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Structure and estrogenic activity of new lignans from *Iryanthera lancifolia*

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Dedicated to Professor Miguel Yus on the occasion of his 60th birthday.

Abstract—Five new dibenzylbutane type lignans (**1**–**5**) were isolated from the stem bark of *Iryanthera lancifolia*. Their structures were determined by extensive 1D and 2D NMR spectroscopic studies and chemical evidence. Seventeen of the isolated compounds were tested for their estrogenic activities in the estrogen responsive human breast cancer cell line MCF-7 BUS using the E-Screen proliferation assay. Cell proliferation was evaluated by the SRB assay to calculate the estrogenic parameters. The majority of the compounds induced a mitogenic response. This effect, given as Relative Proliferative Effect (RPE) to reference estrogen 17β-estradiol (E_2), ranged between 14% and 84%.

1. Introduction

The Myristicaceae family is composed of 18 genera and its species are spread through Asian, African, and American tropical forest.[1] Among the species occurring in the Amazon rain forest, 80% belong to *Virola* and *Iryanthera* genera.[2] There have been reports of Amazonian natives using some *Irianthera* species for medicinal healing.[3–6] The crushed leaves of *Iryanthera* species are used by the amazonian indians for healing seriously infected wounds and cuts, and the latex from the bark of *Iryanthera* species is mixed with warm water for treating gastric infections.[2] Previous chemical studies carried out on *Iryanthera* species have led to the isolation of lignans,[7,8] flavonolignans,[9–12] flavonoids,[13,14] γ-lactone derivatives,[15,16] chalcones,[17] dihydrochalcones,[10–13,15,17–21] 1,3-diarylpropanes[22], and tocotrienols.[23] With respect to *Iryanthera lancifolia* Ducke[24] only chemical studies have been carried out on the pericarps.[23,25] Herein we report the first phytochemical study of the stem bark of *I. lancifolia*. In this sense, we describe the isolation and structure elucidation of five new lignans (**1**–**5**). We have also isolated sixteen known compounds oleiferin C (**6**),[26] oleiferin D (**7**),[26] machilin A (**8**),[27] austrobailignan 6 (**9**),[28] (8R, 7′S, 8′S)-4,4′-bis-(hydroxy)-6,7′-cyclolignan (**10**),[18] cagayanine (**11**),[29] licarine B (**12**),[30] licarine A (**13**),[31] perseal F,[32] eupomatenoide 3 (**14**),[33] iryantherine K,[23] 2′, 4′-dihydroxy-4,6′-dimethoxy-dihydrochalcone (**15**),[19] 2′,4′-dihydroxy-6′-methoxy-3,4-methylenedioxi-dihydrochalcone (**16**),[20] 7-hydroxy-3′,4′-methylenedioxy-flavone (**17**),[34] sargacromenol,[35] and acetate of β-sitosterol-tetraacetyl-β-D-glucopyranoside.[36]

Lignans have shown to bind competitively to the estrogen receptor (ER) in vitro[37] and regulate ER transcriptional activity in several cellular systems.[38–40] This potential capacity to modulate the estrogenic signaling could be, almost in part, responsible for the suggested health-promoting effects of lignans derived from epidemiological and chemical intervention studies in the areas of hormone-dependent cancer and cardiovascular diseases.[41–43]

Keywords: Lignans; Dibenzylbutanes; *Iryanthera*; Estrogenic activity; E-Screen.
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Different methods have been used to evaluate the estrogenic activity of phytoestrogens.[44] Among all the short-terms assays to identify the estrogenic character of diverse-origin molecules, E-Screen is one of the most sensitive and reproducible in vitro human models.[45,46] The E-Screen proliferation assay uses the human breast cancer cell line MCF-7 BUS to assess the proliferation ability of any given drug. In this assay, it is possible to calculate the degree of proliferation (Relative Proliferative Effect, RPE) and estrogenic potency (Relative Proliferative Potency, RPP).[47] Our study applies it to lignan-type compounds for the first time.

2. Results and discussion

2.1. Structures of lignans (1–5)

Repeated chromatography on silica gel and Sephadex LH-20 of the EtOH extract from the stem bark of *I. lancifolia* yielded five new lignans (**1**–**5**), along with the other constituents of known structure mentioned above.

Compound **1** was isolated as an amorphous solid with a molecular formula $C_{19}H_{22}O_3$. Its IR spectrum revealed the presence of hydroxyl group (3400 cm^{-1}) and aromatic rings (1613 cm^{-1}). Its ^1H NMR spectrum showed the existence of two equivalent doublet methyls at δ 0.81 (J = 6.6 Hz). One singlet at δ 5.93 (2H) suggested the presence of one methylenedioxy group. The ^1H NMR spectrum also displayed seven aromatic protons [δ 6.54 d (1H, J = 7.9 Hz), 6.58 bs (1H), 6.70 d (1H, J = 7.7 Hz), 6.73 d (2H, J = 8.2 Hz), 6.96 d (2H, J = 8.2 Hz)] and three multiplets at δ 1.73 (2H), δ 2.35 (2H) and δ 2.56 (2H). One broad singlet at δ 4.69 interchangeable with D_2O was also detected which corresponds to a hydroxyl group. The ^{13}C NMR spectrum revealed the existence of aromatic nucleus and from the rest of signals it was deduced that **1** was a lignan. The analysis of ^{13}C NMR, HMQC, and HMBC spectra allowed the unequivocal assignment of all carbons. The presence of two doublet methyls with the same chemical shift in the ^{13}C and ^1H NMR spectra and the existence of two benzylic CH_2 evidenced that **1** presents a lignan skeleton of the type dibenzylbutane, with 4-hydroxyphenyl and 3,4-methylenedioxyphenyl (piperonyl) as aromatic nucleus. The relative stereochemistry of C-8 and C-8′ (*trans*) was established by comparison of the chemical shifts of the carbons C7–C7′/C8–C8′/C9–C9′ of compound **1** with those of *trans* and *cis* dihydroguaiaretic acids of known configuration [48–50] (Fig. 1).

All data mentioned above allowed us to propose the structure of **1** as *trans*-4′-hydroxy-3,4-methylenedioxy-8,8′-lignan previously non-described in the chemical bibliography. The absolute configuration has not been determined since we have not found suitable crystals for X-ray diffraction.

Compound **2** was isolated as an amorphous solid. A molecular formula of $C_{18}H_{20}O_2$ was established for **2** from its HREIMS. Analysis of the IR spectrum of **2** suggested the presence of hydroxyl group (3400 cm^{-1}) and aromatic nucleus (1610 cm^{-1}). The ^1H NMR spectrum displayed resonances for a doublet methyl at δ 1.07 and a singlet methyl at δ 1.80 typical of a vinylic methyl. Also, the ^1H NMR spectrum showed signals for aliphatic hydrogens at δ 2.45 (m, 1H) and at δ 2.64 (m, 2H), one singlet at δ 6.10 assignable to a vinyl hydrogen, and four doublets corresponding to two aromatic AB systems at δ 6.72 (J = 8.1 Hz), δ 6.76 (J = 8.1 Hz), δ 7.01 (J = 8.7 Hz), and δ 7.05 (J = 8.7 Hz), characteristic of two 1,4-disubstituted aromatic rings. Two broad singlets at δ 4.79 and δ 4.89 interchangeable with D_2O corresponding to two hydroxy groups were also detected. All these data together with the information from the ^{13}C NMR spectrum pointed out that **2** is an unsaturated dibenzylbutane lignan. The unequivocal assignments of all protons and carbons were achieved by the analysis of the COSY, ROESY, HMQC, and HMBC spectra (see Fig. 2). The configuration (*E*) was established because of the NOE effect detected between H-7 and H-8′ and also because we did not observe NOE effect between the Me-9 and the singlet corresponding to H-7. Consequently, the structure of **2** was assigned as (*E*)-4,4′-dihydroxy-7-en-8,8′-lignan.

Compound **3** was isolated as an amorphous solid with negative optical activity ($[\alpha]_D^{20}$ − 2.5, *c* 0.2, $CHCl_3$) and its molecular formula was established by HREIMS as $C_{19}H_{20}O_4$. The IR spectrum showed characteristic signals for hydroxy groups (3200 cm^{-1}), carbonyl group (1718 cm^{-1}), and aromatic nucleus (1601 cm^{-1}). The ^1H NMR spectrum showed resonances for two doublet methyls at δ 0.83 (J = 6.5 Hz) and δ 1.23 (J = 6.9 Hz), one methylenedioxy group (δ 5.90, s), one hydroxy group (δ 5.41, bs), two multiplets at δ 2.80 (1 H) and δ 2.15 (2H), and one quintuplet at δ 3.83 (J = 6.6 Hz, 1H). In the downfield region, were observed two aromatic protons belonging to a 1,4-disubstituted aromatic ring and three hydrogens characteristic of a 1,3,4-trisubstituted aromatic ring. The main difference in the ^{13}C NMR spectrum with respect to compounds **1** and **2** was the presence of a carbonyl carbon at δ 203.3. All data mentioned above indicated that **3** is a dibenzylbutan lignan which presents a carbonyl group next to one of the two aromatic rings. The HMBC correlations between the carbonyl group and the doublet at δ 7.88 (J = 8.8 Hz, 2H) and with the doublet methyl at δ 1.23 ratified the structure depicted in Figure 3 for **3**. We proposed the stereochemistry (8*S*,8′*R*) by biogenetic reasons and also by comparison of the ^{13}C NMR shifts of **3** with those of oleiferin D [(8*S*,8′*R*)-4-hydroxy-3-methoxy-3′,4′-methylenedioxy-7-one-8,8′-lignan], isolated in our phytochemical study whose absolute stereochemistry was determined by X-ray analysis.[26] Taking in to account all data mentioned above, the structure of **3** was assigned as (8*S*,8′*R*)-4-hydroxy-3′,4′-methylenedioxy-7-one-8,8′-lignan.

Compound **4**, with molecular formula $C_{20}H_{22}O_5$ and $[\alpha]_D^{20}$ − 75.2 (*c* 0.2, $CHCl_3$), was isolated as an amorphous solid. The ^1H NMR spectrum of **4** displays signals for two doublet methyls at δ 0.56 (J = 6.9 Hz) and

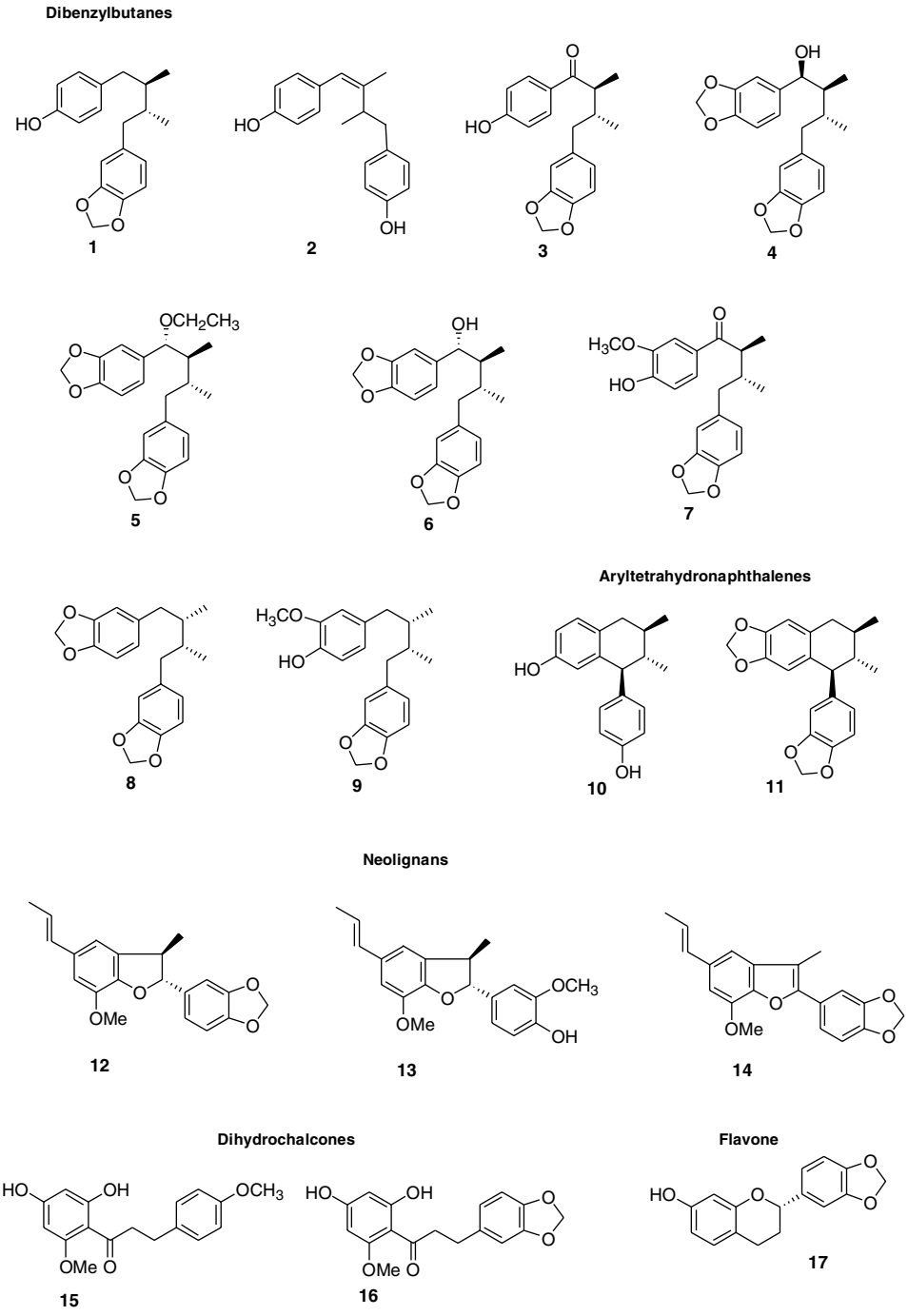

Figure 1. Structures of new lignans **1**–**5** and compounds tested for estrogenic activity.

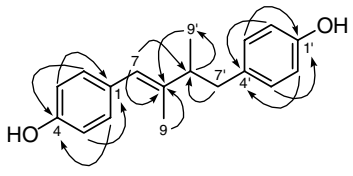

Figure 2. Selected HMBC correlations for compound **2**.

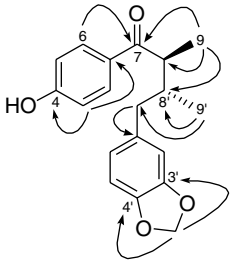

Figure 3. Selected HMBC correlations for compound **3**.

δ 0.85 (J = 6.4 Hz), and two singlets attributable to methylenedioxy groups at δ 5.93 and δ 5.95. The remaining signals are four aliphatic hydrogens as multiplets at δ 1.80 (1H) and δ 2.45 (3H), respectively, and six aromatic protons as multiplet centered at δ 6.70. The main difference with respect to the preceding lignans was the presence of a double doublet at δ 4.30 (J_1 = 1.9 Hz, J_2 = 9.5 Hz), characteristic of a proton geminal to a secondary alcohol. Compound **4** showed spectroscopic data very similar to those of oleiferin C[26] [(7*S*,8*S*,8′*R*)-3,4:3′,4′-bis(methylenedioxy)-7-ol-8,8′-lignan; $[\alpha]_D^{25}$ + 44.0 (*c* 0.8, $CHCl_3$)] but the optical rotation was different, so compound **4** is a diastereoisomer of oleiferin C. We tried to confirm the epimeric stereochemistry of the secondary alcohol at C-7 by ^{1}H NMR using the method of Riguera et al.,[51] but we did not obtain reliable results. Taking in to account all these data together with the value of the optical rotation and biogenetic considerations the structure of **4** was determined as (7*S*,8*S*,8′*R*)-7-hydroxy-3,4:3′,4′-*bis*-(methylenedioxy)-8,8′-lignan.

Compound **5** was obtained as an amorphous solid. The HREIMS of **5** gave a molecular ion at *m/z* 370.1797 corresponding to a molecular formula of $C_{22}H_{26}O_5$. The ^{1}H and ^{13}C NMR spectra were also very close to those of oleiferin C,[26] the main difference was the presence of an additional ethoxy group, and consequently, compound **5** is a dibenzylbutane with two piperonyl groups and an ethoxy substituent at C-7. This consideration was ratified by COSY and HMBC correlations. Considering that EtOH was used in the extraction process, it seems probable that the metabolites in the plant are in hydroxyl form, rather than their corresponding ethyl ethers. To verify that **5** was an artifact, 15 mg of oleiferin C dissolved in 2 ml of EtOH was treated with traces of H_2SO_4. The mixture was stirred for 24 h and then the solvent was removed under vacuum, and after purification using preparative TLC, the obtained derivative showed identical spectroscopic data and $[\alpha]_D^{20}$ to compound **5**. All of these results allowed us to establish the structure of **5** as (7*R*,8*S*,8′*R*)-7-ethoxy-3,4:3′,4′-dimethylenedioxy-8,8′-lignan.

2.2. Estrogenic activity

Phytoestrogen is a general term to define several different families of vegetable kingdom-derived natural products, which show weak estrogenic or antiestrogenic activity toward mammals. Such compounds include flavonoids[52] (kaempferol and quercetin), isoflavonoids[53] (genistein, daidzein, formonetin, and equol), lignans[54] (enterolactone and enterodiol), mycotoxins,[55,56] (zearelenol), and stilbenes[57,58] (resveratrol). In general, plant lignans such as pinoresinol, lariciresinol, syringaresinol, secoisolariciresinol, and matairesinol are not estrogenic themselves. They are converted by the microflora of the mammalian gut to 'mammalian lignans', which have estrogenic character.[59,60] Recently, it has been reported that the lignan nordihydroguaiaretic acid (NDGA) exhibits estrogenic activity in vivo as well as in vitro, in an unique way.[39] During the last years, other tetrahydrofuran type lignans also have shown estrogen-like properties in CV-1 cells transfected with human estrogen receptor (ERα).[38]

With these antecedents and due to the resemblances of some isolated lignans with the structure of dihydroguaiaretic acid, we decided to assay for estrogenic activity of the new lignans together with some of the known compounds. The results of proliferation were estimated after 6 days treatment of cells with natural products or E_2 (reference estrogen). The estrogenic parameters RPE and RPP were calculated and are given in Table 3.

The most active compounds have hydroxyl groups in one or two aromatic rings. Among the dibenzylbutane lignans, compounds **1**, **2**, and **5** showed the highest proliferative effects, with RPEs of 69%, 74%, and 84%, respectively. Dibenzylbutanes **1**, **3** show the same aromatic substitution pattern, therefore the presence of carbonyl function at C-7 (derivative **3**) decreases the activity. The presence of an ethoxy group at C-7 produces a significant increase of the proliferative activity (compare the most active compound **5** with **6**). The stereochemistry of the oxygenated group at C-7 is also an

Table 1. ^{1}H NMR ($CDCl_3$, *J* in Hz in parentheses) of lignans **1**–**5**

Proton	1	2	3	4	5
2	6.58 s	7.01 d (8.6)	7.88 d (8.8)	6.61–6.80 m	6.63 s
3	—	6.72 d (8.1)	6.88 d (8.7)	—	—
4	—	4.79 sa	—	—	—
5	6.54 d (7.9)	6.72 d (8.1)	6.88 d (8.7)	6.61–6.80 m	6.74 m
6	6.70 d (7.7)	7.01 d (8.6)	7.88 d (8.8)	6.61–6.80 m	6.62 d (7.9)
7	2.35 m	6.10 s	—	4.3 dd (1.9, 9.5)	3.88 d (8.7)
8	1.73 m	—	3.38 q (6.6)	1.80 dt (7.1, 9.5)	1.67 m
9	0.81 d (6.6)	1.80 s	1.23 d (6.9)	0.56 d (6.9)	0.97 d (6.8)
2′	6.73 d (8.2)	—	6.55 s	6.61–6.80 m	6.44 s
3′	6.96 d (8.2)	7.05 d (8.7)	—	—	—
4′	—	6.76 d (8.1)	—	—	—
5′	6.96 d (8.2)	4.89 sa	6.68 d (7.8)	6.61–6.80 m	6.67 m
6′	6.73 d (8.2)	6.76 d (8.1)	6.51 d (7.8)	6.61–6.80 m	6.43 d (6.1)
7′	2.56 m	7.05 d (8.7)	2.15 m	2.50 m	2.40 m
8′	1.73 m	2.64 m	2.80 m	2.42 m	1.51 m
9′	0.81 d (6.6)	2.45 m	0.83 d (6.5)	0.85 d (6.4)	0.75 d (6.8)
OCH_2O	5.93 s	—	—	5.93 s	5.82 s
				5.95 s	5.96 s

Table 2. ^{13}C NMR ($CDCl_3$) of lignans **1**–**5**[a]

Carbon	1	2	3	4	5
1	135.5 s	131.4 s	160.1 s	138.4 s	136.0 s
2	109.3 d	130.1 d	131.2 d	106.9 d	107.8 d
3	147.4 s	114.8 d	115.8 d	147.8 s	147.7 s
4	145.4 s	153.5 s	160.1 s	147.0 s	146.7 s
5	107.9 d	114.9 d	115.8 d	108.0 d	107.3 d
6	121.7 d	130.1 d	131.2 d	120.4 d	120.9 d
7	40.5 t	124.1 d	203.3 s	77.2 d	85.5 d
8	38.1 d	141.0 s	45.67 d	42.7 d	41.6 d
9	13.8 q	14.9 q	14.30 q	10.0 q	10.0 q
1′	100.6 t	133.4 s	135.1 s	135.2 s	135.0 s
2′	133.8 s	130.1 d	109.9 d	108.0 d	109.3 d
3′	114.9 d	114.8 d	147.9 s	147.5 s	147.4 s
4′	130.0 d	153.5 s	146.0 s	145.5 s	145.4 s
5′	153.5 s	114.9 d	108.4 d	109.4 d	107.6 d
6′	130.0 d	130.1 d	122.4 d	121.8 d	121.7 d
7′	114.9 d	41.0 t	38.58 t	41.8 t	41.5 t
8′	41.1 t	45.5 d	39.31 d	33.9 d	35.1 d
9′	38.1 d	18.9 q	18.06 q	13.0 q	14.1 q
OCH_2O	—	—	101.1 t	101.0 t	100.6 t
				100.7 t	100.9 t

[a] Data based on HMBC, HMQC, and DEPT spectra.

Table 3. Quantitative evaluation of compounds **1**–**17** in the E-Screen bioassay[a]

Test compound	MOEC[b] (μM)	PE[c] Mean ± SD	RPP[d] (%)	RPE[e] (%) means ± SD
17β-Estradiol	0.001	8.00 ± 0.95	100	100
1	10	5.8 ± 0.3	0.01	68.7 ± 16
2	10	6.2 ± 0.4	0.01	73.7 ± 23
3	10	4.51 ± 0.3	0.01	50.1 ± 13
4		n.a.	0	0
5	10	6.9 ± 0.5	0.01	84.0 ± 21
6	10	4.4 ± 0.2	0.01	47.8 ± 12
7	10	4.0 ± 0.4	0.01	42.8 ± 16
8	10	1.9 ± 0.1	0.01	13.5 ± 5.0
9		n.a.	0	0
10	10	6.4 ± 0.2	0.01	77.8 ± 14
11	10	2.2 ± 0.1	0.01	17.9 ± 8.0
12		n.a.	0	0
13	10	2.8 ± 0.2	0.01	26.4 ± 9.0
14		n.a.	0	0
15	10	6.6 ± 0.6	0.01	80.7 ± 15
16	10	2.5 ± 0.3	0.01	22.1 ± 10
17	10	5.7 ± 0.3	0.01	67.1 ± 16

[a] Lignans without effect on MCF-7 BUS proliferation are indicated as not active (n.a.).
[b] MOEC is the minimal dose of test compound to induce a maximal cell proliferation.
[c] PE is the ratio of the highest cell number obtained with the lignan or E_2 to that of the estrogen-free control.
[d] RPP is the ratio of MOEC of the E_2 to that of phytoestrogen expressed as a percentage.
[e] RPE is the ratio between the PE-1 of the phytoestrogen and the PE-1 of E_2 expressed as a percentage.

important factor of the proliferative activity (**4** vs **5**). Furthermore, methyl groups in *syn* disposition decrease the activity (compounds **8** and **9**). In short, this preliminary structure–activity relationship (SAR) analysis indicates that the substitution at C-7, the relative stereochemistry of the methyl groups, and the oxygenated substituents on the aromatic rings play an important role in the estrogenic activity for the dibenzylbutane lignans.

As far as the two arylnaphthalenes are concerned, the one bearing two hydroxyl groups on the aromatic ring (**10**) exhibited higher activity than the one with two methylenedioxy groups (**11**). The neolignan **13** showed a modest proliferative activity on MCF-7 BUS cells, whereas neolignans **12** and **14** were inactive. The dihydrochalcone **15** and the flavone **17**, both having hydroxyl groups, displayed high estrogenic activity. However, dihydrochalcone **16** showed a modest proliferative activity.

In terms of potency, all active compounds induced the maximal proliferative effect at the minimal dose of 10 μM (MOEC 10 μM) and had the RPP of 0.01% relative to E_2. Therefore, the active phytoestrogens were 10,000 times less potent than E_2 to induce maximal cell proliferation.

We have shown how *I. lancifolia* is an important source of metabolites with estrogenic activity and, therefore, the stem bark of *I. lancifolia* would be effective as preventive agent in cardiovascular and estrogen-dependent diseases.

3. Experimental

3.1. General experimental procedures

IR spectra were obtained using a Bruker IFS28/55 spectrophotometer. Optical rotations were measured with a Perkin-Elmer 241 automatic polarimeter. ^1H and ^{13}C spectra were recorded in $CDCl_3$ or C_5D_5N on a Bruker spectrometry at 300 and 75 MHz, respectively, with TMS as the internal reference. The 2D NMR experiments were conducted on a Bruker WP-400 SY NMR spectrometer in $CDCl_3$ at 400 MHz. Optical rotations were measured with a Perkin-Elmer 241 automatic polarimeter; $[\alpha]_D^{20}$ are given in 10^{-1} deg cm^2g^{-1}. UV spectra were collected with a Perkin-Elmer model 550-SE and IR spectra on a Bruker IFS 55 FT-IR spectrometer. High- and low-resolution mass spectra were obtained on a VG Autospec spectrometer. Macherey-Nagel polygram Sil G/UV_{254} and preparative TLC SIL G-100UV_{254} foils were used for TLC. Silica gel (0.2–0.063 mm) and Sephadex LH-20 were used for column chromatography.

3.2. Plant material

Stem bark of *I. lancifolia* was collected in Maynas, Loreto Region (Perú), in May 2003, and was identified by the botanist Joaquina Albán. A voucher specimen (voucher number JA 14800) was deposited in the Herbarium of the Museo de Historia Natural, Universidad Nacional Mayor de San Marcos (Lima, Perú).

3.3. Extraction and isolation

Dried stem bark of *I. lancifolia* (2.02 kg) was extracted with EtOH (3 L) in a Soxhlet apparatus. 40 g of the

total extract (98 g) was chromatographed on silica gel using, as eluent, mixtures of *n*-hexane/EtOAc of increasing polarity. Seven fractions, A-G, were separated, studied, and chromatographed on Sephadex LH-20, eluting with *n*-hexane/$CHCl_3$/MeOH (2:1:1) and column chromatography or preparative TLC silica gel, using as solvent mixtures of *n*-hexane–EtOAc. Fraction A yielded cagayanin[29] (12 mg), eupomatenoid 3[33] (2.3 mg), machilin A[27] (1.2 mg), and **5** (9 mg). Licarin B[30] (11 mg) was isolated from fraction B. Fraction C yielded austrobailignan 6[28] (4 mg) and **1** (9.6 mg). Fraction D afforded licarin A[31] (103 mg), oleiferin C[26] (35 mg), oleiferin D[26] (34 mg), perseal F[32] (1 mg), 7-hydroxy-3′,4′-methylenedioxy-flavone[34] (9.4 mg), **3** (2.4 mg), and **4** (2.3 mg). Fraction E yielded sargacromenol[35] (3.7 mg), 2′,4′-dihydroxy-4,6′-dimethoxy-dihydrochalcone[19] (6.5 mg), 2′,4′-dihydroxy-6′-methoxy-3,4-methylenedioxy-dihydrochalcone[20] (24 mg), (8*R*,7′*S*,8′*S*)-4,4′-bis(hydroxy)-6,7′-cyclolignan[18] (16.5 mg) and **2** (9 mg). Iryantherin K[23] (45 mg) was isolated from fraction F and fraction G yielded 25 mg of 1,3,4-trihydroxybenzene and 16 mg of β-sitosterol-β-D-glucoside acetate.[36]

3.4. 4′-hydroxy-3,4-methylenedioxy-8,8′-lignan (1)

Amorphous solid; $[\alpha]_D^{20} - 22.9$ (*c* 0.5, $CHCl_3$); UV (EtOH) λ_{max} ($\log\varepsilon$) 286 (3.7), 226 (4.0) nm; IR ($CHCl_3$) γ_{max} (film) 3400, 2960, 2926, 2874, 1613, 1513, 1504, 1489, 1441, 1379, 1359, 1246, 1189, 1173, 1097, 1040, 935, 852, 810, 758 cm^{-1}; EIMS *m/z* (%) 298 (M^+, 43), 135 (100), 107 (52), 77 (14); HREIMS *m/z* 298.1554 (calcd for $C_{19}H_{22}O_3$ 298.1569); ^1H NMR ($CDCl_3$, 300 MHz) δ 4.54 (1H, s, OH-4′), for the rest of signals see Table 1; ^{13}C NMR ($CDCl_3$, 75 MHz), see Table 2.

3.5. (*E*)-4,4′-dihydroxy-7-en-8,8′-lignan (2)

Amorphous solid; $[\alpha]_D^{20} - 46.2$ (*c* 0.3, $CHCl_3$); UV (EtOH) λ_{max} ($\log\varepsilon$) 286 (3.7), 226 (4.0) nm; IR ($CHCl_3$) γ_{max} (film) 3400, 2925, 1702, 1611, 1511, 1449, 1373, 1234, 1173, 1107, 1042, 1015, 991, 938, 898, 865, 849, 825, 784, 771, 716 cm^{-1}; EIMS *m/z* (%) 268 (M^+) (5), 212 (7), 161 (100), 107 (38), 77 (6); HREIMS *m/z* 268.1474 (calcd for $C_{18}H_{20}O_2$ 268.1463); ^1H NMR ($CDCl_3$, 300 MHz) δ 4.59 (1H, s, OH-3); 4.68 (1H, s, OH-3′), for the rest of signals see Table 1; ^{13}C NMR($CDCl_3$, 75 MHz), see Table 2.

3.6. (8*S*,8′*R*)-4-hydroxy-3′,4′-methylenedioxy-7-ona-8,8′-lignan (3)

Amorphous solid; $[\alpha]_D^{20} - 2.5$ (*c* 0.2, $CHCl_3$); UV (EtOH) λ_{max} ($\log\varepsilon$) 283 (3.5), 216 (3.5) nm; IR ($CHCl_3$) γ_{max} (film) 3300, 2925, 2370, 1602, 1581, 1504, 1489, 1442, 1377, 1284, 1247, 1221, 1188, 1171, 1122, 1075, 1040, 971, 930, 849, 798, 766 cm^{-1}; EIMS *m/z* (%) 312 (M^+, 2), 162 (100), 150 (15), 121 (20), 93 (4), 77 (5); HREIMS *m/z* 312.1490 (calcd for $C_{19}H_{20}O_4$ 312.1362); ^1H NMR ($CDCl_3$, 300 MHz) δ 5.41 (1H, bs, OH-4), for the rest of signals see Table 1; ^{13}C NMR ($CDCl_3$, 75 MHz) see Table 2.

3.7. (7*S*,8*S*,8′*R*)-7-hydroxy-3,4:3′,4′-dimethylenedioxy-8,8′-lignan (4)

Amorphous solid; $[\alpha]_D^{20} - 75.2$ (*c* 0.2, $CHCl_3$); UV (EtOH) λ_{max} ($\log\varepsilon$) 286 (3.3), 234 (3.4) nm; IR ($CHCl_3$) γ_{max} (film) 2960, 2924, 2854, 1504, 1488, 1441, 1380, 1247, 1189, 1124, 1096, 1040, 931, 860, 810, 772 cm^{-1}; EIMS *m/z* (%) 342 (M^+, 11), 267 (11), 238 (16), 162 (44), 151 (100), 135 (30), 93 (25), 77 (10); HREIMS *m/z* 342.1544 (calcd for $C_{20}H_{22}O_5$ 342.1467); ^1H NMR ($CDCl_3$, 300 MHz) see Table 1; ^{13}C NMR ($CDCl_3$, 75 MHz) see Table 2.

3.8. (7*R*,8*S*,8′*R*)-7-ethoxy-3,4:3′,4′-dimethylenedioxy-8,8′-lignan (5)

Amorphous solid; $[\alpha]_D^{20} + 43.2$ (*c* 0.9, $CHCl_3$); UV (EtOH) λ_{max} ($\log\varepsilon$) 287 (3.9), 235 (4.0) nm; IR ($CHCl_3$) γ_{max} (film) 2965, 2927, 2876, 1731, 1503, 1487, 1441, 1399, 1378, 1358, 1323, 1246, 1188, 1120, 1085, 1041, 938, 858, 809, 772 cm^{-1}; EIMS *m/z* (%) 370 (M^+, 5), 324 (81), 267 (23), 238 (31), 210 (8), 179 (100), 162 (12), 151 (18), 135 (14); HREIMS *m/z* 370.1797 (calcd for $C_{22}H_{26}O_5$ 370.1780); ^1H NMR ($CDCl_3$, 300 MHz) see Table 1; ^{13}C NMR ($CDCl_3$, 75 MHz) see Table 2.

3.9. Biological assay

3.9.1. Cell culture. Estrogen receptor-positive human breast adenocarcinoma MCF-7 BUS cells were kindly provided by Dr. Olea-Serrano of Granada University (Spain). The MCF-7 BUS cell line was routinely cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% of heat-inactivated fetal bovine serum, antibiotics (100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin), and glutamine (4 mM). Cells were grown at 37 °C in a humidified atmosphere of 95% containing 5% CO_2. Medium was renewed 2–3 times per week.

3.9.2. Charcoal–dextran stripped human serum preparation. In order to minimize the estrogenic activity of serum, steroid hormones were stripped from fetal bovine serum (FBS) by treatment with charcoal and dextran, using an existing modified protocol.[61] The charcoal–dextran stripped FBS (CD-FBS) was filtered and stored at −20 °C until used.

3.9.3. Estrogenic assay (E-Screen test). The potential estrogenic activity of the samples was assessed by measuring the induction of cell proliferation under estrogen-free culture conditions using MCF-7 BUS cell line by slightly modified method of Soto et al.[62,63] The induction of cell proliferation was monitored using the sulforhodamine B (SRB) protein-staining procedure of Skehan et al.,[64] a semiquantitative measure of total cell density.

For the assay, confluent MCF-7 BUS cells were harvested, resuspended in full medium, counted, and seeded into 24-well plate at a density of 20,000 cells/well. After 24 h, the cells were washed

with Phosphate Buffer Solution (PBS) with Ca^{2+} and Mg^{2+}. The test compounds were dissolved in DMSO and serial diluted (1 nM–10μM) into estrogen-free medium (phenol red-free DMEM containing 5% CD-FBS supplemented with 4 mM glutamine, 20 mM HEPES, and 0.1% sodium bicarbonate), with a final DMSO concentration of 0,1% in all cases (four wells per concentration per product preparation). In each experiment, a dose–response curve (1 pM–10 nM) of E_2, a negative control (cell treated only with estrogen-free medium) and vehicle control (estrogen-free medium plus final vehicle concentration) run simultaneously with the samples was included. After 6 days, the cells were fixed and stained using a SRB protocol. The fixed dye was resuspended and read at 492 nm using BioTek's PowerWave XS Absorbance Microplate Reader. Values were corrected for background optical density (OD) from wells only containing medium. The relationship between OD and cell number was previously established by performing different cell inocula and counting half of the wells with a flow cytometer and half with SRB protocol.

To evaluate potential cytotoxicity, the lower product dilution assayed was also tested in the presence of 10 nM E_2 to induce maximal cell proliferation. If maximal proliferation was achieved, it was interpreted as a lack of toxicity. If the cell number was less than the positive control, it was used to qualify the product as cytotoxic. The coincubation of the natural compounds at 10 μM with E_2 did not affect the maximal cell proliferation when compared to E_2 alone (positive control), indicating that all compounds assayed are devoid of cytotoxic activity (data not shown).

The mean cell number from each experiment was normalized to the steroid-free control cultures to correct for differences in the initial seeding density. Values given are means ± the SE for 12 independent observations (three replicates of assays using quadruplicate wells per treatment group).

3.9.4. Estrogenic parameters. The endpoint of the E-Screen test is the cell number relative to the estrogen-free control (negative control). The estrogenic activity of lignans was calculated with the following parameters.[65–67]

MOEC: The lowest concentration of test compound needed for maximal cell yield. It is estimated from the dose–response curve constructed from doses versus activity using SigmaPlot version 4 from Windows software.

3.9.5. Proliferative effect (PE). Ratio of the highest cell yield obtained with the phytoestrogen or E_2 to that of the negative control.

$$PE = \frac{\text{Cell Number } (\textit{Phytoestrogens or } E_2)}{\text{Cell Number } (\textit{Negative control})}$$

3.9.6. Relative Proliferative Effect (RPE). This parameter is the ratio of the maximum proliferation with the phytoestrogen to that of E_2, expressed in percentage.

$$RPE = \frac{\text{PE} - 1(\textit{Phytoestrogens})}{\text{PE} - 1(E_2)} \times 100$$

Thus, the RPE indicates whether or not the test compound induces (a) a proliferative response quantitatively similar to that one obtained with E_2; that is, full agonist (RPE = 100), or (b) a proliferative effect significantly lower than that obtained with E_2, that is, partial agonist (RPE < 100). Finally, RPE describes the efficacy of the test compound relative to that of E_2.

3.9.7. Relative proliferative potency (RPP). The ratio of concentration of E_2 and that of the phytoestrogen that was necessary to obtain MOEC, expressed in percentage. Thus, the RPP describes the potency of the test compound relative to that of E_2.

$$RPP = \frac{\text{MOEC } (E_2)}{\text{MOEC } (\textit{Phytoestrogens})} \times 100$$

The RPP value for E_2 is 100.

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