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nor-Lignans from the leaves of *Styrax ferrugineus* (Styracaceae) with antibacterial and antifungal activity☆

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Abstract

Chemical examination of the leaves of *Styrax ferrugineus* yielded 5-[3″-(β-D-glucopyranosyloxy)propyl]-7-methoxy-2-(3′,4′-dimethoxyphenyl) benzofuran, along with the known *nor*-lignans 5-(3″-hydroxypropyl)-7-methoxy-2-(3′,4′-methylenedioxyphenyl) benzofuran, 5-(3″-hydroxypropyl)-7-methoxy-2-(3′,4′-dimethoxyphenyl)benzofuran, 5-[3″-(β-D-glucopyranosyloxy)propyl]-7-methoxy-2-(3′,4′-methylenedioxyphenyl)benzofuran and the lignan, dihydrodehydrodiconiferyl alcohol. All arylpropanoids isolated showed antibacterial and antifungal activities. The structures of the isolates were established by spectroscopic analysis.

Keywords: *Styrax ferrugineus*; Styracaceae; *nor*-Lignans; Antifungal activity; Antibacterial activity

1. Introduction

Styracaceae is a family constituted of small trees and shrubs, mostly native to tropical and subtropical regions (Pio Correa, 1931). The genus *Styrax* is different from other genera of this family due to the production of resinous material, usually secreted when the barks and trunks are injured by sharp objects (Pio Correa, 1931). This resin, in the past considered a miraculous remedy in several parts of Asia and America, has been used in traditional medicine to treat inflammatory diseases (Costa, 1968). Chemical studies on several Styracaceae plant species have revealed them to be a rich source of arylpropanoids and triterpenoids (Giesbrecht et al., 1985; Takanashi and Takizawa, 1988; Anil, 1980; Segal et al., 1967, 1964; Ulubelen et al., 1978; Kawai and Sugimota, 1940; Kitagawa et al., 1974; Ulubelen, 1976; Nakano et al., 1967).

From the leaves of *Styrax ferrugineus* Ness et Mart., an endemic species of the Cerrado region, a new 5-[3″-(β-D-glucopyranosyloxy)propyl]-7-methoxy-2-(3′,4′-dimethoxyphenyl)benzofuran (**4**) and the known *nor*-lignans 5-(3″-hydroxypropyl)-7-methoxy-2-(3′,4′-methylenedioxyphenyl)benzofuran (**1**), 5-(3″-hydroxypropyl)-7- methoxy-2-(3′,4′-dimethoxyphenyl)benzofuran (**2**), 5-[3″-(β-D-glucopyranosyloxy)propyl]-7-methoxy-2-(3′,4′-methylenedioxyphenyl)benzofuran (**3**) and the lignan dihydrodehydrodiconiferyl alcohol (**5**), together with ursolic and oleanolic acids, sitosterol and stigmasterol, have been isolated. Compounds **1–2** showed inhibitory properties against the microorganisms, *Candida albicans, Cladosporium cladosporioides* and *Staphylococcus aureus*, whereas compounds **3–5** inhibited only *S. aureus* and *C. albicans*. The structures of these arylpropanoids were elucidated by various NMR experiments including COSY, DEPT, HMQC, and HMBC (see Section 3 and Tables 1 and 2).

2. Results and discussion

The dried and powdered leaves of *Styrax ferrugineus* were extracted with a mixture of CH_2Cl_2—CH_3OH (2:1 v/v). Evaporation gave an extract, which showed

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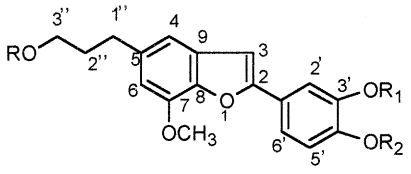

	R	R_1	R_2
1	H	$-CH_2-$	
2	H	CH_3	CH_3
3	Glu	$-CH_2-$	
4	Glu	CH_3	CH_3

antimicrobial activity, detected by inhibition of *C. sphaerospermum, C. albicans* and *S. aureus* (Table 3). The bioactive extract was suspended in H_2O and then partitioned with *n*-hexane, $CHCl_3$ and EtOAc (4×300 ml each). Silica gel chromatography of the $CHCl_3$ solubles (2.27 g) followed by purification on preparative HPLC afforded the new compound homoegonol glucoside (**4**) and the known *nor*-lignans (**1**) (Takanashi et al., 1974), (**2**) (Giesbrecht et al., 1985) and (**3**) (Takanashi and Takizawa, 1988), and the lignan (**5**) (Miyase et al., 1989). The compounds were identified by chromatographic and spectral comparison with those of authentic samples. In addition were also isolated ursolic and oleanolic acids (Mahato and Kundu, 1994) as a mixture, sitosterol and stigmasterol (Kojima et al., 1990). The triterpenes and sterols were identified by comparison with authentic samples and spectroscopic data. A literature search revealed the absence of ^{13}C NMR spectral data for 5-(3″-hydroxypropyl)-7-methoxy-2-(3′,4′-methylenedioxyphenyl)benzofuran (**1**) and 5-(3″-hydroxypropyl)-7-methoxy-2-(3′,4′-dimethoxyphenyl)benzofuran (**2**). Therefore, complete assignment of the ^{13}C NMR spectra for these compounds was undertaken and achieved with the aid of DQF-COSY, DEPT, HMQC and HMBC experiments. The ^{13}C NMR spectral data for **1** and **2** are given in Table 2 and some important HMBC correlations are presented in Fig. 1.

Compound **2** was obtained earlier from the seeds of *S. officinalis* (Segal et al., 1967) after acid hydrolysis and subsequent purification of a glucosidic fraction from bark trunk wood of *S. camporum* (Giesbrecht et al., 1985). In this paper we describe the structural elucidation of compound (**4**) as well as the antimicrobial activities of all lignoids isolated.

Compound **4** was obtained as a white amorphous powder. The molecular formula $C_{26}H_{32}O_{10}$ was deduced from ES-MS *m/z* $[M+Na]^+$ 527, ^1H and ^{13}C NMR spectroscopic data. Its IR spectrum recorded hydroxyl (3550–3200 cm^{-1}), unsaturated (1650 cm^{-1}) and aromatic (1550, 870 cm^{-1}) absorptions, and its UV absorbance

Table 1
^1H NMR spectral data (400 MHz) of **2** and **4**[a]

Proton	**4**[b,c]	COSY
3	7.17 *s*	
4	7.11 *d* (1.9)	H-6
6	6.77 *d* (1.9)	H-4
2′	7.41 *d* (1.9)	H-6′
5′	7.05 *d* (8.0)	H-6′
6′	7.44 *dd* (1.9; 8.0)	H-2′,6′
1″	2.75 *t* (7.5)	H-2″
2″	1.86 *dt* (7.8)	H-1″,3″
3″	3.66 *t* (7.8)	H-2″,1″
1‴	4.11 *d* (8.0)	H-3″
2‴	4.33 *m*	H-1‴,3‴
3‴	3.45 *m*	H-2‴,4‴
4‴	3.62 *t* (9.0)	H-3‴,5‴
5‴	3.64 *ddd* (9.0, 5.0, 3.0)	H-4‴,6‴
6‴	4.23 *dd* (12.0, 5.0)	H-6_a‴,H-5‴
	4.20 *dd* (12.0, 3.0)	
OCH_3-3′	3.83 *s*	
OCH_3-4′	3.88 *s*	
OCH_3-7′	3.99 *s*	

[a] Chemical shifts are expressed in δ values. *J* values (Hz) are in parentheses.
[b] DMSO-d_6
[c] $CDCl_3$.

Table 2
^{13}C NMR spectral data of *nor*-lignans **1**, **2** and **4**

Carbon	**1**[a]	**2**[b]	**4**[c]
2	156.06 *s*	156.35 *s*	156.12 *s*
3	100.32 *d*	100.27 *d*	101.12 *d*
4	112.28 *d*	112.28 *d*	112.12 *d*
5	137.50 *s*	137.49 *s*	137.85 *s*
6	107.64 *d*	107.24 *d*	107.64 *d*
7	144.75 *s*	144.78 *s*	144.36 *s*
8	141.70 *s*	142.41 *s*	141.80 *s*
9	131.01 *s*	131.14 *s*	130.49 *s*
1′	124.88 *s*	123.52 *s*	122.72 *s*
2′	105.51 *d*	108.16 *d*	108.88 *d*
3′	148.02 *s*	149.54 *s*	148.83 *s*
4′	144.75 *s*	149.13 *s*	149.10 *s*
5′	108.58 *d*	111.27 *d*	112.22 *d*
6′	119.14 *d*	118.07 *d*	118.78 *d*
1″	32.40 *t*	32.45 *t*	31.83 *t*
2″	34.63 *t*	34.69 *t*	31.50 *t*
3″	62.27 *t*	62.23 *t*	67.88 *t*
1‴	–	–	103.01 *d*
2‴	–	–	73.54 *d*
3‴	–	–	76.82 *d*
4‴	–	–	70.10 *d*
5‴	–	–	76.86 *d*
6‴	–	–	61.08 *t*
OCH_3-7	56.44 *q*	55.60 *q*	55.41 *q*
OCH_3-3′	–	55.51 *q*	55.40 *q*
OCH_3-4′	–	55.41 *q*	55.60 *q*
$-OCH_2O-$	101.28 *t*	–	–

[a] Recorded at 50 and 100 MHz in $CDCl_3$.
[b] Recorded at 50 and 100 MHz in $CDCl_3$.
[c] Recorded in DMSO at 100 MHz. Multiplicities of carbons were determined by a DEPT experiment.

Table 3
Antimicrobial activity of *nor*-lignoids **1–4** and lignan **5**

Compounds	*S. aureus*[a]	*C. albicans*[a]	*C. sphaerospermum*[b]
Crude extract	200	800	750
1	10	10	5
2	10	12	10
3	15	15	NA[c]
4	20	20	NA
5	20	15	NA
Chloramphenicol	5	5	–
Nystatin	–	–	1
Mycostatin	5	5	–

[a] MIC (μg/ml) = minimum inhibition concentration.
[b] Minimum amount (μg) required for the inhibition of fungal growth on TLC plates.
[c] NA = inactive at 20 μg.

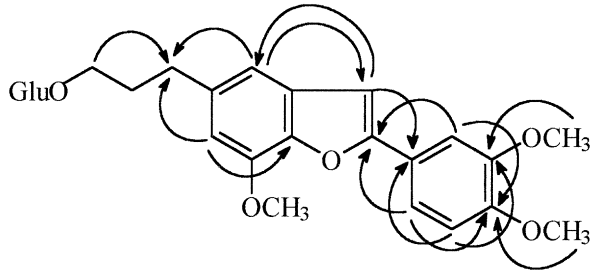

Fig. 1. HMBC correlations observed for **4**.

(MeOH) was at 312.0 and 216.5 nm. The ^{1}H and ^{13}C NMR spectroscopic signals (Tables 1 and 2) were assigned by interpretation of the COSY, DEPT 135, HMQC and HMBC spectra. Comparison of the ^{1}H and ^{13}C NMR spectra of **4** with those described for **2** indicated that **4** is a glucoside derivative of compound **2**. The ^{1}H NMR spectrum of **4** displayed four doublets at δ 6.77 (1H, *d*, $J = 1.9$ Hz), 7.11 (1H, *d*, $J = 1.9$ Hz), 7.05 (1H, *d*, $J = 8.0$ Hz) and 7.41 (1H, *d*, $J = 1.9$ Hz), and a double doublet at δ 7.44 (1H, *dd*, $J = 1.9$; 8.0 Hz) attributable to aromatic protons. In addition were observed a singlet at δ 7.17 (1H, *s*) corresponding to H-3, three singlets due to methoxyl groups at δ 3.99, 3.88 and 3.83 (3H each, *s*), two triplets due to methylene protons at δ 2.75 (2H, *t*, $J = 7.5$ Hz) and 3.66 (2H, *t*, $J = 7.8$ Hz) and a double triplet due to a methylene group at δ 1.86 (2H, *dt*, $J = 7.8$ Hz). This ^{1}H NMR spectroscopic pattern accommodated the same characteristics as that of compound **2**. Further analysis of the ^{1}H NMR spectroscopic data showed a two-proton spin-system consisting of a doublet at δ 4.11 (1H, *d*, $J = 8.0$ Hz) and several signals at δ 4.33–3.45 m, indicative of a glucosyl moiety in compound **4**. The ^{13}C NMR spectrum of **4** (Table 2) suggested the presence of a *nor*-lignan with similar skeleton to **2**. The main difference observed in the ^{13}C NMR spectrum of **4** was due to the presence of the signals at δ 103.01 (CH), 76.82 (CH), 76.86 (CH), 73.54 (CH), 70.10 (CH) and 61.08 (CH_2) which confirmed a glucosyl moiety. The ^{1}H—^{1}H COSY relationship permitted assignment of aromatic proton chemical shifts and also supported the substitution pattern. ^{1}H–^{13}C-one bond (HMQC) data were used to assign carbon resonances (Table 2) to their attached protons. ^{1}H–^{13}C-three-bond (HMBC) correlations from the H-1″ protons resonances at δ 2.69 to the aromatic carbons resonance at δ 107.64 and 112.12 identified the later as C-4. The ^{3}J HMBC couplings of the C-2, C-3′ and C-4′ quaternary carbons with H-2′ and H-6′ proton signals further supported the 1,3,4-trisubstituted pattern for the aromatic moiety linked to the benzofuran moiety of compound **4**. Other significant correlations for confirmation of **4** are depicted in Fig. 1.

The extract and compounds isolated from *S. ferrugineus* were evaluated for antifungal and antibacterial activities. The crude extract showed antifungal and antibacterial activities against *C. sphaerospermum, C. albicans* and *S. aureus*, respectively. Compounds **1–2** exhibited antifungal and antibacterial activities against *C. sphaerospermum, C. albicans* and *S. aureus*, whereas compounds **3–5** inhibited only *S. aureus* and *C. albicans*. The MIC values for these strains, together with those typically used for comparison, are shown in Table 3. The activity against the pathogenic fungus *C. sphaerospermum* could be predictive for the activity against *C. albicans*. In conclusion, the activities of compounds **1–5** may (partly) explain known properties of this plant species, which is popularly used to treat wound infections in several Brazilian regions.

3. Experimental

3.1. Instrumentation and chromatography materials

Optical activities were measured on a Polamat A (Carl Zeiss) polarimeter, whereas IR spectra were recorded on Perkin–Elmer 1710 spectrometer. NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 on a Bruker AC 200 at 200 MHz for ^{1}H and 50 MHz for ^{13}C or Varian Unity 400 NMR instrument at 400 MHz for ^{1}H and 100 MHz for ^{13}C, using TMS as internal standard. The DEPT experiments were performed using polarization transfer pulses of 90° and 135°, respectively. The ES-MS spectra were obtained at 70 eV on a VG Platform II and EIMS spectra on a VG 70 E-HF instrument. For column chromatography, silica gel 60, 70–230 and 230–400 mesh, were used as needed. TLC analysis was carried out on precoated silica gel 60 F_{254} plates, with spots visualized by anisaldehyde 2% in H_2SO_4 after heating.

3.2. Plant material

Styrax ferrugineus Ness et Mart. Leaves were collected at Campininha Farm, Mogi-Guaçú, SP, Brazil, by

M.C.M. Young in November 1995. A voucher specimen has been deposited in the herbarium of the Botanic Garden of São Paulo, Brazil.

3.3. Bioassay

The crude extract and purified lignoids were assayed for antimicrobial activity against the bacterium *S. aureus* ATCC 12228, the fungus *C. sphaerospermum* (Penzig) SPC 491 and the yeast *C. albicans* ICB 12. Stock solutions of crude extracts were dissolved at a concentration of 2 mg/ml in the solvent of extraction. Pure compounds were freshly prepared at 1 mg/ml in an appropriate solvent and were diluted to 100, 50, 30, 10, 5, 1.0 μg/ml. 10 μl of these solutions were applied on precoated TLC plates by means of graduated capillaries. Antibiotic samples mycostatin and chloramphenicol, 30 μg/ml) and the solvent used were also tested as positive and negative controls, respectively. For the antifungal bioautography assay, TLC plates were developed in an appropriate solvent system and thoroughly dried for complete removal of solvents (Homans and Fuchs, 1970). The chromatograms were sprayed with the spore suspension of *C. sphaerospermum* in a nutritive medium and incubated for 72 h in darkness in a moistened chamber at 27°C. Clear inhibition zones appeared against a dark background. Agar dilution assays with *C. albicans* and *S. aureus* were carried out Sabouraud agar medium (Rahalison et al., 1991). The media were plated in Petri dishes and cell suspensions of *C. albicans* and *S. aureus* in distilled water were spread over agar. Incubation was at 30°C for 24 h.

3.4. Extraction

Dried and pulverized leaves (3.5 kg) of *Styrax ferrugineus* Ness et Mart. were exhaustively extracted with a mixture of CH_2Cl_2–CH_3OH (2:1, v/v) at room temperature. After removal of the solvent in vacuo, a crude extract (23 g) was obtained. The concentrate was then diluted with CH_3OH–H_2O (8:2) and successively extracted with hexane, $CHCl_3$ and EtOAc. After removal of the solvent, each extract yielded 2.01, 2.27 and 1.57 g, respectively. The $CHCl_3$ extract (2.27 g) was fractionated by column chromatography on a silica gel flash (77.9 g, 230–400 mesh, Merck) using a CH_2Cl_2–CH_3OH gradient to yield 93 fractions. Fr. 26 (29 mg) yielded pure 5-(3″-hydroxypropyl)-7-methoxy-2-(3′,4′-methylenedioxyphenyl)benzofuran (**1**), and Fr. 34 (137 mg) yielded 5-(3″-hydroxypropyl)-7-methoxy-2-(3′,4′-dimethoxyphenyl)benzofuran (**2**). Fr. 40–41 (39.4 mg) was fractionated by micron silica gel flash CC (Merck) using CH_2Cl_2–CH_3OH (95:5) as eluent to afford 11 Frs. Frs. 5–9 gave a mixture of ursolic and oleanolic acids (21 mg). Frs. 52–55 (50 mg) were submitted to medium pressure silica flash CC (11.6 g, 230–400 mesh, Merck) using Hex–EtoAc–CH_3OH (6:2:2) as eluent to afford 20 Frs. Frs 6–12 furnished dihydrodehydiconiferyl alcohol (**5**) (14 mg). Fr. 65 (43.6 mg) after silica gel 60 G (15.0 g, Merck) CC, using a CH_2Cl_2–CH_3OH gradient yielded 22 Frs. Fr. 20 (13.8 mg) gave a mixture of **3**+**4**, which was further purified by reversed-phase HPLC (RP-18 column) using a mixture of CH_3OH–H_2O (4:6) as eluent to afford compounds **3** (4.0 mg) and **4** (5.8 mg).

3.4.1. 5-(3″-Hydroxypropyl)-7-methoxy-2-(3′,4′-methylenedioxyphenyl)benzofuran (*1*)

White amorphous powder; IR ν_{max} (KBr) 3432, 2926, 1606, 1476, 1232, 1148, 1037, 931 cm^{-1}; ES-MS $[M+Na]^+$ *m/z* 349, ES-MS $[M+K]^+$ *m/z* 365. Identical to literature; (Takanashi et al., 1974). For ^{13}C NMR ($CDCl_3$) spectral analysis, see Table 2.

3.4.2. 5-(3″-Hydroxypropyl)-7-methoxy-2-(3′,4′-dimethoxyphenyl)benzofuran (*2*)

White powder; IR ν_{max} (KBr) 3484, 2929, 1603, 1513, 1478, 1346, 1222, 1142 cm^{-1}; ES-MS $[M+Na]^+$ *m/z* 365, ES-MS $[M+K]^+$ *m/z* 381; Identical to literature; (Giesbrecht et al., 1985) for ^{13}C NMR ($CDCl_3$) spectral analysis see Table 2.

3.4.3. 5-[3″-(β-D-Glucopyranosyloxy)propyl]-7-methoxy-2-(3′,4′-methylenedioxyphenyl)benzofuran (*3*)

White powder, IR ν_{max} (KBr) 3580, 2940, 1600, 1480, 920 cm^{-1}, ES-MS $[M+Na]^+$ *m/z* 511; ES-MS $[M+K]^+$ *m/z* 527; Identical to literature (Takanashi and Takizawa, 1988).

3.4.4. 5-[3″-(β-D-Glucopyranosyloxy)propyl]-7-methoxy-2-(3′,4′-dimethoxyphenyl)benzofuran (*4*)

White powder, $[\alpha]_D^{25}$ −(22.23° (*c* 0.8, CH_3OH); IR ν_{max} (KBr) 3484, 2928, 1609, 1477, 910 cm^{-1}; ES-MS $[M+Na]^+$ *m/z* 527, ES-MS $[M+K]^+$ *m/z* 543; for ^1H NMR and ^{13}C NMR (DMSO-d_6) spectral analysis, see Tables 1 and 2.

*3.4.4.1. Hydrolysis of **4**.* A solution containing glucoside **4** (2.0 mg) in 3 M HCl (5 ml) was heated until reflux began, this being maintained for 4 h. The reaction mixture was cooled to room temperature, neutralized with 1% NaOH and extracted with $CHCl_3$ (3×10 ml). The reaction mixture was purified on CC silica gel using CH_2Cl_2–CH_3OH (99:1) as eluent to obtain **2** (1.0 mg) which showed the same R_f [0.5 CH_2Cl_2–CH_3OH (98:2)], IR and ^1H of authentic 5-(3″-hydroxypropyl)-7-methoxy-2-(3′,4′-dimethoxyphenyl)benzofuran (**2**). Identification of the glucose obtained as a product of the reaction was confirmed after reaction of the aqueous fraction with an enzymatic kit based on glucose oxidase, this being specific for glucose and yielding a positive response (Trinder, 1969).

*3.4.5. Dihydrodehydrodiconiferyl alcohol (**5**)*

Colorless oil; $[\alpha]_D^{25}$ −15.0°, (*c* 0.78, $CHCl_3$); lit (Miyase et al., 1989) $[\alpha]_D^{25}$ −17.7°, (*c* 0.48, acetone). ^{13}C NMR ($CDCl_3$) 87.9 (C-2), 53.8 (C-3), 116.0 (C-4), 127,8 (C-5), 112.5 (C-6), 135.4 (C-7), 144.2 (C-8), 133.1(C-9), 135.4 (C-1′), 108.9 (C-2′), 146.5 (C-3′), 146.7 (C-4′), 114.3 (C-5′), 119.4 (C-6′), 32.0 (C-1″), 34.6 (C-2″), 62.2 (C-3″), 56.0 (-OCH_3-7), 56.0 (OCH_3-3′), 63.9 (-CH_2OH-3).

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