A New Hypoxia Inducible Factor-2 Inhibitory Pyrrolinone Alkaloid from Roots and Stems of *Piper sarmentosum*

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A new trimethoxycinnamoyl-2-pyrrolinone alkaloid, langkamide (1), along with the known compounds pipartline (2) and 3,4,5-trimethoxycinnamic acid (3) were isolated from the roots and stems of the shrub *Piper sarmentosum* Roxb. The structures were established by spectroscopic analyses and comparison of their spectral data with values reported in the literature. The compounds were tested for their ability to modulate hypoxia inducible factor-2 (HIF-2) transcription activity and all three showed HIF-2 inhibitory activity with EC₅₀ values of 14.0, 4.8, and 60.6 μM, respectively, for compounds 1, 2, and 3.

**Key words** *Piper sarmentosum; hypoxia inducible factor-2; pyrrolinone alkaloid; Piperaceae*

Hypoxia-inducible factors regulate oxygen homeostasis and overexpression leads to up-regulation of genes that are associated with carcinogenesis and tumor progression.² Hypoxia-inducible factor-2 (HIF-2) is a heterodimeric transcription factor composed of HIF-2α dimerized with a constitutively expressed HIF-1β/aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-2α regulates oxygen homeostasis by binding to hypoxia response elements (HRE) in the promoters of target genes. Under normal oxygen conditions prolyl and asparaginyl residues on the HIF-2α subunit are hydroxylated promoting binding to the von Hippel Lindau (VHL) E3 ligase complex, which causes ubiquitination and proteasomal degradation of HIF-2α. Under hypoxic conditions, hydroxylation is inhibited resulting in stabilization of HIF-2α and transcriptional activation of downstream genes,² many of which are important in renal cell carcinoma oncogenesis.³ Although small-molecule inhibitors of HIF-1 have been identified,² there have been fewer efforts to discover inhibitors of HIF-2 and therefore targeting the HIF-2 pathway may be an approach for the discovery and development of potential new anticancer agents, especially for renal cell carcinoma.

A high throughput assay to identify inhibitors of HIF-2 was developed and used to screen extracts from the National Cancer Institute’s natural products repository.⁵ An extract from *Piper sarmentosum* Roxb. showed activity in the screen and was selected for further chemical investigation. This paper reports the isolation and structure elucidation of langkamide (1), a new 2-pyrrolinone alkaloid, along with the known compounds, pipartline (2) and 3,4,5-trimethoxycinnamic acid (3) (Fig. 1), and the HIF-2 inhibitory activity of these three compounds.

**Results and Discussion**

The high resolution-electrospray ionization (HR-ESI)-MS of 1 showed a [M+H]⁺ ion at *m/z* 304.1180 indicating a molecular formula of C₁₆H₁₇NO₅ with nine double bond equivalents. The ¹³C-NMR and heteronuclear single quantum coherence (HSQC) spectra showed 16 carbon resonances including three methoxyl groups, six sp³ methines, one sp³ methylene, and six quaternary sp² carbons, two of which were carbonyls. Methoxyl ¹H-NMR signals at δ 3.89 (6H, s) and 3.88 (3H, s), along with an aromatic signal at δ 6.85 (2H, s), indicated a symmetrically substituted trimethoxybenzene moiety. Two olefinic protons at δ 7.82 (1H, d, *J* = 13.0 Hz, H-8) and 7.92 (1H, d, *J* = 13.0 Hz, H-7) showed a strong correlation spectroscopy (COSY) correlation and the coupling constant of 13.0 Hz indicated that the double bond had a Z configuration. This was supported by comparison with the corresponding E olefin in pipartline which had a coupling constant of 15.6 Hz. The olefinic proton at δ 7.82 (H-8) showed heteronuclear multiple bond connectivity (HMBC) correlations to resonances of the trimethoxybenzene moiety at δ 130.6 (C-9) and 106.1 (C-10, C-14), and to a carbonyl at δ 165.8 (C-6) which linked these fragments and thus established the presence of a cinnamoyl moiety.

Two mutually coupled olefinic protons at δ 7.36 (1H, dt, *J* = 5.0, 1.5 Hz, H-4) and 6.22 (1H, dt, *J* = 5.0, 1.5 Hz, H-3) had HMBC correlations to a conjugated carbonyl at δ 170.9 (C-2). They also showed modest COSY correlations to two methylene protons at δ 4.54 (2H, *t*, *J* = 1.5 Hz, H₂-5). The proton and carbon NMR data accounted for 8 double bond equivalents and a mass of 289 amu, leaving a difference of 14 amu and one double bond equivalent. This suggested the presence of a 2-pyrrolinone ring, and the ¹³C resonance at δ...
51.6 for the C-5 methylene was fully consistent with nitrogen substitution at this position. The structure of 1 was assembled by linking the cinnamoyl and pyrrolinone moieties through the nitrogen atom, and a literature search revealed that compound 1 differs from a known dihydrocinnamoyl 2-pyrrolinone compound from *Piper demeranum* by the presence of the $\Delta^7,8$ olefinic bond.$^9$

Compound 2 was identified as piparticlate by comparison of its spectroscopic data with literature values, and it differed from 1 in the size of the amide ring.$^{20}$ Compound 3 was identified as 3,4,5-trimethoxycinnamic acid by comparing its HR-ESI-MS and NMR data with corresponding values reported in the literature.$^9$

The compounds were evaluated for the ability to inhibit HIF-2.$^9$ All three compounds inhibited HIF-2 transcriptional activity with EC$_{50}$ values of 14.0, 4.8, and 60.6 $\mu$M, respectively, for 1, 2, and 3. Compounds 2 and 3 showed moderate cytotoxicity with IC$_{50}$ values of 61.4 and 78.4 $\mu$M, while compound 1 showed no cytotoxicity at the highest dose tested (66 $\mu$M). Other agents, that have been shown to inhibit HIF-2$\alpha$, include ibuprofen$^{10}$ which inhibited endogenous HIF-2$\alpha$ in a dose dependent manner in the renal 786-0 cell line and Neovastat ($E$-941)$^{11}$ which inhibited HIF-2$\alpha$ expression in lung tissue in asthmatic BALB/c mice.

### Experimental

**General** Optical rotations were measured on a Perkin-Elmer 241 polarimeter. UV spectra were recorded on a Varian Cary 50 UV–vis spectrophotometer. NMR data were collected using a Bruker Avance III 600 NMR spectrometer in CDCl$_3$ at 600 MHz for 1H and 150 MHz for 13C. MS spectra were measured with an Agilent Technologies 6510 Q-TOF LC/MS. Column chromatography was performed using Sephadex LH-20 (Amersham Biosciences).

**Plant Material** Roots and stems of *Piper sarmentosum* (Piperaceae) were collected in Langkat Province, North Sumatra, Indonesia in July 1991 by Willem de Wilde and B. E. E. de Wilde-Duyfes. The plant was identified by Dr. Max van Balgooy, Leiden Herbarium, The Netherlands and a voucher specimen (collection number U44Z1794) is maintained at the University of Illinois at Chicago.

**Extraction and Isolation** The plant material (3.2 kg) was ground and extracted by immersion in CH$_2$Cl$_2$–MeOH (1:1) for 15 h in a Soxhlet apparatus.$^12$ The solvent was removed and the plant material was immersed for 15 h in methanol (MeOH). The combined extracts were reduced to dryness in vacuo to give 8.7 g of crude extract. A portion of this extract (1.05 g) was treated with 10 $\mu$l of the test medium containing XTT and 0.63% phenazine methosulfate (PMS) in an incubator for 2 h at 37 $^\circ$C. Metabolically active cells reduce the tetrazolium salt to a colored formazan product whose absorbance was measured at 450 nm.

### Bioassays

Details of the HIF-2 and XTT assays have been previously described.$^13$ The renal clear cell carcinoma cell line, 786-0, was engineered with five copies of a HIF-2 hypoxia response element (HRE) linked to a luciferase output signal. The cell line was propagated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and penicillin–streptomycin (at 100 IU/ml, 100 $\mu$g/ml, respectively). G418 (0.75 $\mu$g/ml) and hygromycin B (200 $\mu$g/ml) were used for routine selection and cell lines were maintained at 37 $^\circ$C in a humidified incubator under a 5% CO$_2$, 20% O$_2$ atmosphere.

Cells were trypsinized, spun down and resuspended in phenol red-free DMEM supplemented with 10% fetal bovine serum (FBS). 384-well plates were seeded at 5000 cells per well in 27 $\mu$l medium and incubated overnight. Stock solutions of test compounds were prepared in dimethylsulfoxide (DMSO) and diluted with medium to prepare test compound dilutions at 10 times the final concentrations. For the test compounds, 3 $\mu$l was added to each well along with 30 $\mu$l of SteadyLite luciferase assay reagent (PerkinElmer, MA, U.S.A.) and luminescence measurements were made after 15 min of incubation at room temperature. In the presence of an inhibitor, HIF-2 did not bind to the HRE, resulting in a decrease of the luciferase signal in the cells.

Since cytotoxicity can also decrease the luciferase signal, a 2,3-bis[2-methoxy-4-nitro-5-sulphonyl]-2H-tetrazolium-5-carboxanilide (XTT) colorimetric assay was run concurrently to eliminate false-positives. After 24 h incubation with test compounds or DMSO control, compounds cells were treated with 10 $\mu$l of the test medium containing XTT and 0.63% phenazine methosulfate (PMS) in an incubator for 2 h at 37 $^\circ$C. Metabolically active cells reduce the tetrazolium salt to a colored formazan product whose absorbance was measured at 450 nm.

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### References


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