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KINASE INHIBITORS FROM *POLYGONUM CUSPIDATUM*

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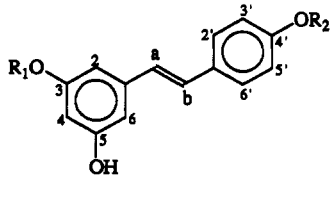
ABSTRACT.—Bioassay-directed fractionation of a medicinal plant, *Polygonum cuspidatum* (Polygonaceae), has led to the discovery of a hydroxystilbene, resveratrol [**1**], as an inhibitor of a protein-tyrosine kinase ($p56^{lck}$) partially purified from bovine thymus. Both trans and cis isomers of resveratrol possess comparable protein-tyrosine kinase inhibitory activity. Comparison of the IC_{50} values of resveratrol for protein-tyrosine kinase inhibitory activity with those of piceid (resveratrol-O^3-β-glucoside) [**2**] and resveratrol-$O^{4'}$-β-glucoside [**3**] shows the requirement of free hydroxyl groups on both phenyl rings for the protein-tyrosine kinase inhibition. Protein kinase C inhibitory analysis suggests the requirements of two free hydroxyl groups on one phenyl ring only.

Numerous cytotoxic compounds have been isolated from natural sources using a cytotoxicity-based screening system. Most of these compounds lack selectivity in attacking tumor cells and display minimal therapeutic indices. It is, therefore, essential to envisage more specific screening approaches for discovering new anticancer drugs with greater selectivity. One of the biochemical mechanism-based screening systems used by our natural product anticancer drug discovery group for selecting active plants and for directing the fractionation and purification of active constituents is based on the inhibition of protein-tyrosine kinase (PTK) activity. PTKs are a large group of enzymes that catalyze the transfer of the γ-phosphate of ATP to the hydroxyl group of tyrosine on many key proteins which can then induce the cascade of altered cell parameters characteristic of transformed cells (1–5).

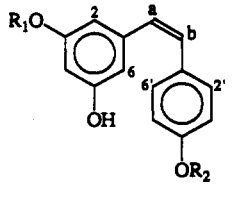
Our previous studies of the dried roots of a Chinese medicinal plant, *Polygonum cuspidatum* Sieb. and Zucc. (Polygonaceae), led to the isolation of a new class of PTK inhibitor, derived from the basic skeleton of anthraquinone (6). We now report the isolation of a second class of PTK inhibitors, namely stilbenes, from this medicinal plant.

RESULTS AND DISCUSSION

As part of an ongoing study to discover and evaluate kinase inhibitors from plants, we observed that the MeOH fraction of *P. cuspidatum* roots exhibited inhibitory activity (IC_{50} <80 μg/ml) against the $p56^{lck}$ PTK partially purified from bovine thymus. Fractionation of the extract monitored by PTK inhibitory activity yielded resveratrol (3,4′,5-trihydroxystilbene) [**1**]. Two glucosides of resveratrol, piceid (resveratrol-O^3-β-glucoside) [**2**] and resveratrol-$O^{4'}$-β-glucoside [**3**], were also isolated from the aqueous fraction of the plant material. Structure-activity relationships for these natural stilbenes



1 $R_1=R_2=H$
2 $R_1=$β-glc, $R_2=H$
3 $R_1=H$, $R_2=$β-glc



4 $R_1=R_2=H$
5 $R_1=$β-glc, $R_2=H$
6 $R_1=H$, $R_2=$β-glc

as well as their cis isomers **4–6**, prepared by photochemical isomerization (λ=365 nm) of the corresponding trans isomers, were investigated for PTK and protein kinase C (PKC) inhibitory activities.

Structures of stilbenes **1–3** were determined by comparing their mp and spectral data to those reported in the literature. The ^{1}H-nmr chemical shifts of stilbenes **1–6** are given in Table 1. The assignments of the proton chemical shifts of the glucose moieties of stilbenes **2**, **3**, **5**, and **6** were based on the previous ^{1}H-nmr study of simple diglucopyranoses (7) and spin decoupling experiments. In order to distinguish the chemical shifts of olefinic H_a and H_b protons, lowest energy conformers of stilbenes **1–3** were constructed, employing the Alchemy II molecular modeling software program (Tripos Associates Inc., St. Louis, MO). The study showed that H_a is closer to H-2′ than H_b is to H-6′, as shown in Table 2. NOe experiments were therefore used in assigning the chemical shifts of H_a and H_b. On irradiation of H-2′ and H-6′ of resveratrol [**1**], the protons at δ 6.87 (d) and δ 7.00 (d) were enhanced by 22.6% and 9.8%, respectively. This established the δ 6.87 signal for the chemical shift of H_a. Similar experiments were performed on stilbenes **2** and **3**, and the results are given in Table 2. However, the olefinic proton resonances for the cis isomers could not be distinguished by the nOe experiments. The corresponding Overhauser effects were not evident (<3%) due to the significant contribution of the relaxation process between two cis phenyl rings. The molecular modeling study indicated that the distance between H-6 and H-6′ is less than 2.8 Å for the cis isomers. A series of decoupling experiments were carried out to determine the long-range couplings between H_a and H-2 (H-6) or between H_b and H-2′ (H-6′). These results allowed us to assign clearly the olefinic protons (Table 1).

Resveratrol [**1**] and piceid [**2**] have previously been isolated from other *Polygonum* species (8–10). Resveratrol [**1**], which is considered to be a phytoalexin, occurs in numerous other plant genera, e.g., *Veratrum* (11), *Arachis* (12), *Trifolium* (13), *Vitis* (14), *Rheum* (15), *Gnetum* (16), *Eucalyptus* (17) and *Scirpus* (18). In addition to *Polygonum*, piceid is reported to occur in *Picea* (19) and *Eucalyptus* (20) species. The stilbene glycoside **3** was previously isolated only from rhubarb (*Rheum rhizoma*) (21,22). This is the first report of the presence of resveratrol and two of its glycosides (**2** and **3**) in one plant. Natural stilbenes occur mostly in the more stable trans form, but a few cases have been reported of naturally occuring cis stilbenes (23,24): e.g., stilbene **4** has been isolated from ground nut (*Arachis hypogaea*), but only uv and ms data have been documented (12). Cis stilbenes **5** and **6** are new compounds.

Stilbene derivatives have a variety of biological and pharmacological activities. Resveratrol [**1**] possesses antibacterial and antifungal activities (25,26). There is also evidence to suggest that resveratrol and piceid have lipid lowering action in rats with hyperlipemia (27) and that resveratrol induces platelet hypoaggregation in rats (28). Such effects in humans from drinking wine were attributed to the presence of resveratrol in the skin of grapes (29,30). Piceid [**2**] offers some protection from liver injury in rats fed with peroxidized oils (31). The effect of resveratrol and other stilbenes on gastric H^+,K^+-ATPase has also been recently evaluated (32).

As shown in Table 3, resveratrol [**1**] possessed the highest PTK inhibitory activity of the three stilbenes isolated from this plant, revealing the importance of the free phenolic groups for the observed activity. In order to evaluate the inhibitory specificity, we also measured its inhibition of PKC, a serine/threonine protein kinase (4,33,34). Resveratrol inhibited PKC with a potency comparable to its inhibition of PTK (Table 3). Unlike the inhibition of PTK activity, compound **3** inhibited PKC with significantly better potency than resveratrol [**1**], indicating that PKC inhibition lacks the requirement for all phenolic groups to be free. The near absence of PKC inhibitory activity by compound **2**, however, argues for a requirement for two free phenolic groups on the same

TABLE 1. Nmr Data[a] for Stilbenes **1–6**.

Proton	Compound					
	1	**2**	**3**	**4**	**5**	**6**
H-2	6.53 d, J=2.0	6.80 t, J=2.0	6.54 d, J=2.0	6.27 d, J=2.0	6.43 t, J=2.0	6.26 d, J=2.2
H-4	6.25 t, J=2.0	6.46 t, J=2.0	6.27 t, J=2.0	6.21 t, J=2.0	6.41 t, J=2.0	6.22 t, J=2.2
H-6	6.53 d, J=2.0	6.66 t, J=2.0	6.54 d, J=2.0	6.27 d, J=2.0	6.48 s	6.26 d, J=2.0
H-2′	7.41 d, J=8.5	7.41 d, J=8.5	7.48 d, J=8.5	7.13 d, J=8.5	7.13 d, J=8.5	7.19 d, J=8.6
H-6′	7.41 d, J=8.5	7.41 d, J=8.5	7.48 d, J=8.5	7.13 d, J=8.5	7.13 d, J=8.5	7.19 d, J=8.6
H-3′	6.82 d, J=8.5	6.82 d, J=8.5	7.04 d, J=8.5	6.69 d, J=8.5	6.72 d, J=8.5	6.91 d, J=8.6
H-5′	6.82 d, J=8.5	6.82 d, J=8.5	7.04 d, J=8.5	6.69 d, J=8.5	6.72 d, J=8.5	6.91 d, J=8.6
H_a	6.87 d, J=16.5	6.90 d, J=16.5	6.95 d, J=16.0	6.31 d, J=12.0	6.34 d, J=12.0	6.39 d, J=12.0
H_b	7.01 d, J=16.5	7.08 d, J=16.5	7.04 d, J=16.0	6.41 d, J=12.0	6.44 d, J=12.0	6.45 d, J=12.0
Sugar moiety[b]						
H-1		4.92 d, $J_{1,2}$=7.7	4.97 d, $J_{1,2}$=7.6		4.74 d, $J_{1,2}$=8.0	4.94 d, $J_{1,2}$=7.5
H-2		3.44 t, $J_{2,3}$=8.8	3.46 t, $J_{2,3}$=7.7		3.35 m, $J_{2,3}$=7.0	3.44 m, $J_{2,3}$=8.1
H-3		3.50 m, $J_{3,4}$=9.4	3.50 m, $J_{3,4}$=8.3		3.43 m, $J_{3,4}$=7.2	3.49 m, $J_{3,4}$=9.0
H-4		3.43 t, $J_{4,5}$=8.8	3.44 t, $J_{4,5}$=8.3		3.34 m, $J_{4,5}$=7.0	3.43 m, $J_{4,5}$=8.6
H-5		3.52 m, $J_{5,6a}$=2.5 $J_{5,6b}$=6.0	3.53 m, $J_{5,6a}$=2.5 $J_{5,6b}$=5.5		3.43 m, $J_{5,6a}$=3.0 $J_{5,6b}$=5.0	3.52 m, $J_{5,6a}$=2.0 $J_{5,6b}$=5.0
H_a-6		3.90 dd, $J_{6a,6b}$=12.0	3.88 dd, $J_{6a,6b}$=11.9		3.77 dd, $J_{6a,6b}$=12.5	3.85 dd, $J_{6a,6b}$=11.5
H_b-6		3.70 dd	3.69 dd		3.65 dd	3.67 dd

[a]δ values are in Me_2CO-d_6 and are relative to solvent shift δ_H=2.04 ppm. Under J couplings the use of s, d, t, and m refers to singlet, doublet, triplet, and multiplet.

[b]Mutual coupling constants given only once, at their first occurrence in table.

TABLE 2. NOe of Olefinic Protons of Stilbenes **1–3**.

Compound	NOe %		Distance (Å) between	
	H_a	H_b	H_a and H-2	H_b and H-6′
1	22.6, δ 6.87	9.8, δ 7.01	2.015	2.397
2	23.7, δ 6.90	15.3, δ 7.08	2.031	2.390
3	22.3, δ 6.95	14.2, δ 7.04	2.094	2.413

ring. From the data presented in Table 3, it is also seen that cis isomers possess comparable inhibitory activity in both PTK and PKC assays.

We previously showed that emodin, an anthraquinoid PTK inhibitor isolated from the same plant, is a competitive inhibitor of the enzyme with respect to ATP (6). On the other hand, piceatannol (3,4,3′,5′-tetrahydroxy-*trans*-stilbene) inhibited PTK by competing with the tyrosine-containing peptide or protein substrate for binding to the enzyme (35). Resveratrol is, thus, most likely a competitive inhibitor with respect to protein substrate. It is interesting that a single plant simultaneously contains both ATP and protein-substrate inhibitors. Resveratrol [**1**] is not a very potent PTK or PKC inhibitor. However, this is the first time that a stilbene PTK inhibitor was directly isolated from a natural source guided by protein kinase assay. Compound **1** may serve as an important lead for further chemical modifications and total syntheses to produce more potent and specific PTK or PKC inhibitors.

TABLE 3. Kinase Inhibitory Activity of Stilbenes **1–6**.[a]

Compound	IC_{50} (μg/ml)	
	PTK	PKC
1	6×10^{1}	4×10^{1}
2	2×10^{2}	2×10^{2}
3	2×10^{2}	3×10^{0}
4	5×10^{1}	3×10^{1}
5	5×10^{2}	2×10^{2}
6	$>8\times10^{2}$	6×10^{0}

[a]Standard inhibition of PTK by emodin, $IC_{50}=10\pm5$ μg/ml. Standard inhibition of PKC by trifluroperazine, $IC_{50}=30\pm20$ μg/ml.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Mp's were determined on a Fisher-Johns melting point apparatus and were uncorrected. Ir spectra were obtained on a Perkin-Elmer 1600 series FTIR. Uv spectra were obtained on a Beckman DU-7 spectrophotometer. ^{1}H-nmr spectra were determined with a Varian VXR-500S instrument. Fabms were measured on a Finnigan 4000 spectrophotometer. Si gel 60 (230–400 mesh) and C-18 bonded Si gel (LRP-2, Whatman) were used for cc. A Hitachi L-6200 hplc system with a Hitachi L-4000 uv detector and an IBM C-8 bonded (10×250 mm, 5 μ) Si gel column were used for hplc.

PLANT MATERIAL.—The roots of *P. cuspidatum* were collected by Dr. C.-T. Chang (Union Chemical Laboratory, Industrial Technology Research Institute, Hsinchu, Taiwan) in Taiwan. A sample was identified by the Taiwan Forestry Herbarium (Taiwan Forestry Research Institute, Taipei, Taiwan), where a voucher specimen is deposited.

EXTRACTION AND FRACTIONATION.—The dried ground roots of *P. cuspidatum* (4.8 kg) were extracted with 95% EtOH at room temperature. After vacuum evaporation of EtOH, the residue (600 g) was partitioned between CH_2Cl_2 and H_2O (1:1). The H_2O fraction was lyophilized to produce a residue (220 g),

which was inactive in the PTK assay. The residue of the CH_2Cl_2 extract (130 g) was further partitioned between hexane and 90% aqueous MeOH (1:1). The MeOH fraction (95 g) exhibited strong PTK inhibitory activity ($IC_{50} < 80$ μg/ml).

The active 90% MeOH fraction (25 g) was chromatographed on a Si gel flash column. Elution with $CHCl_3$/MeOH (5–50%) afforded seven fractions (A–G). Resveratrol [**1**] (42 mg) was obtained by recrystallization of a portion of the residue (50 mg) obtained after concentrating the active fraction C (62 mg) ($IC_{50} < 80$ μg/ml).

The aqueous fraction of *P. cuspidatum* contained two other stilbene analogues indicated by blue fluorescence under uv light (λ 365 nm). The aqueous fraction (45 g) was dissolved in MeOH-H_2O (1:9) and successively extracted with CH_2Cl_2, EtOAc, and *n*-BuOH. The residue of the EtOAc extract (10 g) was chromatographed on a Si gel flash column. Elution with $CHCl_3$/MeOH (5–100%) afforded 16 fractions (A–P). Repeated chromatography of fraction I (3 g) on a C-18 Si gel flash column eluting with MeOH/H_2O (20–50%), yielded **2** (1.5 g) and **3** (103 mg). These glucosides were further purified by hplc employing a C-8 column and eluting with MeOH-H_2O (6:4).

PROTEIN-TYROSINE KINASE (PTK) INHIBITION.—$P56^{lck}$ was partially purified from bovine thymus through the butyl-agarose chromatography step and was assayed by the procedures described previously using angiotensin I and [γ-^{32}P]ATP as substrates (36). All assays contained 8% DMSO, which was used as a solvent for all inhibitory compounds. Emodin ($IC_{50}=10\pm5$ μg/ml) was used as a reference compound.

PROTEIN KINASE C (PKC) INHIBITION.—Protein kinase C activity was assayed using PKC activity partially purified from rat brain, consisting primarily of the alpha and beta isoforms. Assay for inhibition of PKC's histone kinase activity employed conditions modified from those originally described (37,38). Phosphatidylserine (8 μg/ml) and phorbol esters (3 nM), but no calcium, were included for PKC activation, concentrations which only resulted in activation of PKC to 30 to 50% of its maximal activity. This was done to allow for detection of PKC activators as well as inhibitors in the same assay. Each sample to be assayed was dissolved in DMSO, diluted with DMSO, mixed with a solution of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) in DMSO, and dispensed into duplicate assay tubes to give the indicated final concentration of the sample in the assay. Normally, dose-response analysis utilized ten-fold dilutions yielding 0.16 to 160 μg/ml of test sample. The final concentration of DMSO in the assay was 2.4%. To each of these dispensed volume of DMSO solutions was added 50 μl of a mixture in 20 mM tris-Cl buffer, pH 7.4, containing phosphatidylserine (1 μg). EGTA (125 nmol), glycerol (5 mg), and rat brain PKC. The reactions were started by the addition of 75 μl of a mixture in the same buffer of [γ-^{32}P]ATP (2.5 nmol, specific activity approximately 2000 cpm/pmol), 1.25 μmol $MgCl_2$, 625 nmol *p*-nitrophenylphosphate, and 30 μg lysine-rich histone. After incubation at 30° for 10 min, a 50-μl aliquot of each reaction was removed and applied to Whatman P81 phosphocellulose paper. This was washed in distilled H_2O four times, rinsed with Me_2CO and Et_2O, and air-dried. Radioactivity on each paper was determined by counting Cerenkov radiation in a scintillation counter.

In each experiment two control agents (staurosporine and trifluroperazine) were included to assure assay quality. Duplicate no-enzyme blanks also were done, and their value was subtracted from all assay results before any further calcuations. The IC_{50} for each agent was determined as the concentration calculated (by logarithmic interpolation) to inhibit PKC activity by 50%. Trifluroperazine ($IC_{50}=30\pm20$ μg/ml) was used as a reference compound.

PHOTOCHEMICAL ISOMERIZATION OF TRANS STILBENES TO CIS STILBENES.—A solution of trans stilbene isomer (25 mg) in DMSO (4 ml) in a quartz cuvette was irradiated by uv light (λ 365 nm) for 24 h. The conversion was generally 75–80%, and the cis isomer was separated from the trans isomer by hplc employing the C-8 column and eluting with MeOH-H_2O (1:1).

Resveratrol [**1**].—Pale yellow crystals (aqueous MeOH): mp 260° [lit. (39) 261°]; uv λ max (EtOH) nm (log ε) 219 (4.29), 305 (4.02), 320 (3.34); ir ν max (KBr) cm^{-1} 3200–3300, 1589, 1507, 1150, 966, 831; ^{1}H nmr see Table 1; fabms *m/z* $[M]^-$ 228.

Piceid (resveratrol-O^3*-β-glucoside)* [**2**].—Pale yellow crystals (aqueous MeOH): mp 135–137° [lit. (40) 130–140°]; uv λ max (EtOH) nm (log ε) 217 (3.30), 306 (4.21), 314 (4.12), 320 (4.14); ir ν max (KBr) cm^{-1} 3200–3400, 1596, 1512, 1075, 838; ^{1}H nmr see Table 1; fabms *m/z* $[MH]^+$ 391.

Resveratrol-$O^{4'}$*-β-glucoside* [**3**].—Off-white crystals (aqueous MeOH): mp 258° [lit. (22) 253–254°]; uv λ max (EtOH) nm (log ε) 211 (4.23), 304 (4.25), 315 (4.17); ir ν max (KBr) cm^{-1} 3200–3400, 1601, 1511, 1083, 837; ^{1}H nmr see Table 1; fabms *m/z* $[MH]^+$ 391.

cis-*Resveratrol* [**4**].—White powder (aqueous MeOH): mp 170–174°; uv λ max (EtOH) nm (log ε) 216 (3.38), 230 (3.21), 290 (3.15); ir ν max (KBr) cm^{-1} 3300–3400, 1599, 1234, 1074, 836; ^{1}H nmr see Table 1; fabms *m/z* $[MH]^+$ 229.

cis-*Piceid (*cis-*resveratrol*-O^3-β-*glucoside)* [**5**].—White powder (aqueous MeOH): mp 126–129°; uv λ max (EtOH) nm (log ε) 216 (3.27), 286 (3.18); ir ν max (KBr) cm^{-1} 3250–3450, 2356, 1600, 1071; ^1H nmr see Table 1; fabms *m/z* $[MH]^+$ 391.

cis-*Resveratrol*-$O^{4'}$-β-*glucoside* [**6**].—White powder (aqueous MeOH): mp 140–143°; uv λ max (EtOH) nm (log ε) 204 (3.41), 274 (3.32); ir ν max (KBr) cm^{-1} 3186–3427, 1594, 1508, 1398, 1071, 865; ^1H nmr see Table 1; fabms *m/z* $[MH]^+$ 391.

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