae *Phanerochaete velutina*. All these compounds showed anti-*Helicobacter pylori* activity, as described by Dekker et al.,[2] with compound **1** exhibiting twice as much potency as the other spiroacetal compounds. The peculiarity of all these derivatives was their selectivity; in fact when tested against a panel of other microorganisms they did not show any antibacterial activity.[2]

Spirolaxine **1** has also been reported to exhibit cholesterol-lowering activity.[3] More recently we reported spirolaxine activity towards endothelial cells (BMEC and HUVEC) and a variety of tumor cell lines (i.e., LoVO and HL60).[4–6]

Owing to the interest shown in such molecules, and with the future development of a chiral synthetic route to compound **1** and its derivatives in mind, we decided to determine the stereochemistry of the four stereocenters of **1**. We were able to reisolate the metabolite, but found some difficulty in the crystallization of both the natural compound and a number of its derivatives (methyl ether, acetate or benzoate). HPLC analysis showed the presence of a minor isomer: after preparative HPLC, which gave pure spirolaxine, we were able to obtain crystals suitable for X-ray analysis.

Results and Discussion

Relative Configuration

Single-crystal X-ray analysis of **1** was performed in order to confirm its molecular structure and in particular to assign the relative configurations of the four chiral carbon atoms present in the molecule and labelled in Figure 1 as C7, C6′, C10′ and C13′. The relative configurations of these four centres were found to be the same. In Figure 1 they all appear as *R**, which is consistent with evidence from circular dichroism (CD) and other data (see section Absolute Configuration).

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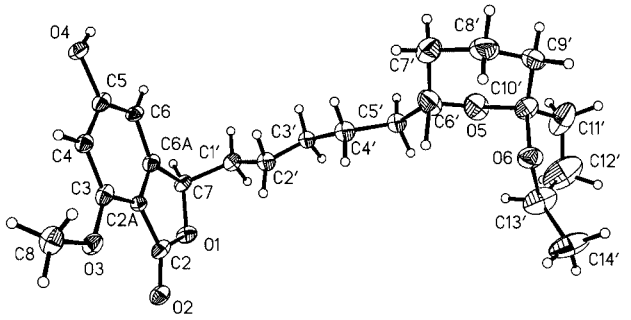


Figure 1. View of the ordered independent molecule of **1** showing an arbitrary atomic labelling scheme.

Crystallization of spirolaxine always generated twinned crystals instead of single crystals, one half of the twin being related to the other by the interchange of the *a* and *c* axes.

Two independent molecules are present in the unit cell: one of the two molecules shows conformational disorder along the methylene chain (see Figure 2). This form of disorder is associated with two possible conformations of the aliphatic chain: a planar *all-trans* conformation, as observed in the nondisordered molecule, and a mixed *trans-gauche* sequence of torsion angles, as observed in the main conformer (site occupation factor of 60%) of the disordered molecule.

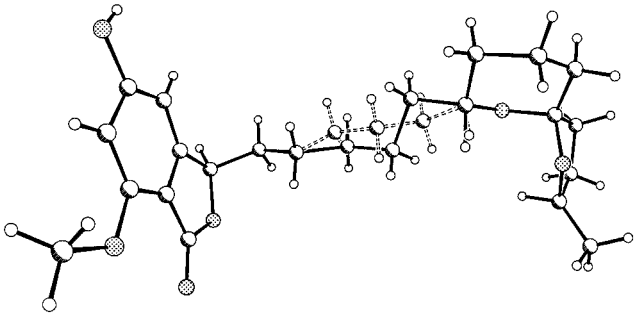


Figure 2. View of the disordered independent molecule of **1**.The dashed bonds link atoms with an occupation factor of 40%.

The pyranosyl ring in both the molecules exhibits the expected $^4C_1$ chair conformation [ring-puckering parameters:[7] Q_T = 0.505(14) and 0.541(14) Å, θ_2 = 6.0(1) and 5.5(1)°, and φ_2 = 4(1) and 0(1)° for the two independent molecules, respectively]. The five-membered ring adopts the envelope conformation [ring-puckering parameters: q_2 = 0.29(1) and 0.36(1)° and φ_2 = 168 (4) and 166(3)° for the two independent molecules, respectively].

The crystal structure shows the formation of well-defined layers: the molecules are positioned with the side-chain nearly parallel to the *b* axis, while the plane defined by the nearly coplanar atoms of the phthalide group are oriented parallel to the *ac* plane (Figure 3). The crystal packing is stabilized by intermolecular O4–H···O2 hydrogen bonds with the connecting phthalide planes translated by a *c* lattice vector. A network of weaker intra- and intermolecular C–H···O-type hydrogen bonds provides additional crystal stabilization.

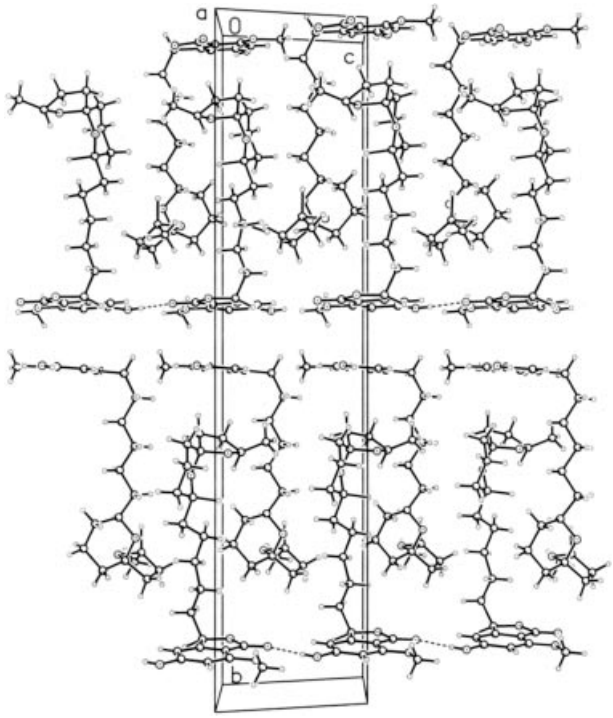


Figure 3. A portion of the crystal packing of **1** viewed down the *a* axis, showing the hydrogen bonds that link the adjacent phthalide groups along the [001] direction.

Absolute Configuration

In order to predict the absolute configuration at C-7 in spirolaxine **1** (and consequently the chirality of the carbon atoms C-6′, C-10′ and C-13′) we performed a CD calculation employing the DeVoe treatment of the coupled-oscillator theory.[8] Spirolaxine **1** is a suitable molecule for this treatment given its strong electric-dipole-allowed UV transitions [$\lambda_{EtOH,max.}$ = 216, 259 nm (ε = 27550, 13800 L mol^{-1} cm^{-1}), plus a weaker band at 291 nm (ε = 6300 L mol^{-1} cm^{-1})]. These electronic transitions are commonly observed in "benzoate" chromophores.[9]

The ground-state conformation of **1** (with all the stereocenters having the *R* configuration) was calculated by using the semi-empirical AM1 Hamiltonian,[10] while its electronic transitions were calculated by means of the semi-empirical ZINDO method,[11] which can be considered an improved version of the previously largely used CNDO/S program.[12] In this way we predicted a set of transitions for **1** at 211 (f = 0.65), 218 (f = 0.72) and 255 nm (f = 0.39), plus a long-wavelength transition at 294 nm (f = 0.03), in good agreement with the experimental UV spectrum of spirolaxine. The frequency, the intensity and the directions of these three electric-dipole transitions were used in the DeVoe calculations (note that the weak transition at 294 nm, being partly of n–π* origin,[9] was neglected in the CD calculations). To these transitions we added an oscillator for each C–H, C–C and C–O bond to account for the far-UV transitions of the aliphatic and the acetalic moieties of the spirolaxine molecule. The parameters defined elsewhere[13]

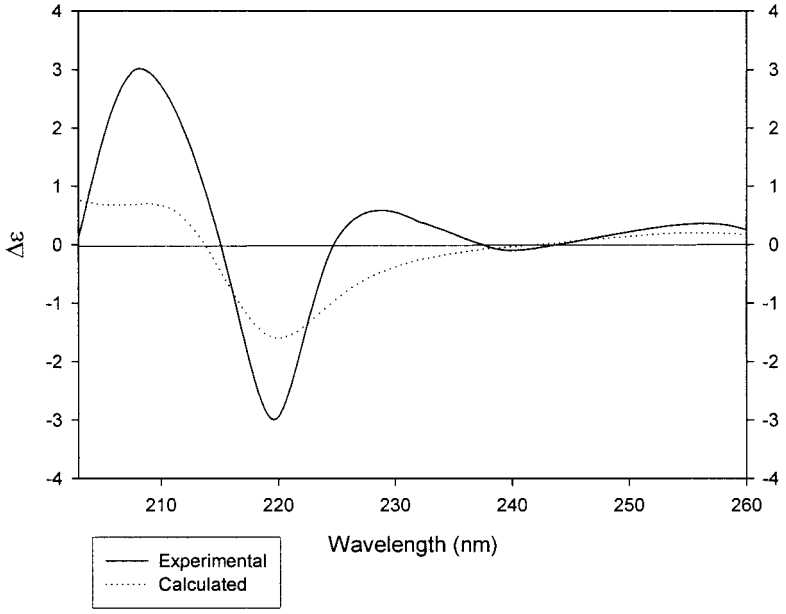
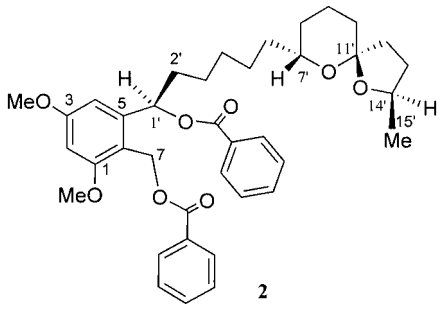


Figure 4. The calculated and experimental CD spectra of spirolaxine **1**.

were used to reproduce the C–C and C–H electric-dipole transitions, while the parameters of ether C–O transitions were again calculated by means of the ZINDO method.

The result is shown in Figure 4; it can be seen that the DeVoe-calculated CD spectrum matches the experimental spectrum well in the 205–255 nm region, both in the signs and in the intensities of the bands. Only the weak CD effect observed at about 235 nm ($\Delta\varepsilon$ = +0.4 L mol^{-1} cm^{-1}) does not have a theoretical counterpart: in the calculated spectrum this CD band appears to merge into the much more intense negative band at 220 nm.

Compound **1** was next methylated to obtain **1a**. Treatment of this compound with excess diisobutylaluminium hydride (DIBAH) and then with benzoyl chloride/pyridine caused reduction of the phthalide nucleus to give the dibenzoate derivative **2** (see Expt. Sect.).



An extensive CD calculation, similar to that described above, on a molecule as large as **2** was not feasible. We therefore performed a DeVoe calculation that included only the two long-axis benzoate transitions (which occur at 227 nm): this is equivalent to the application of the dibenzoate chirality rule, which has been successfully applied a number of times in the prediction of the absolute configuration of natural products.[14] After structure minimization using the AM1 Hamiltonian, the DeVoe-simplified calculation could reproduce the observed experimental CD spectrum of **2** (Figure 5; solvent: EtOH), which is dominated by a strong negative exciton couplet in the 210–240 nm region. The predicted negative couplet results from the *M* (negative) helicity of the dibenzoate system.

In conclusion, the *R* configuration at C-7 in spirolaxine is well-supported by CD calculations; consequently the absolute chirality of the four chiral centers in **1** is 7*R*, 6′*R*, 10′*R* and 13′R.

As further confirmation of the chirality sequence, the absolute configuration at C-14′ of phanerosporic acid, obviously related in the biogenetic pathway to spirolaxine, was established as *R*.[15]

Origin of the Strain

The DNA sequence of the fungal strain that produces spirolaxine **1** (obtained in 1989 from Centraal Bureau voor Schimmel Cultures, CBS n. 578.63) was determined by us and has been deposited at the GenBank; the CBS has now reclassified the strain 578.63 as *Phanerochaete crysosporium*. In our hands this new strain, which possesses the same DNA sequence, was unable to produce the metabolite **1**.[16]

The American Type Cultures Collection (ATCC) deposited the CBS strain as *P. pruinosum* with the serial number ATCC 1515.

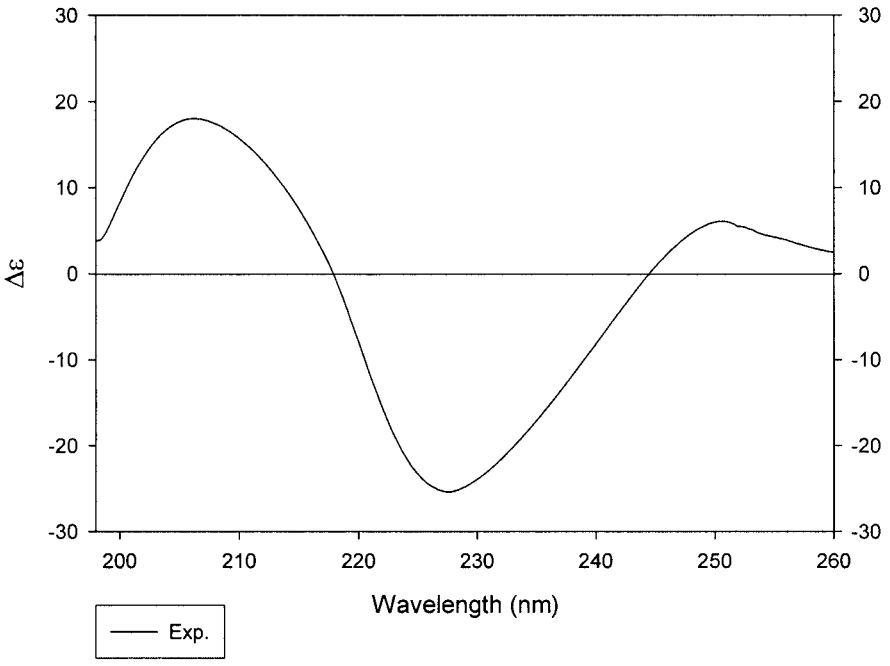


Figure 5. The experimental CD spectrum of compound **2**.

Experimental Section

General Remarks: UV absorption spectra of the compounds under study were measured in 95% EtOH. Mass spectra were obtained with a Finnigan-MATT-TSQ 70ev spectrometer. NMR spectra were measured with a Bruker ARX 400 spectrometer operating at 400 MHz with Me_4Si as the internal standard. CD spectra were recorded with a Jasco 500A dichrograph. HPLC analyses were performed using a LiChroCART RP-18 column (Merck) on an Agilent 1100 instrument. Flash column chromatography was performed on Merck silica gel, TLC and PLC with Merck HF_{254} silica gel. The purity of the products was checked by TLC, NMR spectroscopy and MS and was deemed sufficient for the purpose of structural determination.

Isolation and Purification of Compound 1: Spirolaxine **1** was isolated from MPGA (malt peptone glucose agar, 20:4:20:15 g L^{-1}) cultures (30 Roux flasks) of *Sporotrichum laxum* as described previously.[1] The crude EtOAc extracts (1.5 g) were purified by flash chromatography on a column of silica gel using CH_2Cl_2/MeOH (25:1) as eluent. PLC using EtOAc/hexane (1:1) as eluent gave compound **1** (250 mg); HPLC: water/acetonitrile (40:60); flow rate = 0.5 mL min^{-1}; retention time = 12.62 min. Pure compound **1** (20 mg) was subjected to chromatography on a KR 100-10-C-18 (Kromasil) preparative column and successively crystallized slowly from acetone. CD (EtOH, c = 0.02): λ = 208, 220, 232, 255 and 283 nm ($\Delta\varepsilon$ = +1.8, –1.8, +0.24. +0.23 and –0.7 L mol^{-1} cm^{-1}).

Compound 2: Compound **1** (100 mg) was methylated with MeI/K_2CO_3/acetone to obtain 7-methylspirolaxine **1a**. EI-MS: m/z = 418 $[M]^+$. DIBAH (4.4 mL of a 1 M hexane solution) was added to a solution of **1a** (70 mg) in dry CH_2Cl_2 (5 mL) at 0 °C. After stirring for 5 h the mixture was poured into ice/dilute HCl and the product extracted with EtOAc. Removal of the solvent left a crude oil which was dissolved in dry pyridine (1 mL) and treated with benzoyl chloride (0.5 mL). Usual work up and PLC in CH_2Cl_2/MeOH (50:1) gave compound **2** as an oil (35 mg). EI-MS: m/z (%) = 630 (8) $[M]^+$, 386 (70), 286 (28) and 105 (100). UV: λ = 203, 261 and 339 nm (ε = 22360, 10730 and 5100 L mol^{-1} cm^{-1}). CD (EtOH, c 0.018): λ = 206, 228, 253 and 347 nm ($\Delta\varepsilon$ = +18.03, –25.4. +5.3 and +2.65 L mol^{-1} cm^{-1}). ^{1}H NMR ($CDCl_3$): δ = 8.1–7.3 (m, 10 H, ArH), 6.64 and 6.43 (d, J = 2.2 Hz, 2 H, H-2 and H-4), 6.13 (dd, J = 8.8 and 4.6 Hz, 1 H, H-1′), 5.68 and 5.60 (d, J = 12 Hz, 2 H, H-7), 4,11 (m, 1 H, H-14′), 3.81 (s, 6 H, 2×OMe), 3.62 (m, 1 H, H-7′), 2.2–1.0 (m, 20 H, 10×CH_2), 1.22 (d, J = 6.2 Hz, 3 H, H-15′) ppm.

X-ray Crystallography: Many attempts were required to crystallize compound **1**, and a number of different crystallization procedures were tested. The crystals used in the diffraction analysis were grown from acetone solution. All the crystals examined were found to be twinned. Diffraction data were collected on a Siemens P4 diffractometer using graphite-monochromated (Cu-K_α) radiation (λ = 1.54179 Å) and the $\theta/2\theta$ scan technique. Unit cell parameters were determined by least-squares refinement of the optimized setting angles of 58 reflections in the range $6.7 \leq \theta \leq 26.1°$. A total of 6160 reflections, 5310 unique (R_{int} = 0.045), were collected up to θ = 63.5°. Three standard reflections were monitored every 250 measurements to check the stability of the crystal. No significant intensity decay was observed.

Crystal data: $C_{23}H_{32}O_6$, M = 404.5, colorless prismatic crystal (0.5×0.4×0.2 mm), monoclinic, space group $P2_1$, a = 8.084(3), b = 35.117(5) and c = 8.115(3) Å, β = 107.46(5)°, V = 2197.6(9) $Å^3$, Z = 4, D_c = 1.223 g cm^{-3}, $F(000)$ = 872, μ ($Cu_{K\alpha}$) = 0.71 mm^{-1}.

Structure Solution and Refinement: The molecular structure was solved by direct methods (using the SIR97 program[17]) but at the beginning it was impossible to refine the model to an acceptable R value and the quality of structural parameters was relatively poor because of the twinned nature of the crystals. In the end, with the data of a particularly favorable crystal, refinement turned out to be possible using the SHELXL program[18] after determining the mutual orientation and the fractional contribution of each of the components of the twinned crystal. It was found that the two individual components of the twins were related by the interchange of the a and c axes according to the matrix (001 010 100). Taking this twinning-law into account substantially improved the refinement

and the R value dropped from 18 to 8%. A ratio of 0.65:0.35 between the volume of the two components was obtained by least-squares refinement. Two independent molecules are contained in the unit cell; one of these shows conformational disorder along the methylene chain. Owing to this form of disorder, the convergence of the refinement was not completely satisfactory. The hydrogen atoms were located at idealized positions with isotropic temperature factors (1.2 or 1.5 times the temperature factors of the attached atom). The last refinement on F^2, with anisotropic temperature factors for non-hydrogen atoms, was carried out separately on each of the two molecules. The final stage of the refinement, for the 542 total parameters, converged to $R_1 = 0.0764$ for 3774 observed reflections [$I > 2\sigma(I)$] and to $R_1 = 0.1133$ for all 5310 unique reflections. The final difference map showed maximum and minimum residual peaks of 0.183 and –0.170 e Å^{-3}, respectively.

With no atoms heavier than oxygen in the structure, the absolute configuration could not be reliably determined because of the absence of significant anomalous dispersion effects. Indeed, for these structures the absolute structure parameter x-Flack[19] is at best weakly indicative of the correct absolute configuration. In the case of spirolaxine the Flack parameter has an inconclusive value of 0.3 (0.6) due to the twinning found in the single crystal used for the data collection and to the disorder found in the molecular structure.

CCDC-250914 contains the crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Details of Calculations: Ground-state geometry minimizations were performed by using the AM1 semi-empirical method[20] as provided in Gaussian98.[21] The electronic transitions were calculated by means of the ZINDO-1 program,[11] again within the Gaussian98 package, using the molecular geometries previously optimized by the AM1 method. The maximum number of states (10) was used in all ZINDO computations. CD theoretical calculations were carried out by means of a computer program based on the DeVoe equations.[22] For details of the geometrical and the spectroscopic input data, see ref.[8].

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