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Anti-HIV activity of dibenzylbutyrolactone-type lignans from *Phenax* species endemic in Costa Rica

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Abstract

Previously, we isolated two new dibenzylbutyrolactone-type lignans, named phenaxolactones **1** and **2**, from the leaves of *Phenax angustifolius* Wedd. (Urticaceae). In this investigation three new dibenzylbutyrolactone lignans (phenaxolactones **3**–**5**), together with phenaxolactone **1**, and flavones vitexin (**6**), isovitexin (**7**), were isolated from *Phenax rugosus* Wedd. leaves collected in Santa Ana, Costa Rica. The structures were elucidated using 1D and 2D NMR spectroscopy as well as mass spectrometry. Phenaxolactones **1**–**5** and flavones **6** and **7** were evaluated for their inhibitory activity against HIV-1_{MN} in infected C8166 cells. The most promising compound was phenaxolactone **1** with an EC50 value of 3.0 μM, no cytotoxicity at 112 μM and a therapeutic index value of 37.3.

Introduction

HIV-AIDS is a major cause of mortality and due to the potential for development of resistance to existing therapies, discovery of new therapeutic agents is crucial. Investigations into the molecular processes of HIV infection have led to the identification of a number of macromolecular targets for drug design, such as HIV-1 reverse transcriptase, protease, and integrase enzyme, and regulatory proteins. Other targets include proteins that are involved in virus attachment and fusion. The viral surface protein gp120 interacts with the cellular receptor CD4 (Dalgleish et al 1984). This causes a change in the conformation of gp120 (Wyatt et al 1998), which binds to a second cellular receptor of the family of chemokine receptors (LaBranche et al 2001), allowing the HIV protein gp41 to penetrate the cell membrane, leading to membrane fusion (Yachou & Sekaly 1999).

We report the isolation and structure elucidation of the new dibenzylbutyrolactone-type lignans from plants and their inhibitory activities against HIV-infection in cell cultures.

Phenax is a genus of the family Urticaceae consisting of aproximately12 species, which are very well distributed throughout Tropical America, although some of them have become naturalized in the Asian tropics (Burger 1977). The individuals are shrubs or herbs and, occasionally, small trees. These species grow wild in Costa Rica and have been used traditionally as insecticidal agents (Rastrelli et al 1998). Previously, we isolated two new dibenzylbutyrolactone-type lignans, named phenaxolactones **1** and **2**, from the leaves of *Phenax angustifolius* Wedd. (Urticaceae) (Rastrelli et al 2001). In this investigation three new dibenzylbutyrolactone lignans named phenaxolactones **3**–**5**, together with phenaxolactone **1** and flavones vitexin (**6**) and isovitexin (**7**), have been isolated from *Phenax rugosus* Wedd. leaves collected in Santa Ana, Costa Rica.

Lignans are found in the roots, stems, bark, fruit and seeds of many plant species and are derived from dimerization of phenylpropanoid units at the central carbons of their side chains. Lignans of the podophyllotoxin and dibenzylbutyrolactone series have received considerable interest recently as antiviral agents. There have been several modes of antiviral activity associated with lignans: tubulin binding (inhibition of tubulin polymerization interferes with the formation of the cellular cytoskeleton and with some critical viral processes), reverse transcriptase inhibition, integrase inhibition

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and topoisomerase inhibition. Whereas podophyllotoxin and its derivatives were the most prominent representatives of the tubulin binding lignans (Hammonds et al 1996), inhibition of reverse transcriptase was observed for various classes of lignans, such as dibenzylbutyrolactones, dibenzylbutanes, dibenzylcyclooctadienes, and aryltetralins (Charlton 1998). Dibenzylbutyrolactones derived from arctigenin were active as inhibitors of viral integrase (Eich et al 1996), whereas, the plant lignan nordihydroguaiaretic acid was found to suppress HIV-1 replication in infected cells by preventing proviral transcription and HIV Tat-transactivated transcription (Gnabre et al 1995).

Materials and Methods

General experimental procedures

Optical rotations were measured on a Perkin-Elmer 192 polarimeter equipped with a sodium lamp (589 nm) and a 1-cm microcell. UV spectra were obtained with a Beckman DU 670 spectrophotometer and IR spectra with a Bruker IFS-48 spectophotometer. A Bruker DRX-600 spectrometer, operating at 599.19 MHz for ^{1}H and 150.858 for ^{13}C, using the UXNMR software package was used for NMR experiments in CD_3OD. ^{1}H-^{1}H DQF-COSY (Double Quantum Filtered COSY), ^{1}H-^{13}C HSQC and HMBC experiments were obtained using conventional pulse sequences. The selective excitation spectra, 1D TOCSY (Davis & Bax 1985), were acquired using waveform generator-based GAUSS shaped pulses, mixing times ranging from 100 to 120 ms, and MLEV-17 spin-lock field of 10 kHz preceded by a 2.5-ms trim pulse; chemical shifts were expressed in δ (ppm) referring to solvent peaks: δ_H 3.34 and δ_C 40.0 for CD_3OD. Electrospray ionization mass spectrometry (ESI-MS) in the positive and negative ion mode was performed using a Finnigan LC-Q Deca instrument from Thermoquest (San Jose, CA) equipped with Excalibur software. Samples were dissolved in MeOH and infused in the ESI source by using a syringe pump; the flow rate was 3 μL min^{-1}. The capillary voltage was 5 V, the spray voltage was 5 kV, and the tube lens offset at 35 V. The capillary temperature was 220°C. Data were acquired in the MS1 scanning mode (m/z 150–700). Exact masses were measured by a Q-Star Pulsar (Applied Biosystems) triple-quadrupole orthogonal time-of-flight (TOF) instrument. Electrospray ionization was used in TOF mode at 8.500 resolving power. Samples were dissolved in pure MeOH, mixed with the internal calibrant, and introduced directly into the ion source by direct infusion. Calibration was performed on the peaks of CsI and synthetic peptide (TOF positive ion calibration solution, Bachem,) at m/z 132.9054 and 829.5398, respectively. Sodium-containing molecular ions of analytes were measured. HPLC separations were performed on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector and a Waters μ-Bondapak C18 10 μm (300 × 7.8 mm) column.

Plant material

The leaves of *P. rugosus* (HBK) Weddell 1 were collected near Santa Ana, San José, Costa Rica, in February 1999 and identified by Professor Luis Poveda, of the Universidad Nacional. A specimen of the plant (P.r. 3, 1999) used in this study was deposited at the Herbario Juvenal Valerio Rojas, Universidad Nacional.

Extraction and isolation

The powdered, dried aerial parts (550 g) were extracted at room temperature with EtOH/H_2O (7:3) to give 10.65 g lyophilized extract. Part of the extract (8 g) was partitioned between *n*-BuOH and H_2O to afford a *n*-BuOH-soluble portion (4.7 g) which was chromatographed on a Sephadex LH-20 column (100 × 5 cm) using MeOH as eluent. Fractions (9 mL) were collected and checked by TLC (Silica gel, *n*-BuOH/HOAc/H_2O (60:15:25)). Fractions 18–29 (75 mg), containing the crude lignans mixture, were submitted to RP-HPLC on a C-18 μ-Bondapack column (300 × 7.8 mm, flow rate 2.5 mL min^{-1}) using MeOH/H_2O (4:6) as the eluent to yield phenaxolactones **1** (20.2 mg; t_R, 16.7 min), **3** (18.0 mg; t_R, 8.2 min), **4** (17.5 mg; t_R, 14.2 min) and **5** (9.5 mg; t_R, 7.2 min) (see Figure 1). Fractions 58–64 (120 mg) were separated in the same conditions giving vitexin (**6**) (11.0 mg; t_R, 15 min) and isovitexin (**7**) (15.3 mg; t_R, 16 min).

Phenaxolactone ***1***
2-Hydroxy-2-(3′,4′-dihydroxyphenyl)-methyl-3-(3″,4″-dimethoxyphenyl)-methyl-γ-butyrolactone. Amorphous yellow residue. $[\alpha]_D^{25}$: −39.1° (*c* 0.5, MeOH); UV (MeOH) λ_{max} 233 (4.15), 282 (3.75) nm; IR (KBr) ν_{max} 3405, 2916, 2866, 1636, 1564 cm^{-1}. ESI-MS m/z 373 [M-H]$^-$. HR-ESIMS (negative) m/z 373.2345 (calcd for $C_{20}H_{22}O_7$, 373.3038, [M - H]$^-$). ^{1}H and ^{13}C NMR data were consistent with those previously reported (Rastrelli et al 2001).

Phenaxolactone ***2***
2-Hydroxy-2-(4′-*O*-β-D-glucopyranosyl-3′-hydroxyphenyl)-methyl-3-(3″, 4″-dimethoxyphenyl)-methyl-γ-butyrolactone. Amorphous yellow residue isolated from *Phenax angustifolius*. $[\alpha]_D^{25}$: −57.5° (*c* 0.5, MeOH); UV λ_{max} (MeOH) nm 230 (4.08), 280 (3.80); IR (KBr) ν_{max} 3414, 2938, 2850, 1612, 1524 cm^{-1}. ESI-MS m/z 535 [M-H]$^-$, 373 [(M-H)-162]$^-$. HR-ESIMS (negative) m/z 535.3746 (calcd for $C_{26}H_{32}O_{12}$, 535.2226, [M - H]$^-$). ^{1}H and ^{13}C NMR data were consistent with those previously reported (Rastrelli et al 2001).

Phenaxolactone ***3***
2-Hydroxy-2-(3′, 4′-dihydroxyphenyl)-methyl-3-(3″-methoxy, 4″-hydroxyphenyl)-methyl-γ-butyrolactone. Amorphous yellow residue. $[\alpha]_D^{25}$: −38.0° (c 0.5, MeOH); UV λ_{max} (MeOH) nm 232 (6.10), 280 (4.05); IR (KBr) λ_{max} 3405, 2916, 2866, 1636, 1564 cm^{-1}. ^{1}H and ^{13}C NMR data, see Table 1; ESI-MS

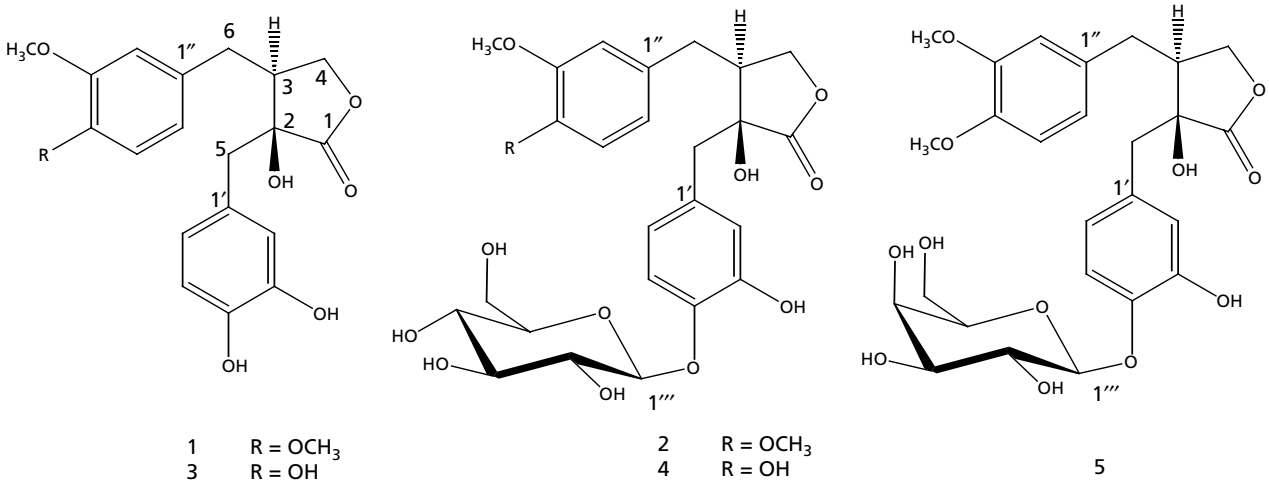

Figure 1 Phenaxolactones **1**–**5** isolated from *Phenax angustifolius* and *P. rugosus*.

m/z 359 [M-H]$^-$. HR-ESIMS (negative) *m/z* 359.5103 (calcd for $C_{19}H_{20}O_7$, 359.4032, [M - H]$^-$).

Phenaxolactone ***4***
2-Hydroxy-2-(4′-*O*-*β*-D-glucopyranosyl-3′-hydroxyphenyl)-methyl-3-(3″-methoxy, 4″-hydroxyphenyl)-methyl-γ-butyrolactone. Amorphous yellow residue. $[\alpha]_D^{25}$: −64.1° (*c* 0.5, MeOH); UV λ_{max} (MeOH) nm 230 (4.12), 280 (4.00); IR (KBr) ν_{max} 3412, 2936, 2854, 1610, 1526 cm^{-1}. ^{1}H and ^{13}C NMR data, see Table 1; ESI-MS *m/z* 521 [M-H]$^-$, 359 [(M-H)-162]$^-$. HR-ESIMS (negative) *m/z* 521.2450 (calcd for $C_{25}H_{30}O_{12}$, 521.1440, [M - H]$^-$).

Phenaxolactone ***5***
2-Hydroxy-2-(4′-*O*-*β*-D-galactopyranosyl-3′-hydroxyphenyl)-methyl-3-(3″,4″-dimethoxyphenyl)-methyl-γ-butyrolactone. Amorphous yellow residue. $[\alpha]_D^{25}$: −56.1° (*c* 0.5, MeOH); UV λ_{max} (MeOH) nm 230 (4.15), 280 (3.95); IR (KBr) ν_{max} 3415, 2935, 2854, 1600, 1524 cm^{-1}. ^{1}H and ^{13}C NMR data, see Table 1; ESI-MS *m/z* 535 [M-H]$^-$, 373 [(M-H)-162]$^-$. HR-ESIMS (negative) *m/z* 535.3012 (calcd for $C_{26}H_{32}O_{12}$, 535.1941, [M - H]$^-$).

Vitexin ***6***
^{1}H and ^{13}C NMR data were consistent with those previously reported (Agrawal 1989; Harborne 1994). ESI-MS *m/z* 431 [M-H]$^-$.

Isovitexin 7
^{1}H and ^{13}C NMR data were consistent with those previously reported (Agrawal 1989; Harborne 1994). ESI-MS *m/z* 431[M-H]$^-$.

Antiviral assays

The anti-HIV activity and toxicity of compounds were assessed in C8166 human T lymphoblastoid cells infected with HIV-1_{MN}. The cells were cultured in RPMI 1640 with 10% fetal calf serum. Forty-thousand cells per microtitre plate well were mixed with fivefold dilutions of compounds before addition of 10 CCID50 units of virus and incubated for five to six days. The inhibition of HIV-infection was assessed by examining syncytia, by estimating antigen gp120 by ELISA (Mahmood & Hay 1992), and by measuring cell viability of virus-infected cells and uninfected cell controls using the XTT-formazan method (Weislow et al 1989).

Virus infectivity assay

The total progeny virus was titrated in microtitre plates using double dilutions of freshly collected supernatants and C8166 cells. The end point was determined by examining syncytia formation and by the XTT-formazan method. The virus titre (CCID50) was expressed as the reciprocal of the dilution that gave a 50% end point. To measure the effects of compounds on virus infectivity, HIV-1_{MN} (10^4–10^5 CCID50) was incubated with test compound at 37°C for 1 h, the virus was serially diluted, and the infectivity end-point determined. In each case the compound was diluted to well below the EC50 such that residual compound did not interfere with the virus titration.

Gp120–sCD4 binding assays

Gp120–sCD4 interaction was measured by ELISA. Soluble CD4 (sCD4) was bound to microtitre plate wells at a concentration of 0.05 μg/well. Various dilutions of compounds were mixed with equal volumes of recombinant gp120 (0.04 μg mL^{-1}) and added to CD4 coated wells. After incubation at 37°C for 3–5 h, the binding of gp120 was detected using human anti-HIV serum and anti-human Ig conjugated to horseradish peroxidase. Using WIACALC (Pharmacia LKB) the percent inhibition was calculated from linear logarithmic plots using three concentrations of gp120 alone as standard.

Table 1 ^{13}C NMR ^{1}H NMR data of phenaxolactones **3**–**5** in CD_3OD[a]

	3		**4**		**5**	
Position	δ_C	δ_H (J_{H-H} in Hz)	δ_C	δ_H (J_{H-H} in Hz)	δ_C	δ_H (J_{H-H} in Hz)
1	180.7		180.5		180.4	
2	77.5		77.3		77.3	
3	44.5	2.48, m	44.7	2.50, m	44.6	2.50, m
4	71.9	4.02, 2H, t (10.0)	71.9	4.02, 2H, t (10.0)	71.8	4.01, 2H, t (10.0)
5	41.9	3.17, d (13.5)	41.8	3.15, d (13.5)	41.8	3.15, d (13.5)
		2.98, d (13.5)		2.96, d (13.5)		2.96, d (13.5)
6	32.2	2.89, dd (13.0, 3.5)	32.2	2.86, dd (13.0, 4.5)	32.2	2.87, dd (13.0, 4.5)
		2.55, dd (13.0, 11.0)		2.53, dd (13.0, 11.5)		2.53, dd (13.0, 11.5)
1′	128.1		132.6		132.9	
2′	114.9	6.74, d (1.5)	115.9	6.82, d (1.5)	115.9	6.83, d (1.5)
3′	146.7		147.3		147.3	
4′	148.7		147.4		147.6	
5′	116.2	6.75, d (8.0)	117.8	7.12, d (8.0)	117.8	7.10, d (8.0)
6′	124.1	6.60, dd (8.0, 1.5)	125.0	6.72, dd (8.0, 1.5)	124.1	6.73, dd (8.0, 1.5)
1″	133.6		133.5		133.4	
2″	114.0	6.62, d (1.5)	113.9	6.74, d (1.5)	113.2	6.74, d (1.5)
3″	151.2		151.1		149.2	
4″	146.2		146.4		150.7	
5″	115.4	6.80, d (8.0)	115.3	6.88, d (8.0)	114.0	6.88, d (8.0)
6″	122.5	6.66, dd (8.0, 1.5)	122.4	6.72, dd (8.0, 1.5)	122.2	6.72, dd (8.0, 1.5)
Glc (in **4**) o Gal (in **5**)						
1‴			102.9	4.89, d (7.5)	101.3	5.05, d (7.5)
2‴			74.8	3.32, dd (9.5, 7.5)	72.2	3.57, dd (9.7, 7.5)
3‴			77.9	3.41, t (9.5)	75.6	3.74, dd (7.5, 3.5)
4‴			71.3	3.50, t (9.5)	71.0	3.49, dd (3.5, 1.5)
5‴			78.2	3.38, m	75.4	3.44, ddd (1.5, 5, 7)
6‴			62.4	3.70, dd (12.2, 4.5)	62.2	3.72, dd (12, 5)
				3.88, dd (12.2, 3.5)		3.48, dd (12, 7)
OMe	56.4	3.77, s	56.4	3.78, s	56.4	3.77, s
					56.4	3.79, s

[a]Chemical shift values are in ppm and *J* values in Hz presented in parentheses. Assignments confirmed by 1D TOCSY, 2D DQF-COSY, HSQC, HMBC experiments.

Table 2 Anti-HIV activity of phenaxolactones **1–5**, vitexin (**6**) and isovitexin (**7**) isolated from *Phenax angustifolius* and *P. rugosus*

Compounds	EC50[a]	TC50[b]	Selectivity index
1	3.0 ± 0.05	112 ± 2	37.3 ± 0.7
2	5.0 ± 0.06	75 ± 1.5	15.0 ± 0.53
3	0.8 ± 0.04	10 ± 0.5	12.5 ± 0.16
4	2.8 ± 0.11	18 ± 1.0	6.4 ± 0.1
5	5.2 ± 0.1	75 ± 2.3	14.4 ± 0.15
6	100 ± 5	500 ± 18	5.0 ± 0.09
7	10.0 ± 0.5	200 ± 10	20.0 ± 0.0
AZT	0.016 ± 0.002	> 1000	$> 10^5$

[a]EC50 = concentration (μM) that reduces by 50% the production of gp120 in infected C8166 cells. [b]TC50 = concentration (μM) that causes 50% cytotoxicity to uninfected C8166 cells. The EC50 and TC50 values were calculated from averages of three independent experiments showing similar results.

Statistical analysis

Comparison of the various properties of the various compounds was performed using the Kruskal–Wallis test, followed by post hoc analysis (Nemenyi's test). In all cases, $P < 0.05$ denoted significance.

Results and Discussion

The dried leaves of *Phenax rugosus* were extracted with EtOH–H_2O (7:3). Fractionation of the dried residue on a Sephadex LH-20 column and final purification by HPLC gave six compounds. Three of them were identified as phenaxolactone **1**, vitexin (**6**) and isovitexin (**7**), on the basis of interpretation of their spectroscopic data and specifically by comparison of their NMR values with those in the literature. The last three compounds were identified as new dibenzylbutyrolactone lignans named phenaxolactones **3**–**5**, on the basis of the evidence outlined below.

The ESIMS of phenaxolactone **3** showed an $[M-H]^-$ ion at m/z 359 $[M-H]^-$ which was 14 mass units lower than that of phenaxolactone **1** and consistent with the molecular formula $C_{19}H_{20}O_7$ also deduced using ^{13}C NMR and DEPT analysis. The 600 MHz ^1H NMR spectrum (Table 1) showed signals for two methylenes, an oxymethylene, a methine and a methoxyl group in the aliphatic region. The aromatic side exhibited proton signals at δ 6.74 (d, J = 1.5 Hz, H-2′), 6.75 (d, J = 8.0 Hz, H-5′) and 6.60 (dd, J = 8.0 Hz, J = 1.5 Hz, H-6′), and a δ 6.62 (d, J = 1.5 Hz, H-2″), 6.80 (d, J = 8.0 Hz, H-5″) and 6.66 (dd, J = 8.0 Hz, J = 1.5 Hz, H-6″), in agreement with the presence of two 1,3,4-trisubstituted aromatic rings. The ^{13}C NMR spectrum (Table 1) indicated the presence of a lactone carbonyl (δ_C 180.7) and 12 aromatic carbon resonances, six of which were fully substituted. The remaining carbons (2 CH_2, 1 CH_2O, 1 CH, 1 C-OH) suggested the structure of a diarylbutyrolactone-type of lignan (Nishibe et al 1980; Abdel-Kader et al 1997). Analysis of the 1D and 2D NMR spectra of phenaxolactone **3** with homo- and heteronuclear direct and long-range correlations allowed the assignments of all ^1H and ^{13}C NMR signals as listed in Table 1. Each proton was assigned by means of the ^1H-^1H DQF-COSY technique, which showed correlations (H_2-4/H-3/H_2-6) within the three spin system containing the H_2-4 resonance (δ 4.02, br t, J = 10.0 Hz), typical of methylene protons of a 2,3-*trans*-γ-butyrolactone (Inagaki et al 1972). The COSY spectrum confirmed the presence of two 1,3,4-trisubstituted phenyl groups. The HSQC spectrum of phenaxolactone **3** indicated all of the protonated carbon correlations, thereby leading to the elucidation of the structural skeleton. Readily identifiable constitutive units of phenaxolactone **3** included a lactone, a CH_2 linked to a quaternary carbon, the CH_2-CH-CH_2OR chain (as also evidenced by the COSY experiment), and a tertiary alcoholic group (C-2). The ^{13}C NMR chemical shift of C-2 (δ_C 77.5) was almost superimposable to that of *trans*-fused butyrolactones such as benchequiol (Estevez-Reyes et al 1993) as well as the previously isolated phenaxosides **1** and **2**, and different from that reported for *cis*-fused butyrolactones such as guayadequiol (Gonzales et al 1990) (δ_C 75.9). Also, the resonances of C-5 and C-6, diagnostic for the distinction between *cis*- and *trans*-fused butyrolactone rings (Nishibe et al 1980), confirmed a 2,3-*trans*-γ-butyrolactone structure for phenaxolactone **3**. The chemical shifts of the aryl carbons and hydrogens indicated a 3′,4′-dihydroxy and a 3″-methoxy,4″-hydroxy substitution on the phenyl groups. This demonstrated that phenaxolactone **3** differed from phenaxolactone **1** only in the substitution of the methoxy group at C-4″with a phenolic function, as suggested by the absence of the signals at δ 3.80 in the ^1H NMR spectra, and at δ 56.50 in the ^{13}C NMR spectra, and by some small $\Delta\delta$ differences in the carbon resonances of ring B. The presence of methoxyl group at C-3″ was confirmed by HMBC data, which indicated that C-3" was coupled to a methoxy singlet (δ 3.78) and to H-2″ and H-5″; C-4″ to both H-2″, H-5″ and H-6″; C-5″ to H-6″. Other diagnostic correlations were observed between signals assigned to H-2″ and H-6″ and C-6, C-1″, C-3″, C-4″, C-5″, this connectivity information confirmed the location of the -OMe groups at C-3″ of ring B and that the 3-methoxy,4-hydroxy group was linked to C-6. Moreover the cross-peaks observed between H-2′ and H-5′ and C-1′, C-3′, C-5′, and C-6′ signals and between H-6′ and C-5, C-1′, C-2′, C-4′, and C-5′ signals supported the 3,4-dihydroxy substitution on ring A. From all these data phenaxolactone **3** was determined as 2-hydroxy-2-(3′,4′-dihydroxyphenyl)-methyl-3-(3″-methoxy,4″-hydroxyphenyl)-methyl-γ-butyrolactone.

The ESIMS of phenaxolactone **4** showed as base peak the $[M-H]^-$ ion at m/z 521 and the fragment at m/z 359 $[(M-H)-162]^-$ in MS/MS spectrum corresponded to the loss of a hexose unit. From the mass and ^{13}C and ^{13}C DEPT NMR data, the molecular formula $C_{25}H_{30}O_{12}$ was deduced for phenaxolactone **4**. Its complete structure was elucidated by 1D and 2D NMR experiments at 600 MHz. The ^1H NMR spectrum of phenaxolactone **4** was similar in chemical shifts and multiplicities of the signals to that of phenaxolactone **3**, except for the downfield shift of H-5′ ($\Delta\delta = +0.37$) and small downfield shifts observed for H-6′ and H-2′ in the aromatic region as well as for the presence of signals attributable to a sugar moiety in the aliphatic region (Table 1). This evidence clearly suggested that phenaxolactone **4** was a glycosyl derivative of phenaxolactone **3**. The glycosidic linkage was determined to be at the C-4′ position based on the cross-peaks due to ^3J long-range coupling between the anomeric proton (δ 4.89, H-1‴) and C-4′ (δ 147.5) in the HMBC spectrum. Also, the observed upfield shift of C-4′ (−1.2 ppm) and downfield shifts of the *ortho*-correlated C-5′ (+1.6 ppm) and the *para*-correlated C-1′ (+4.5 ppm) and C-3′ (+0.6 ppm) carbons, in comparison with those observed in **3**, were indicative of glycosidation at C-4′. These shifts were almost superimposable on those observed in phenaxolactone **2** in comparison with phenaxolactone **1** (Rastrelli et al 2001). The sugar substituent at C-4′ was identified as β-D-glucopyranosyl by a combination of 1D TOCSY, 2D DFQ-COSY, HSQC and HMBC experiments as reported previously (Rastrelli et al 2001). Therefore the structure of phenaxolactone **4** was determined as 2-hydroxy-2-(4′-*O*-β-D-glucopyranosyl-3′-hydroxyphenyl)-methyl-3-(3″-methoxy,4″-hydroxyphenyl)-methyl-γ-butyrolactone.

The ESIMS of phenaxolactone **5** showed as base peak the $[M-H]^-$ ion at m/z 535 and the fragment at m/z 373 $[(M-H)-162]^-$ in MS/MS spectrum corresponded to the loss of a hexose unit. From the mass and ^{13}C and ^{13}C DEPT NMR data, the molecular formula $C_{26}H_{32}O_{12}$ was deduced for phenaxolactone **5**.

NMR data of phenaxolactone **5** were very similar to those of phenaxolactone **2** already reported, except for the part of the spectrum corresponding to the sugar moiety. The sugar substituent at C-4′ was identified as β-D-galactopyranosyl by a combination of 1D TOCSY, 2D DFQ-COSY, HSQC and HMBC experiments. The 1D TOCSY experiments allowed resolution of the overlapped signals of sugars into a subset of individual monosaccharide spectra. In the 1D TOCSY of phenaxolactone **5** the anomeric proton signal ascribable to a β-D-galactose (H-1‴, δ 5.02, d,J = 7.5 Hz) showed connectivities to three meythines (δ3.57, 3.74, 3.49). The coherence transfer to H-5‴ was not obtained because of the small coupling constants between H-4‴ and H-5‴. We also recorded a 2D COSY

spectrum that established the proton sequence within this sugar fragment as H-1 (δ 5.05), H-2 (δ 3.57), H-3 (δ 3.74) and H-4 (δ 3.49) (Table 1). Analysis of the correlated ^{13}C NMR signals in the HSQC spectrum led to the identification of galactopyranose (Table 1). The β-configuration at the anomeric position for the galactopyranosyl unity was easily seen from their relatively large $^{3}J_{H1-H2}$ coupling constants (7.5 Hz). Therefore the structure of phenaxolactone **5** was determined as 2-hydroxy-2-(4′-*O*-β-D-galactopyranosyl-3′-hydroxyphenyl)-methyl-3-(3″,4″-dimethoxyphenyl)-methyl-γ-butyrolactone.

The activity and toxicity of phenaxolactones **1**–**5** isolated from *P. angustifolius* and *P. rugosus* were tested in C8166 cells infected with HIV-1_{MN}. The results of antiviral activities are presented in Table 2. Phenaxolactone **1** demonstrated relatively potent anti-HIV activity with an in-vitro EC50 value of 3.0 μM. It also exhibited a good selectivity index value of 37.3. Replacement of the 4″-OCH_3 group of the ring B with a 4″-OH gave phenaxolactone **3**, another non-glycosylated lignan isolated from *Phenax* species, which showed increased anti-HIV activity with an EC50 value of 0.8 μM but had a lower therapeutic index value of 12.5. Phenaxolactone **4**, the glycosylated derivative of phenaxolactone **3** was less active, EC50 = 2.8 μM. Moderate antiviral activity was observed for phenaxolactones **2** and **5**, the glycosyl derivatives at the C-4′ position of phenaxolactone **1**.

A comparison of the anti-HIV activity of phenaxolactones **1**–**5** showed an interesting structure–activity relationship. Compound **1** with two methoxyl substituent in the C-3 benzoyl moiety demonstrated more significant anti-HIV activity as reflected by the therapeutic index values of 37.5. Derivatives **3** and **4**, hydroxylated at the 4″ position of this benzyl group, had increased anti-HIV activity but were also more cytotoxic, with therapeutic indices 12.5 and 6.4, respectively. Moreover, it was evident that glycosidation at the C-4′ position of C-2 benzoyl moiety reduced anti-HIV activity in all model compounds tested.

A study of the mechanism of action of compound **1** revealed that, like dextran sulfate, phenaxolactone **1** was more effective when added before or at the time of virus infection. This suggested inhibition of an early step in the virus replicative cycle.

Compound **1** was tested for its ability to bind to virus and neutralize its infectivity. It was incubated at 400 μM at 37 °C, with virus at a high titre of 10^5–10^6 TCID50 (tissue culture infecting dose). After 1 h, compound **1** was removed by serial dilutions to a concentration well below its EC50. The residual virus titre was determined by infecting C8166 cells and compared with the control without any compound. Lignan **1** inhibited virus infectivity by 99%, which suggested that it bound to virus and inhibited at an early stage of virus infection.

Further experiments indicated that it inhibited the binding of gp120 to sCD4 in a dose-dependent manner (Table 3). The EC50 value of approximately 50 μM by this assay was much higher than that recorded in cell cultures. The reason for higher activity in cell cultures might have been because it inhibited both binding and fusion of native viral envelope proteins to CD4 and to other chemokine receptors. The gp120/CD4 interaction assay used recombinant proteins in an ELISA format without gp41, which is required for fusion after gp120 binding to cellular receptor CD4. Dibenzylbutyrolactone lignans were described previously as inhibitors of anti-HIV replication in acutely infected H9 cells (Li-Ming et al 1996) and as inhibitors of anti-HIV-1 integrase (Eich et al 1996). Therefore, the lignans studied here might have also interacted with targets other than envelope proteins of HIV.

Table 3 Inhibition of gp120/CD4 interaction by phenaxolactone **1**

Compounds	Concn (μM)	Inhibition (%)
Phenaxolactone **1**	400	100 ± 1.0
	200	96 ± 2.1
	100	78 ± 2.3
	50	51 ± 3.1
	25	36 ± 1.1
Dextran sulfate	20	78 ± 3.2
	4	60 ± 3.5
	0.8	33 ± 2.1

The percent inhibition was calculated as an average of three separate experiments showing similar data.

Previously, some flavonoids have been reported as potential anti-HIV drugs due primarily to their interaction with the surface protein gp120 to prevent the binding of virus to the CD4 receptor and so blocking the infection (Mahmood et al 1993). Moreover, flavones chrysin and acacetin-7-O-β-D-galactopyranoside showed good anti-HIV activity with relatively low toxicity, when tested in acutely infected H9 cells (Hu et al 1994). No data has been reported until now for C-glycosides flavones such as vitexin and isovitexin, therefore, compounds **6** and **7** were tested in C8166 cells infected with HIV-1_{MN}. Isovitexin (**7**) exhibited an in-vitro EC50 value of 10.0 μM with a selective index above 20 (Table 2).

Conclusion

The compounds phenaxolactones **1**–**5** showed an interesting structure–activity relationship that suggested that they could be modified and new compounds synthesized for better and increased efficacy and selectivity. These compounds should be investigated for their potential use as microbicides for the prevention of HIV infection.

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