# Antifungal Flavonoids from *Hildegardia barteri*

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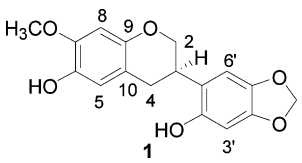
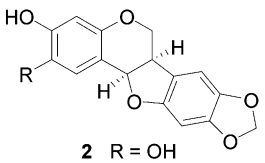
A new isoflavan, (3*R*)-6,2′-dihydroxy-7-methoxy-4′,5′-methylenedioxyisoflavan, hildegardiol (**1**), and two known flavonoids, 2-hydroxymaackiain (**2**) and farrerol (**3**), were isolated from the antifungal root extract of *Hildegardia barteri*. The pterocarpan **2** was largely responsible for the observed antifungal activity.

In the last two decades, the number of immunocompromised hosts with systemic fungal infections has grown alarmingly. Patients with such a defect in their self-defense system are principally those treated with immunosuppressive drugs, such as cancer and organ transplant patients, or those with acquired immunodeficiency syndrome (AIDS). This growing number of patients, coupled with increasing antibiotic resistance in bacteria and fungi, has created an urgent need for new, potent antimicrobial drugs. In our continuing effort to find new antifungal natural products,[1,2] the modest inhibition of azole-resistant *Candida albicans* by an extract of *Hildegardia barteri* (Mast.) Kosterm. (Sterculiaceae), coupled with rather low cytotoxicity against human CEM-SS cells, attracted our attention.

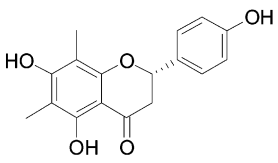
*H. barteri* is a fast-growing African tropical pioneer tree.[3] Its seeds are consumed as raw or roasted nuts, and a nutritional study demonstrated the presence of long chain triglycerides (C-46 to C-50) and cyclopropenoid acids in low concentration.[4] From the organic extract of the roots, we isolated a new isoflavan named hildegardiol (**1**), together with the known pterocarpan 2-hydroxymaackiain (**2**)[5] and the flavanone farrerol (**3**).[6]




**1**




**2** R = OH
**4** R = H




**3**

Our laboratory uses a preliminary analysis/dereplication scheme based on HPLC separation of a crude extract and evaluation by multiple detectors: ELSD, UV/vis, MS, and bioassay of fractions collected in microtiter plates. The UV and mass spectroscopic data of active fractions can be compared against various natural products databases to identify and eliminate quickly and efficiently known, frequently occurring compounds with minimal and/or nonspecific activity. When this methodology was applied to the crude organic extract of the roots of *H. barteri*, fractions 32, 37, and 46 (of 88) showed activity against azole-resistant *Candida albicans*. The UV and MS data for these fractions pointed to the "flavonoid" class of phenolic molecules, but, for rigorous structural elucidation of the active compounds, it was necessary to scale-up the isolation process. This was accomplished by liquid−liquid partitioning, vacuum liquid chromatography on $C_{18}$ bonded phase, gel permeation through Sephadex LH-20, and HPLC on $C_{18}$ bonded phase, yielding **1**−**3**.

Accurate mass measurement of the pseudomolecular ion of **1** gave *m/z* 317.0970, corresponding to a molecular formula of $C_{17}H_{16}O_6$. The presence of four aromatic singlets ($\delta$ 6.74, 6.46, 6.43, and 5.85) in the $^1$H NMR spectra suggested a 6,7,2′,4′,5′-substitution pattern of a flavonoid compound. $^1$H NMR signals at $\delta$ 4.21 (ddd, 1 H), 3.83 (dd, 1 H), 3.48 (m, 1 H), 2.61 (ddd, 1H), and 2.55 (ddd, 1H), in combination with the $^{13}$C NMR and HSQC resonances at $\delta$ 69.8, 32.3, and 30.6, indicated that **1** was an isoflavan.[7] The $^1$H NMR spectra also allowed us to identify the substituents as a methylenedioxy group ($\delta$ 5.30 and 5.29, each a doublet, $J$ = 1.4 Hz), one methoxyl group ($\delta$ 3.10, 3H, s), and two hydroxyl groups ($\delta$ 5.09 and 3.66, both br). These structural features were confirmed by UV absorption in $CH_2Cl_2$ at 232 and 301 nm, characteristic for a 4′,5′-methylenedioxyisoflavan.[8] A prominent ion at *m/z* 151 (in ESIMS), consistent with a retro-Diels−Alder fragmentation, indicated that one hydroxyl group and the methoxyl unit were in ring B.[9]

The order of the substitution on the A ring was determined by selective irradiation and HMBC experiments. A differential NOE experiment showed the interaction between the methoxyl protons and H-8 and the HMBC cross-peaks observed for H-8 with C-6, C-7, C-9, and C-10; in addition, the C-6 hydroxyl proton correlates to C-5, C-6, and C-7. These data established that the A ring substitution was 6-hydroxy-7-methoxy. The results of the HMBC experiment supported the presence of the second hydroxyl group at C-2′ (C-2′-OH to C-1′, C-2′, and C-3′) and the methylenedioxy unit bonded to C-4′ and C-5′ (H-3′ to C-1′,

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**Table 1.** Spectrum of Antifungal Activity of **1**−**3**[a]

| test organisms/ # strains | resistance/ sensitivity | fluconazole | voriconazole | amphotericin B | **1** | **2** | **3** |
|---|---|---|---|---|---|---|---|
| *C. albicans*/3 | azole-sens | 0.12 | 0.007 | | >128 | 128 | >32 |
| *C. albicans*/3 | polyene-res | | | 3 | >128 | 128 | >32 |
| *C. glabrata*/2 | polyene-res | | | 4−8 | >128 | 32 | >32 |
| *C. albicans*/2 | azole-res | 256 | 4−16 | | 64−>128 | 32−128 | >32 |
| *C. glabrata*/2 | azole-res | 256 | 8 | | 64−>128 | 32 | >32 |
| *C. krusei*/2 | azole-res | 128−256 | 1 | | >128 | 128 | >32 |

[a] $IC_{50}$ values are reported in units of $\mu$g/mL for standards and test compounds.

C-2′, C-4′, and C-5′). A C-2′-oxygen substituent is in agreement with the biosynthetic pathways to plant-derived isoflavans.[7]

The C-3 chirality of isoflavans can be determined more reliably using circular dichroism, because optical rotation measurements alone often give insufficient or ambiguous results.[10] Studies have shown that isoflavans with different A and B ring substitution modify markedly the shape of CD curves.[11] The combination of different conformations of the *O*-heterocyclic ring due to steric effects and the sign of the third sphere contribution determined by the spectroscopic moments is responsible for the diversity of curve shapes.[12] Therefore, the presence of oxygenation on both aromatic rings in **1** results in two determinant regions at 240 ($^1L_a$) and 280−300 ($^1L_b$) nm. Compound **1** displayed a negative and positive Cotton effect at $^1L_a$ and $^1L_b$, respectively, indicating the absolute stereochemistry of C-3 as *R*.

Comparison of the spectroscopic data of **2** with literature reports indicated the only substantial difference to be in the magnitude of the optical rotation from the reported value (−25°).[5] Pterocarpans are found in nature only in a 6a,11a-*cis* configuration, which indicates that only one levorotatory compound could be possible.[10] With the absence of a hydroxyl group at C-2, (−)-maackiain (**4**) constitutes a reasonable reference compound. Chang et al. reported $[\alpha]_D$ −175.0 for this compound,[13] while we obtained a very comparable $[\alpha]_D$ −196 for **2**.

Compound **3** was identified by comparison with spectroscopic data reported in the literature (UV, MS, $^1$H NMR, and $[\alpha]_D$).[6,14]

The isoflavans constitute one of the smaller groups of flavonoids. These are mainly substituted with hydroxyl and methoxyl groups, most commonly at positions 7, 2′, and 4′ and less often at 6, 8, and 3′. The presence of a methylenedioxy unit is uncommon in natural isoflavans, and there are only two previously reported compounds with this characteristic.[15,16] To our knowledge, this is the first isoflavan with a conventional skeleton isolated from a plant outside the family Fabaceae. Dean and co-workers reported the first non-Fabaceae isoflavonoid, but with a bridged ring structure, from a Meliaceae species.[17]

Compound **2** was previously isolated only once, from *Cladrastis platycarpa* (Leguminosae).[5] The presence of similar substituents in equivalent positions in compounds **1** and **2** and the co-occurrence of these compounds in *H. barteri* exemplify the close biosynthetic relationship between pterocarpans and isoflavans.

Compound **3** was first reported from *Rhododendron farrerae* and is a common constituent of the genus *Rhododendron*.[6,18] The existence of a C-methyl substitutent at C-6 and/or C-8 is fairly common in flavanones, but their presence is unusual in the Sterculiaceae.[19]

One of the known functions of flavonoids is their role in protecting plants against microbial attack. In particular, isoflavonoids, flavans, or flavanones have often been identified as antifungal agents.[20] Compounds **1**−**3** were tested against a panel of 14 strains of *Candida*; the results are presented in Table 1. Hildegardiol (**1**) showed minimal activity against two of six azole-resistant strains and was inactive against all other test strains; farrerol (**3**) was inactive against all test strains at the highest concentration tested (32 $\mu$g/mL, because of limited supply). The pterocarpan **2** exhibited the best, albeit still quite modest, activity, but only against azole- or polyene-resistant strains, not against azole-sensitive strains. All these compounds, and probably minor congeners, additively account for the activity observed in the crude extract (77% inhibition at 250 $\mu$g/mL).

## Experimental Section

**General Experimental Procedures.** UV experiments were done on a Perkin-Elmer Lambda 20 UV/vis spectrometer. IR spectra were obtained with a Perkin-Elmer Spectrum 2000 FT-IR spectrometer. CD spectra were recorded with a Jasco J-720 spectropolarimeter. $^1$H and $^{13}$C NMR analyses, including 2D experiments, were conducted on a Varian 500 MHz INOVA instrument. A Thermo Finnigan TSQ Quantum AM triple quadrupole mass spectrometer with PEG as internal standard was used for HR-MS. Preparative HPLC was performed on a Waters 600 pump controlled by MassLynx software. Postcolumn detection was accomplished by a parallel arrangement of Micromass ZMD electrospray ionization (ESI) mass spectrometer (cone voltage = 30), a Waters 996 photodiode array (PDA), a Sedex 75 evaporative laser light scattering detector (ELSD), and a Kratos Spectroflow 980 spectrofluorimeter.

**Plant Material.** *Hildegardia barteri* was collected by R. Gereau and J. Lovett of the Missouri Botanical Garden, under contract to the NCI, in the Iringa Region, Tanzania, in January 1989. A voucher specimen (Q66T0984) is maintained at the Missouri Botanical Garden.

**Extraction and Isolation.** The dried woody roots (stripped of bark) were finely ground in a hammer mill; 552 g of ground root was placed into a 3 L borosilicate glass percolator and steeped in $CH_2Cl_2$−MeOH (1:1) for 16 h at room temperature. After draining, the marc was covered briefly with MeOH, which was then drained and combined with the $CH_2Cl_2$−MeOH extract. Solvent was removed by rotary evaporation at <40 °C, followed by high-vacuum drying, resulting in a yield of 37.57 g. Crude extract (10 g) was partitioned with 250 mL of hexane−MeCN−MeOH−$H_2O$ (20:17:1:2); the polar phase was washed with hexane (3 × 250 mL). An aliquot of the polar phase (4.46 g) was processed by VLC using $C_{18}$ bonded phase, affording four fractions upon elution with MeCN−$H_2O$ (1:4), MeCN−MeOH−$H_2O$ (17:1:2), MeOH, and $CH_2Cl_2$. The second fraction (1.62 g) was subjected to gel permeation on Sephadex LH-20 with a solvent gradient from hexane−$CH_2Cl_2$−MeOH (2:5:1) to $CH_2Cl_2$−MeOH (1:1). Fractions 8−21 (521 mg) contained compounds **1**−**3**. A portion of this fraction (80 mg) was purified by gradient HPLC [$C_{18}$ Dynamax 60 Å, 8 $\mu$m, 2.1 × 25 cm, MeCN−20 mM $NH_4OAc$ pH 4, 0−5 min isocratic at 3:7, 5−25 min gradient to 1:1, 25−45 min gradient to 1:0, and isocratic to 60 min, flow rate 15 mL/min] using an automatic collector set in negative mode to yield **1** (27 mg) and two fractions enriched with compounds **2** and **3**. Finally, two HPLC purification processes were done to obtain these compounds in high-purity level [**2** (7.9 mg): gradient $C_{18}$ Dynamax 60 Å,

8 $\mu$m, 2.1 × 25 cm, MeOH−$H_2O$, 0−10 min isocratic at 55:45, 10−30 min gradient to 1:0, flow rate 10 mL/min; **3** (1.7 mg): gradient $C_{18}$ Dynamax 60 Å, 8 $\mu$m, 1 × 25 cm, MeOH−$H_2O$, 0−25 min isocratic at 6:4, 25−30 min gradient to 1:0, then isocratic, flow rate 3.5 mL/min].

**Hildegardiol (1):** pale tan gum; UV ($CH_2Cl_2$) $\lambda_{max}$ (log $\epsilon$) 232 (2.35), 301 (3.92) nm; CD ($CH_2Cl_2$) $[\theta]_{267}$ −1.4, $[\theta]_{289}$ −5.2, $[\theta]_{297}$ 0.6, $[\theta]_{307}$ 12.9, $[\theta]_{322}$ 0.1; IR (NaCl) $\nu_{max}$ 3401, 1633, 1510, 1167, 1036, 933 $cm^{-1}$; ESIMS *m/z* 315 ($M - H^-$, 100), 151 (30); $^1H$ NMR ($C_6D_6$) $\delta$ 6.74 (H-5, 1H, s), 6.46 (H-6′, 1H, s), 6.43 (H-8, 1H, s), 5.85, (H-3′, 1H, s), 5.30 and 5.29 (O−$CH_2$−O, ea 1H, d, $J$ = 1.4 Hz), 5.09, (C-6 OH, 1H, s), 4.21 (H-2$\beta$, 1H, ddd, 10.2, 3.4, 1.8), 3.83 (H-2$\alpha$, 1H, dd, 10.2, 9.6), 3.66 (C-2′ OH, 1H, br s), 3.48 (H-3, 1H, m), 3.10 ($OCH_3$, s), 2.61 (H-4$\alpha$, 1H, ddd, 16.0, 10.1, 0.7), 2.55 (H-4$\beta$, 1H, ddd, 16.0, 5.6, 1.5); $^{13}C$ NMR ($C_6D_6$) $\delta$ 148.3 (C-9), 148.2 (C-2′), 146.7 (C-4′), 146.0 (C-7), 142.1 (C-5′), 140.3 (C-6), 120.2 (C-1′), 114.9 (C-5), 114.1 (C-10), 107.5 (C-6′), 101.0 (O−$CH_2$−O), 100.4 (C-8), 98.2 (C-3′), 69.8 (C-2), 55.2 ($OCH_3$), 32.3 (C-3), 30.6 (C-4).

**Antimicrobial and Hemolytic Activities.** Initial biological screening and analysis were performed using clinical isolates in a broth microdilution assay using general procedures described earlier.[1] Follow-up antimicrobial assays were performed by Shawn Messer, Dan Diekema, and Michael Pfaller at the University of Iowa Carver College of Medicine, using 11 recent drug-resistant and three recent drug-sensitive clinical isolates of *Candida* sp. and two quality control strains, *C. krusei* ATCC 6258 and *C. parapsilopsis* ATCC 22019. Analysis of antifungal activity was performed in a broth microdilution assay using RPMI 1640 buffered with MOPS, following the NCCLS guidelines.[21] The results are presented in Table 1 as $IC_{50}$ values.

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## References and Notes

(1) Klausmeyer, P.; Chmurny, G. N.; McCloud, T. G.; Tucker, K. D.; Shoemaker, R. H. *J. Nat. Prod.* **2004**, *67*, 1732−1735.
(2) Klausmeyer, P.; McCloud, T. G.; Tucker, K. D.; Cardellina, J. H., II; Shoemaker, R. H. *J. Nat. Prod.* **2005**, *68*, 1300−1302.
(3) Omolodun, O. O.; Cutter, B. E.; Krause, G. F.; McGinnes, E. A. *Wood Fiber Sci.* **1991**, *23*, 419−435.
(4) Inglett, G. E.; Cavins, J. F.; Spencer, G. F. *Econ. Bot.* **1973**, *27*, 128−130.
(5) Mizuno, M.; Tanaka, T.; Katsuragawa, M.; Saito, H.; Iinuma, M. *J. Nat. Prod.* **1990**, *53*, 498−499.
(6) Arthur, H. R. *J. Chem. Soc.* **1955**, 3740−3742.
(7) Grosvenor, P. W.; Gray, D. O. *Phytochemistry* **1996**, *43*, 377−380.
(8) Dewick, P. M. In *The Flavonoids−Advances in Research*; Harborne, J. B., Mabry, T. J., Eds.; Chapman and Hall: New York, 1982; pp 535−536.
(9) Mabry, T. J.; Markham, K. R. In *The Flavonoids, Part 1*; Harborne, J. B., Mabry, T. J., Mabry, H., Eds.; Academic Press: New York, 1975; p 81.
(10) Slade, D.; Ferreira, D.; Marais, J. P. J. *Phytochemistry* **2005**, in press.
(11) Versteeg, M.; Bezuidenhoudt, B. C. B.; Ferreira, D. *Tetrahedron* **1999**, *55*, 3365−3376.
(12) Snatzke, G.; Kajtár, M.; Werner-Zamojska, F. *Tetrahedron* **1972**, *28*, 281−288.
(13) Chang, L. C.; Gerhäuser, C.; Song, L.; Farnsworth, N. R.; Pezzuto, J. M.; Kinghorn, A. D. *J. Nat. Prod.* **1997**, *60*, 869−873.
(14) Youssef, D. T. A.; Ramadan, M. A.; Khalifa, A. A. *Phytochemistry* **1998**, *49*, 2579−2583.
(15) Ingham, J. L.; Dewick, P. M. *Phytochemistry* **1980**, *19*, 1767−1170.
(16) Bojase, G.; Wanjala, C. C. W.; Majinda, R. R. T. *Phytochemistry* **2001**, *56*, 837−841.
(17) Dean, F. M.; Monkhe, T. V.; Mulholland, D. A.; Taylor, D. A. H. *Phytochemistry* **1993**, *34*, 1537−1539.
(18) Harborne, J. B.; Williams, C. A. *Phytochemistry* **1971**, *10*, 2727−2744.
(19) Bohm, B. A. In *The Flavonoids. Advances in Research Since 1986*; Harborne, J. B, Ed.; Chapman and Hall: New York, 1994; pp 408−419.
(20) Harborne, J. B.; Williams, C. A. *Phytochemistry* **2000**, *55*, 481−504.
(21) National Committee for Clinical Laboratory Standards. In NCCLS document M27-P, NCCLS, Villanova, PA, 1992.

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