Isolation and Structure Elucidation of New PKCα Inhibitors from *Pinus flexilis*

Kit K. Lee,[†] Brian D. Bahler,[†] Glenn A. Hofmann,[‡] Michael R. Mattern,[‡] Randall K. Johnson,[‡] and David G. I. Kingston*,[†]

Department of Chemistry, Virginia Polytechnic and State University, Blacksburg, Virginia 24061-0212, and Department of Biomolecular Discovery, SmithKline Beecham Pharmaceuticals, P.O. Box 1539, King of Prussia, Pennsylvania 19406-0939

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Bioassay-guided fractionation of the CH_2Cl_2−MeOH extract of *Pinus flexilis* using an assay for protein kinase C (PKC) inhibitory activity led to the isolation of the two new bioactive diarylheptanoids (3*R*)-1,7-bis(3,4-dihydroxyphenyl)-3-(β-D-glucopyranosyl)heptan-3-ol (**1**) and its aglycon (3*R*)-1,7-bis(3,4-dihydroxyphenyl)heptan-3-ol (**2**), together with the three known bioactive compounds, hirsutenone (**3**), oregonin (**4**), and hirsutanonol (**5**). The IC_{50} values of compounds **1**−**5** in the PKC assay were 1.4, 1.6, 1.4, 8.6, and 4.6 μg/mL, respectively.

In continuation of our studies on the isolation and structure elucidation of potential anticancer agents from plants,[1] we elected to add an assay for inhibition of the enzyme protein kinase C (PKC) to the yeast assay for DNA-damaging agents described previously.[2] PKC has emerged as an attractive target for anticancer treatment because it is one of the key elements of the signal transduction pathway and is intimately involved in cell-growth regulation and tumor promotion;[3] it is the primary receptor for tumor-promoting phorbol esters.[4] A number of naturally occurring PKC inhibitors have been isolated, including phenylethanoid glycosides such as verbascoside,[5] stilbenes,[6] isoflavanoids such as amorphaquinone,[7] and various microbial metabolites,[8] most notably the alkaloids staurosporine[9] and balanol.[10] Some of these inhibitors, including staurosporine and sphingosine, inhibit tumor promotion,[11] which implies that inhibitors of PKC may have significant therapeutic value. A review of naturally occurring PKC inhibitors has appeared.[12]

As a part of our collaborative work in finding new PKC inhibitory agents, a CH_2Cl_2−MeOH (1:1) extract of the stem and stem bark of *Pinus flexilis* James (Pinaceae) showed activity in our assay for PKC inhibitory activity. The crude extract (527 mg) was passed through a polyamide column in methanol to remove tannins, which interfere with the PKC assay. One of the resulting detannated fractions exhibited PKC-inhibitory activity, and this fraction was subjected to SiO_2 column chromatography, followed by reversed-phase HPLC. Two new diarylheptanoids, **1** (14.3 mg, 2.7%) and **2** (1.4 mg, 0.26%), and the three known diarylheptanoids, hirsutenone (**3**, 13.6 mg, 2.6%), oregonin (**4**, 33.9 mg, 6.4%), and hirsutanonol (**5**, 1.1 mg, 0.21%), were obtained. Their IC_{50} values in the PKC assay are 1.4, 1.6, 1.4, 8.6, and 4.6 μg/mL, respectively.

The molecular formula for compound **1** was determined to be $C_{25}H_{35}O_{10}$ by HRFABMS. Its ^{1}H NMR spectrum (Table 1) revealed the presence of two 1,2,4-trisubstituted phenyl groups and a β-pyranosyl unit in which the anomeric proton signal appeared as a doublet with $^3J_{1,2} = 7.8$ Hz.[13] The assignment of the sugar as a glucopyranosyl unit was supported by the observation of signals for oxygenated carbons at δ 103.2 (d, C-1‴), 75.3 (d, C-2‴), 78.2 (d, C-3‴), 71.8 (d, C-4‴), 77.8 (d, C-5‴), and 62.9 (t, C-6‴). It was confirmed by connectivities deduced from COSY, HMBC, and HETCOR data (Table 1) and by the coupling constants observed for H-2‴ and H-3‴. The remaining seven carbon atoms were connected linearly based on their 2D NMR data (Table 1). The β-glucopyranose moiety was assigned to the C-3 position of the aglycon on the basis of a three-bond C,H correlation of the anomeric proton (H-1‴) with the C-3 carbon signal of the aglycon. The two trisubstituted phenyl groups were assigned to the C-1 and C-7 positions on the basis of 2D NMR data (Table 1). These results led to the gross structure of compound **1**. The absolute configuration of the glucose was assigned as D on the basis of analogy with the structure of similar compounds.

The ^{1}H and ^{13}C NMR spectra of **2** were similar to those of **1**, except that the signals corresponding to the glucose moiety were lacking. The molecular formula for compound **2** was determined to be $C_{19}H_{24}O_5$ by ^{13}C NMR and EIMS (M^+ at 332). Based on the 2D NMR data (Table 1), the structure of **2** was assigned as the aglycon of **1**.

The absolute configuration of **1** was established by ^{13}C NMR spectroscopy. Comparison of the ^{13}C NMR chemical shifts of **1** with those of **2** (Table 1) revealed that a larger glycosidation shift at C-4 (−4.1 ppm) than that at C-2 (−3.2 ppm) was observed in *d*-C_5D_5N. Application of the glycosidation shift rule[14] to these shifts indicated the configuration at C-3 of the glycoside **1** to be *R*. The validity of this application in this series has recently been confirmed by X-ray crystallographic analyses for some related diarylheptanoid analogues.[15] On the basis of the above results and the assumption that glucose is a member of the commonly found D-series, the structure of **1** was assigned as (3*R*)-1,7-bis(3,4-dihydroxyphenyl)-3-(β-D-glucopyranosyl)heptan-3-ol.

The small amount of **2** available prevented the determination of an optical rotation value for it with either Na or Hg light sources, but its CD spectrum was measured and compared to that of the similar diarylheptanoid analogue (3*R*)-1,7-diphenylheptan-3-ol (**6**).[16] Both of them exhibited only negative CD maxima, and both gave two strong negative maxima at 255−285 nm. Based on the above evidence, the structure of **2** was assigned as (3*R*)-1,7-bis(3,4-dihydroxyphenyl)heptan-3-ol; this conclusion

* To whom correspondence should be addressed. Tel.: (540) 231-6570. Fax: (540) 231-7702. E-Mail: dkingston@vt.edu.
[†] Virginia Polytechnic Institute and State University.
[‡] SmithKline Beecham Pharmaceuticals.

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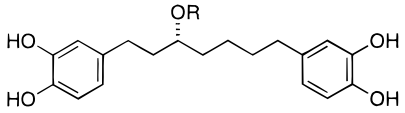
Table 1. ^{1}H NMR, ^{13}C NMR, and HMBC Data for Compounds **1** and **2** in MeOH-d_4[a]

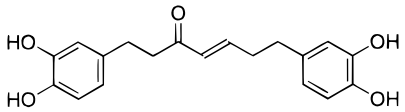
	compound **1**				compound **2**			
position	^{1}H	^{13}C[b]	^{13}C[c]	HMBC	^{1}H	^{13}C[b]	^{13}C[c]	HMBC
1	2.54 (t, 7.6)	31.7 (t)	31.3	C-1′, C-2′	2.58 (m)	32.4 (t)	32.3	C-1′
2	1.78, 1.72 (m, m)	37.9 (t)	37.7	C-1, C-3, C-1′	1.60, 1.54 (m, m)	40.5 (t)	40.9	C-1, C-3
3	3.68 (m)	79.4 (d)	78.6		3.49 (m)	71.7 (d)	70.3	
4	1.59 (m)	34.7 (t)	34.4	C-3	1.45 (m)	38.2 (t)	38.5	C-3
5	1.37 (m)	25.6 (t)	25.0	C-6, C-7	1.31 (m)	26.3 (t)	26.1	C-6, C-7
6	1.53 (m)	33.0 (t)	32.7	C-5, C-7	1.43 (m)	32.9 (t)	32.6	C-5, C-7
7	2.43 (t, 7.6)	36.1 (t)	35.7	C-1″, C-2″	2.44 (m)	36.2 (t)	35.8	C-1″
1′		135.7 (s)	[d]			135.7 (s)	[c]	
2′	6.70 (d, 2.0)	116.9 (d)	117.5	C-4′, C-6′	6.65 (d, 2.0)	116.6 (d)	117.1	C-4′, C-6′
3′		146.0 (s)	147.2			146.0 (s)	147.2	
4′		144.0 (s)	145.1			144.1 (s)	145.2	
5′	6.64 (d, 8.1)	116.3 (d)	116.6	C-3′	6.62 (d, 8.1)	116.3 (d)	116.6	C-3′
6′	6.50 (dd, 8.1, 2.0)	120.8 (d)	120.2	C-2′, C-4′	6.49 (dd, 8.1, 2.0)	120.7 (d)	120.0	C-2′, C-4′
1″		135.7 (s)	[d]			135.4 (s)	[c]	
2″	6.59 (d, 2.0)	116.6 (d)	117.0	C-4″, C-6″	6.59 (d, 2.0)	116.5 (d)	117.0	C-4″, C-6″
3″		145.9 (s)	146.9			146.0 (s)	147.2	
4″		144.0 (s)	145.1			144.0 (s)	145.2	
5″	6.64 (d, 8.1)	116.2 (d)	116.5	C-3″	6.61 (d, 8.1)	116.2 (d)	116.5	C-3″
6″	6.46 (dd, 8.1, 2.0)	120.7 (d)	119.9	C-2″, C-4″	6.46 (dd, 8.1, 2.0)	120.7 (d)	119.9	C-2″, C-4″
1‴	4.29 (d, 7.8)	103.2 (d)	103.4	C-3				
2‴	3.18 (dd, 9.2, 7.8)	75.3 (d)	75.5	C-1‴, C-3‴				
3‴	3.35 (dd, 9.2, 8.0)	78.2 (d)	78.4	C-2‴, C-4‴				
4‴	3.31 (m)	71.8 (d)	71.9	C-3‴				
5‴	3.24 (m)	77.8 (d)	77.9	C-4‴				
6‴	3.88 (dd, 11.9, 2.3)	62.9 (t)	63.0					
	3.70 (dd, 11.9, 5.6)							

[a] The one-bond C,H connectivities were obtained from HETCOR for **1** and from HMQC for **2**. [b] The multiplicities of the carbon signals were deduced from DEPT. [c] Spectrum obtained in pyridine-d_5. [d] These signals were concealed underneath the solvent signals (δ 135 ppm).

suggests that compounds **1** and **2** share the same biogenesis. The structures of the three known diarylheptanoids, hirsutenone (**3**),[17] oregonin (**4**),[15] and hirsutanonol (**5**),[15] were assigned on the basis of comparison of their spectroscopic data with data from the literature.



1 R = β–D–glucose
2 R = H



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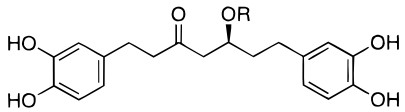
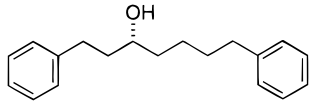


4 R = β-D-xylose
5 R = H



6

More than 70 linear diarylheptanoids have been isolated from Nature. They are known to have a variety of biological activities, such as antifungal activity,[18] inhibition of prostaglandin biosynthesis,[19] and antihepatotoxic activity.[20] To our knowledge, this is the first isolation of diarylheptanoids from *Pinus* species.

Experimental Section

General Experimental Procedures. The optical rotation was measured on a Perkin−Elmer 241 polarimeter, and the CD spectrum was recorded on a JASCO J-720 spectropolarimeter. UV spectra were measured on a Shimadzu UV-1201 instrument. IR spectra were recorded on a Perkin−Elmer FT-IR 1600 instrument. NMR spectra were recorded in CD_3OD on a Varian Unity 400 NMR instrument at 399.951 MHz for ^{1}H and 100.578 MHz for ^{13}C, using standard Varian pulse sequences programs. PKC was provided by SmithKline Beecham Pharmaceuticals. Scintillation countings were performed on a Beckman LS 3800 liquid scintillation system. The exact mass measurement was obtained at the Nebraska Center for Mass Spectrometry.

Plant Material. A sample of stems and stem bark of *Pinus flexilis* James (Pinaceae) (B 632449, PR-8384) was collected in New Mexico in June 1964. A voucher specimen is on deposit in the Herbarium of the National Arboretum, Agricultural Research Service, USDA, Washington, DC.

Plant Extraction and Isolation of Diarylheptanoids 1−5. Stems and stem bark of *Pinus flexilis* were extracted with CH_2Cl_2−MeOH (1:1) to give an active extract (3.47 g), designated HEX 762. The active extract (527 mg) was passed through a polyamide column (15.0 g, 13.0 × 3.2 cm) using MeOH as eluent to yield five detanninated fractions. Fraction 2 (124.1 mg) was active in the PKC assay and was subjected to column chromatography on SiO_2 (8.3 g, 21.5 × 1.3 cm) with 7% and 15% MeOH in CH_2Cl_2 as eluents to give 46 fractions of 6 mL each. Fractions 12−16 yielded hirsutenone (**3**, 13.6 mg, 2.6%), fractions 33−35 contained oregonin (**4**, 33.9 mg, 6.4%), and fractions 37−39 gave compound **1** (14.3 mg, 2.7%). Fractions 19−22 (4.6 mg) were further separated by reversed-phase HPLC (Rainin Dynamax-60 Å C_{18}, 8 μm, 250 × 10 mm, 60% to 80% MeOH−H_2O in 12 min at 2 mL/min) to afford compound **2** (1.4 mg, 0.26%) and hirsutanonol (**5**, 1.1 mg, 0.21%). The structures of compounds **3**−**5** were assigned by ^{1}H and ^{13}C NMR, HMQC, HMBC, and comparison with literature data.

Compound 1: yellow gum-like substance, $[\alpha]_D$ −22° (*c* 0.20, MeOH); UV (MeOH) λ_{max} (log ϵ) 283 nm (3.56); IR (film) ν_{max} 3316 (br), 2929, 2856, 1604, 1526, 1448, 1364, 1284, 1200, 1114, 1077, 1020 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; FABMS (glycerol) *m*/*z* 495.2232 $[M + H]^+$ (calcd for $C_{25}H_{35}O_{10}$, 495.2230).

Compound 2: yellow gum-like substance, UV (MeOH) λ_{max} (log ϵ) 283 (3.48), 206 (4.24) nm; ^1H and ^{13}C NMR data see Table 1; EIMS *m*/*z* 332 $[M]^+$ (9), 314 (4), 163 (14), 149 (18), 136 (21), 123 (100%).

PKC Inhibitory Bioassay. The bioassay for inhibition of PKC was conducted using rhPKCα and [γ-^{33}P]-ATP (Andotek Life Sciences). The reaction buffer consisted of 1.8 mM EGTA, 2.2 mM $CaCl_2$, 0.02 mg/mL glycogen synthetase peptide, 80 μg/mL L-α-phosphatidyl-L-serine, 2 μg/mL 1,3-diolein, 20 mM $MgCl_2$, and 20 mM Tris buffer (pH = 7.5). From the initial samples at 10 μg/μL in DMSO, a series of dilutions was prepared on a microtiter plate, and subsequently 5 μL of each dilution was placed in wells on another plate. The reaction buffer (25 μL), PKC enzyme (10 μL), and ATP (10 μL of 0.05 mM ATP containing 10% [γ-^{33}P]ATP) were added to the plate, and this reaction mixture was incubated at 37 °C for 20 min. The contents of the plates were then individually spotted on Whatman P-81 cation exchange papers (1.8 × 2.0 cm). After being washed by a dilute phosphoric acid solution (0.6%) followed by distilled H_2O rinses, the papers were dried after a quick immersion in Me_2CO and placed in scintillation vials with 10 mL of Scintiverse BD (Fisher Scientific) for counting. The assay was performed at least two times for each compound. IC_{50} values were calculated as concentration of each sample required to give 50% inhibition of the enzyme based on the scintillation counts. Staurosporine (Sigma, St. Louis, MO) was used as a positive control.

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