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Pestacin: a 1,3-dihydro isobenzofuran from *Pestalotiopsis microspora* possessing antioxidant and antimycotic activities

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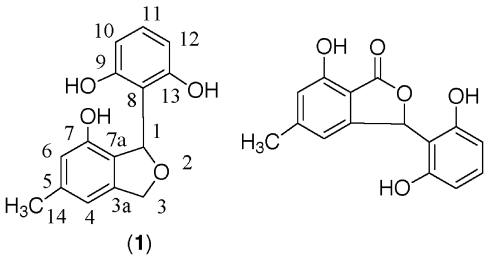
Abstract—*Pestalotiopsis microspora*, an endophytic fungus native to the rainforest of Papua New Guinea, produces a 1,3-dihydro isobenzofuran. This product, pestacin, is 1,5,7-trisubstituted and exhibits moderate antifungal properties and antioxidant activity 11 times greater than the vitamin E derivative trolox. Antioxidant activity is proposed to arise primarily via cleavage of an unusually reactive C–H bond and, to a lesser extent, through O–H abstraction. Isolation of pestacin was achieved by extraction of culture fluid with methylene chloride followed by silica gel chromatography. Structure was established by X-ray diffraction and ^{13}C and ^{1}H NMR. The X-ray data demonstrate that pestacin occurs naturally as a racemic mixture. A mechanism for post-biosynthetic racemization is proposed.

1. Introduction

Endophytic fungi and bacteria are organisms living within the tissues of host plants. Typically, endophytes coexist with their hosts without any pathogenic symptoms. These organisms have proven to be an unusually rich source of novel bioactive natural products. One of the most widely distributed endophytic fungi is *Pestalotiopsis microspora*.[1] This microorganism produces an array of bioactive organic substances including the anticancer drug taxol.[2] However, because of the enormous genetic diversity of *P. microspora*, each individual isolate is generally unique in the substances that it produces. One of the recently described compounds from *P. microspora* is isopestacin, a bioactive isobenzofuranone.[3] Additional analysis of the culture fluid of this particular fungus has now provided a second compound, pestacin (**1**) shown in Figure 1. Pestacin is a 1,3-dihydro isobenzofuran whose structure is reported here from X-ray and NMR analysis. Pestacin occurs naturally as a racemic mixture and exhibits potent antioxidant activity. Herein, we propose a mechanism for antioxidant activity and suggest a post-biosynthetic racemization path.



(**1**)

Figure 1. Pestacin (**1**) showing the numbering used and the related compound isopestacin.

2. Results and discussion

2.1. X-Ray and NMR analyses

The structure of (**1**) was established by X-ray diffraction of a crystal grown from a CH_2Cl_2 solution. Pestacin crystallizes in the $P\bar{1}$ space group and contains one molecule per asymmetric unit (Fig. 2). Three other naturally occurring 1,3-dihydro isobenzofurans have been previously observed, however, (**1**) represents the first 1,5,7 trisubstituted natural product.[4,5] Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication numbers CCDC 194690. Copies of the data may be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (fax: +44-1223-336033 or e-mail: deposit@ccdc.cam.ac.uk). Other crystallographic information is included in Section 4.

Keywords: endophyte; isobenzofuran; antioxidant; pestacin; isopestacin; antifungal; quinone methide.

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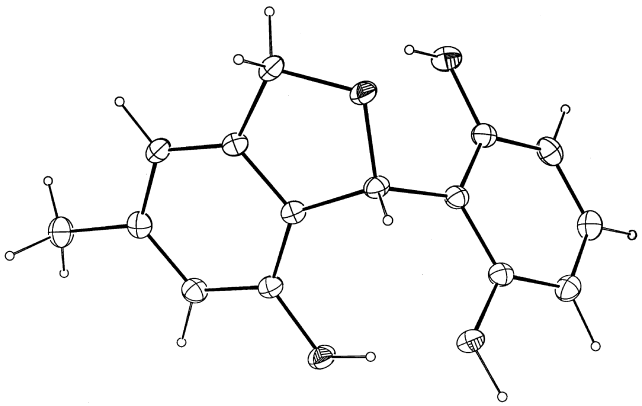

Figure 2. The asymmetric unit of (**1**) showing molecular conformation in the solid.

Table 1. Pestacin ^{13}C and ^{1}H NMR shift assignments

Position	$\delta\,^{13}C$	$\delta\,^{1}H$
1	79.90	6.79 (s)
3	74.39	5.01 (d, J=11.3 Hz) 5.33 (d of d, J=11.3, 2.7 Hz)
3a	142.51	–
4	113.56	6.53 (s)
5	140.78	–
6	116.32	6.44 (s)
7	152.63	–
7a	124.83	–
8	114.31	–
9	157.43	–
10	108.97	6.32 (d, J=8.2 Hz)
11	130.18	6.91 (t, J=8.2 Hz)
12	108.97	6.32 (d, J=8.2 Hz)
13	157.43	–
14	21.39	2.24 (s)

Reference NMR ^{13}C and ^{1}H data were collected using the 2D INADEQUATE and HMQC techniques, respectively, as shown in Table 1. Since other structural data are known from X-ray analysis, these two NMR experiments alone are sufficient to unambiguously establish all shifts. NMR parameters used are included in Section 4.

2.2. Racemization in pestacin

Pestacin occurs naturally as a racemic mixture of 1*S* and 1*R* enantiomers as indicated by its crystallization into a centrosymmetric space group. This racemization likely occurs post-biosynthetically as enzyme mediated reactions occur with stereospecificity. A racemization mechanism that proceeds through a cationic intermediate is thus proposed (Fig. 3). This intermediate has the desirable feature of being stabilized by seven resonance structures. These intermediates are further stabilized by their ability to tautomerize into the three neutral *ortho*-quinone methide-like structures shown in Figure 4 (**1a**–**c**). A similar cationic intermediate has been previously demonstrated to occur in 1-phenyl 1,3-dihydro isobenzofuran,[6] lending support to the proposed racemization mechanism. The racemization of 1-aryl substituted 1,3-dihydro isobenzofurans may also occur generally as suggested by the complete absence of stereochemical data for 1-aryl substituted synthetic products despite numerous studies.[7–13] In addition, isopestacin occurs naturally as a racemic mixture,[3] supporting the generality of this racemization process.

2.3. In vitro antioxidant activity and a proposed mechanism

The similarity of (**1**) to isopestacin, a known antioxidant,[3]

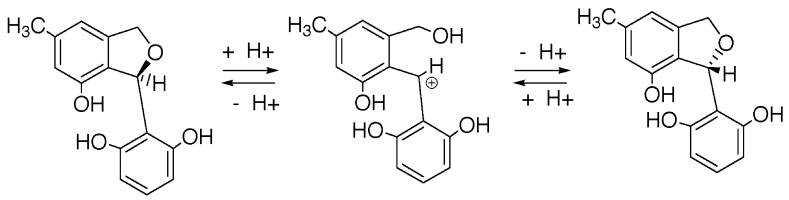

Figure 3. Proposed racemization mechanism in pestacin.

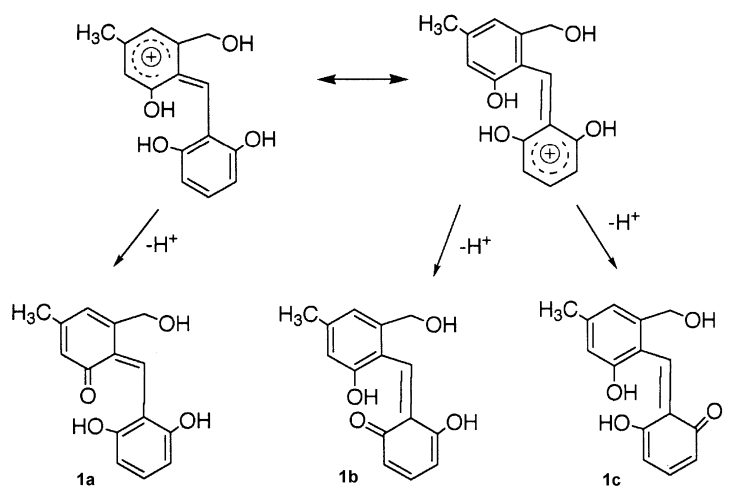

Figure 4. Postulated stabilization of the cationic intermediate in (**1**) involving seven resonance stabilized forms and three neutral *o*-quinone methide-like structures accessible through tautomerization.

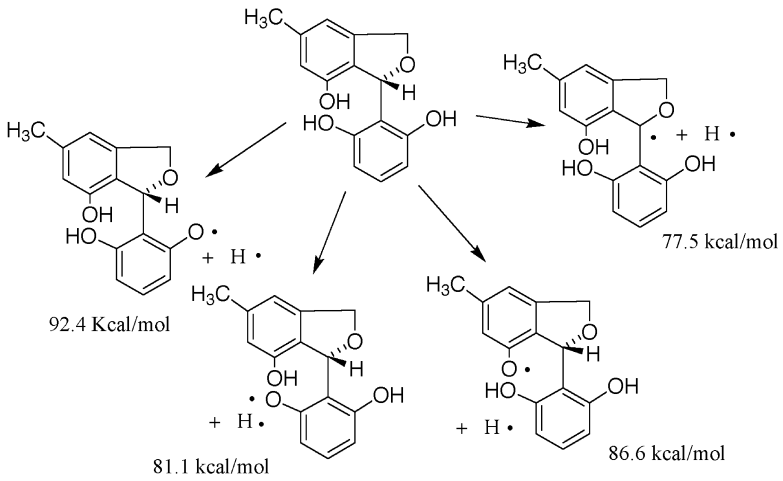

Figure 5. Computed bond dissociation enthalpies for a variety of possible H-abstractions in (**1**). Stereochemistry shown is arbitrary as (**1**) occurs naturally as a racemic mixture.

suggests that (**1**) may also exhibit such activity. Pestacin was thus analyzed using the total oxyradical scavenging capacity (TOSC) assay. This analysis assesses a compound's ability to inhibit a free radical initiator's oxidation of α-Keto-γ-methiolbutyric acid to release ethylene gas.[14,15] Compounds with antioxidant properties thus decrease the production of ethylene gas, as measured by gas chromatography. Compound (**1**) required a 1.7 ± 0.1 mM solution to reduce the effects of the free radical initiator by 50%. In contrast, the water-soluble vitamin E derivative, trolox, required an 18.8 ± 0.9 mM solution to exert the same effect. Pestacin, therefore, appears to be an extremely effective antioxidant as measured by this assay.

A mechanism for antioxidant activity is proposed based on the structure of (**1**). Most phenolic antioxidants are thought to function by interrupting free radical chain reactions to form non-radical products.[16] This chain-breaking step relies upon the relatively low bond dissociation enthalpy (BDE) of the O–H bond in reactions with reactive oxygen species (ROS) as shown below:

$$ROS^{\bullet} + ArOH \rightarrow ROS{-}H + ArO^{\bullet}$$

The $ArO^{\bullet}$ thus generated may then combine with other radicals to form the non-radical species. Presumably, other reactive X–H bonds function in a like manner. Accordingly, measurement or theoretical prediction of O–H BDE's has proven useful in assessing antioxidant activity.[17] Unfortunately, some molecules contain multiple OHs making it difficult to experimentally establish which O–H bond is mechanistically relevant. Further complicating analyses is the presence of other potentially active groups such as a labile C–H bond in curcumin.[18] Only theoretical studies are able to circumvent this difficulty by allowing separate analysis of BDE for each potentially labile bond. Recent progress has made very accurate BDE computations feasible with reported errors of less than ±1 kcal/mol.[19] Such computed BDEs have also been demonstrated to correlate well with experimental antioxidant activity.[17] A mechanism for antioxidant activity of (**1**) is therefore proposed based on calculated BDEs using the medium-level model 2 of DiLabio et al.[19]

The BDEs of several bonds in (**1**) were computed including the O–H bonds at C7, C9, C13 and the C–H bond at C1 (see Fig. 5). The C1–H bond is included in the calculation because of the ease with which this proton is lost in the racemization process previously described. Among model compounds,[17] phenol is regarded as a poor antioxidant with a computed BDE of 87.1 kcal/mol while α-tocopherol, with a BDE of 75.8 kcal/mol, is a very efficient antioxidant. Trolox, which lacks the $C_{16}H_{33}$ phytl tail of α-tocopherol, is estimated to have BDE approximately equal to α-tocopherol since Wright has demonstrated that the removal of this moiety has a negligible effect on BDE.[17] Of the four bonds considered in (**1**), the C1–H has the lowest calculated BDE (77.5 kcal/mol) with a value approximately equal to that of α-tocopherol. Abstraction of this H is also sterically feasible as shown in Figure 2. This unusual result suggests that the antioxidant properties of (**1**) arise largely from the mechanism shown in Figure 6 in which direct abstraction of the hydrogen at C1 occurs. This mechanism is preferred over the next best path (abstraction of the C13O–H proton) at a rate in excess of 400 times that of the closest competitor under the assumption of comparable A factors in the

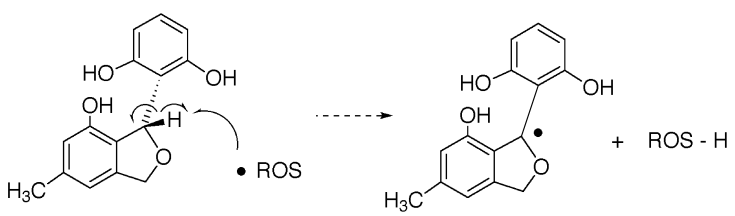

Figure 6. Proposed primary mechanism for antioxidant activity in (**1**). Computed bond dissociation enthalpies indicate that this mechanism is the thermodynamically most favored route for activity in (**1**). However, secondary activity also would be possible via O–H abstraction (see Fig. 7).

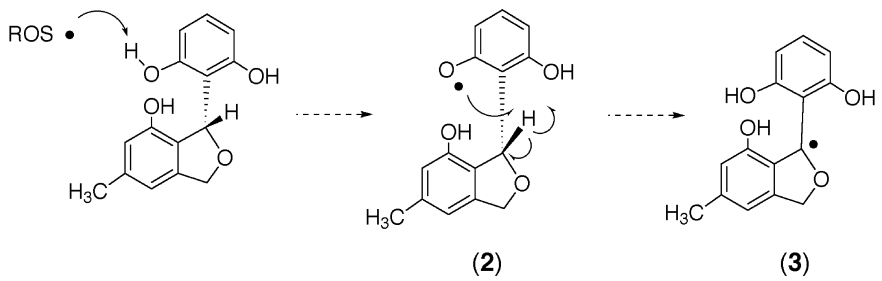

Figure 7. Proposed secondary mechanism of antioxidant activity in (**1**). Additional activity is proposed to occur via direct abstraction of the benzylic hydrogen as shown in Figure 6.

Arrhenius equation. However, the BDEs for H abstraction from O–H groups still suggest that such processes could be viable.

The additional contribution to antioxidant activity from O–H abstraction is most probable at the C13O–H where a BDE (81.1 kcal/mol) is observed. This BDE value is comparable with the well-known antioxidants curcumin[18] and 2-butyl 4-hydroxy anisole.[17] This secondary mechanism involves a postulated reaction with ROS to provide an oxygen radical species (**2**), which is converted to a stabilized, doubly benzylic radical, (**3**). This five-centered radical abstraction mechanism is given in Figure 7.

Activity at other hydroxyl moieties is surprisingly varied. For example, abstraction at the C9O–H, which is structurally similar to C13O–H, is not favored with a BDE of 92.4 kcal/mol. Careful examination of C9O–H cleavage reveals that removal of this hydrogen breaks a hydrogen bond with O2 (see Fig. 2) and thus makes this process unfavorable. It should be noted, however, that the present computations are for the gas phase and inclusion of solvation may make abstraction at this position more feasible. Abstraction of the C7O–H is also unfavored due to hydrogen bonding to the oxygen at C13. According to the work of Wright,[17] the C13O–H should compensate by reorienting to become a hydrogen bond donor with the newly formed C7O$^{\bullet}$ radical. However, the C13O–H bond would have to reorient to a position out of the plane of the aromatic ring, an energetically costly conformation (by roughly 4 kcal/mol). Thus, abstraction of the O–H at C13 appears to be strongly preferred over other hydroxy's when solvation is neglected.

Based on the computed BDE values, we propose a bimodal antioxidant mechanism for (**1**) in which abstraction at the C1–H bond is most probable with additional activity obtained from O–H abstraction, predominantly at the C13O–H position. This view is consistent with the experimental data since abstraction solely from the C1–H position would be expected to yield an activity comparable to α-tocopherol. In fact, the observed activity in (**1**) is 11 times greater. The previously described alternative antioxidant mechanism involving a single electron transfer was not investigated here as Wright has demonstrated that in most cases H-atom transfer is preferred.[17]

2.4. Antifungal activity

Pestacin was analyzed for antifungal activity using previously described methodology.[3] Inhibition of *Pythium ultimum*, an important root-invading pathogen, was observed with a minimum inhibitory concentration of approximately 10 μg/mL. This level of activity is slightly greater than that previously observed for isopestacin.[3] Additional bioactivities are suggested from the known properties of quinone methides as antitumor drugs, antibiotics and DNA alkylators.[20–22]

3. Conclusions

The isolation and structural characterization of the potent antioxidant pestacin (**1**) are reported. The presence of a unique doubly benzylic carbon in (**1**) is postulated to account for both the strong antioxidant activity and the observation of racemization. Future work on synthetic derivatives of (**1**) may hold promise for improving antioxidant and other biological activities. In particular, preparation of derivatives should be possible given the well-documented reactivity of *o*-quinone methides with nucleophiles.[23] Additionally, 1-aryl-1,3-dihydro isobenzofurans are known to undergo electrophilic substitution when treated with alkali metals under proper conditions.[8] Further studies on (**1**) and related 1-aryl-1,3-dihydro isobenzofurans are therefore warranted in an effort to realize the full potential of these efficient antioxidant compounds.

4. Experimental

P. microspora (isolate 12–30) was grown for 3 weeks in still culture at 23°C on the M1D medium.[3] The medium, free of the fungal mycelium, was extracted twice with equal volumes of methylene chloride and dried by rotary evaporation. The residue was dissolved in a minimal amount of chloroform and passed through a column of silica gel (2.5 cm, 40 μm particle diameter). Chloroform was then passed over the column and (**1**) eluted in the 100 mL fraction (±20 mL). Elution of (**1**) was determined by silica gel TLC and fractions containing partially purified (**1**) combined. Final purification was achieved on the column described above using chloroform/acetonitrile (100/1 v/v) with elution of (**1**) occurring at 60–75 mL. Thin layer chromatography of (**1**) was performed on 0.25 mm Merck silica gel plates with the following R_f values for each system; chloroform/methanol (9/1 v/v), 0.59; chloroform/acetonitrile (6/3 v/v), 0.78; choloroform/ethyl acetate (9/1 v/v), 0.32. Visualization of (**1**) on TLC plates was achieved under shortwave UV and also by

spraying with a 1% solution of vanillin in sulfuric acid followed by gentle heating to form a reddish spot.[24] Crystals of (**1**) were obtained from slow evaporation of a methylene chloride solution at 23°C. The yield of (**1**) ranged from 16–20 mg/L of culture fluid.

Pestacin has a molecular weight of 258.08921, as determined by high-resolution mass spectroscopy, consistent with a molecular formula of $C_{15}H_{14}O_4$. An uncorrected melting point of 179–182°C was measured and UV absorption peaks were observed at 232 and 275 nm (in methanol), with molar extinction coefficients of 3480 and 3132, respectively.

X-Ray analysis of (**1**) was performed using a colorless thin plate (0.30×0.23×0.03 mm^3) mounted on a glass fiber with traces of viscous oil. A Nonius Kappa CCD diffractometer using Mo Kα radiation (λ=0.71073 Å) collected 10 frames of data at 200±1 K with an oscillation range of 1°/frame and an exposure time of 20 s/frame.[25] Indexing and unit cell refinement based on all observed reflections from those 10 frames, indicated a triclinic *P* lattice. A total of 3990 reflections (θ_{max}=27.5°) were indexed, integrated and corrected for Lorentz polarization and absorption effects using DENZO-SMN and SCALEPAC.[26] Post refinement of the unit cell gave *a*=4.5431(5) Å, *b*=10.4265(10) Å, *c*=13.5157(16) Å, α=71.102(5), β=84.634(5), γ=87.145(7) and *V*=602.93(11) $Å^3$. Axial photographs and systematic absences were consistent with the compound having crystallized in the triclinic space group $P\bar{1}$. Structure was solved by a combination of direct and heavy atom methods using SIR 97.[27] All of the non-hydrogen atoms were refined with anisotropic displacement coefficients. Hydrogen atoms were located and refined isotropically using SHELXL97.[28]

Carbon and ^1H NMR shift assignments were established using the INADEQUATE and HMQC experiments, respectively, and are consistent with the X-ray structural determination of (**1**). All experiments were performed on 73 mg of (**1**) dissolved in 0.2 mL of CD_3OD and placed in a Shigimi tube designed to center the sample inside the coil with glass plugs susceptibility matched to the solvent extending beyond the coil. Analyses were performed on a Varian INOVA 500 MHz spectrometer. Spectral widths of 20.0 kHz were used in both dimensions of the INADEQUATE analysis with digital resolutions of 156.3 and 0.2 Hz/point acquired in the evolution and acquisition dimensions, respectively. Other parameters included a ^{13}C 90° pulse width of 4.8 μs, a temperature of 26°C, a $^1J_{CC}$ value of 65 Hz, and a collection of 128 evolution increments of 256 scans each. The INADEQAUTE spectrum was referenced to the central line of CD_3OD at 49.15 ppm and all shift assignments established using a computer program designed to enhance signal detection.[29,30] The HMQC spectrum was collected with ^{13}C and ^1H spectral widths of 20.0 and 4.5 kHz, respectively, and digital resolutions of 156.3 and 0.02 Hz/point collected for the ^{13}C and ^1H dimensions. Analysis was performed at 26°C with 128 evolution increments of 64 scans each, collected using ^{13}C and ^1H 90° pulse widths of 15.2 and 4.7 μs, respectively. The spectrum was referenced to the CD_3OD ^1H and ^{13}C signals at 3.31 and 49.15 ppm, respectively.

Antioxidant activity of (**1**) was assessed by dissolving 8.7 mg of (**1**) in 200 μL of DMSO then diluting the solution 1:10, 1:100, and 1:1000 in DMSO. A 10 μL volume of each sample was then combined in a 10 mL vial with 100 μL of 100 mM potassium phosphate buffer, 100 μL of 2 mM α-keto-γ-methiolbuthric acid (KMBA), and 690 μL of H_2O. Control samples were also prepared containing 100 μL of 100 mM potassium phosphate buffer, 100 μL of 2 mM KMBA, and 700 μL of H_2O. The sealed samples were incubated at 37°C for 5 min followed by injection of 100 μL of the free radical initiator, 2,2′-azobis(2-methylpropionamidine) (ABAP). Peroxyl radicals, generated by thermal homolysis of ABAP at 37°C, then oxidized KMBA to produce ethylene gas.[14,15] Ethylene production was monitored by gas chromatography of 1 mL aliquots removed every 12 min over a 96-min period. Chromatographic analysis was performed on a Hewlett–Packard 5890A gas chromatograph equipped with a Supelco 6-foot Porpak Q packed column, a flame ionization detector, and employing helium as a carrier gas (30 mL/min). The inlet, oven and detector temperatures used were 160, 60, and 220°C, respectively. Data analysis was performed on a Hewlett–Packard Chemstation using integrated peak area. All analyses were repeated four times and the results averaged.

All bond dissociation enthalpies were computed according to the MLM2 method of DiLabio et al.[19] Computations were performed using Gaussian 98[31] with parallel processing.

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