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New potential antitumor compounds from the plant *Aristolochia manshuriensis* as inhibitors of the CDK2 enzyme

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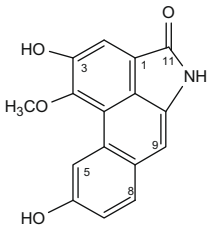
ABSTRACT

The 70% aqueous methanolic extract of the Chinese plant *Aristolochia manshuriensis* was found to contain two novel substituted phenanthrene compounds, SCH 546909 (**1**), and another phenanthrene glycoside (**2**). The structures of **1** and **2** were established based on NMR studies. They were identified as inhibitors of the CDK2 enzyme. Compound **1** was found to be a potent inhibitor of the CDK2 enzyme with an IC_{50} of 140 nM, whereas compound **2** was found to be less active with an IC_{50} of >10 μM.

CDK2 (cyclin-dependent kinase 2) is a serine/threonine kinase, which regulates[1] cell-cycle progression[2] at multiple levels. During G1/S transition, CDK2/cyclin E[3] phosphorylates and inactivates the retinoblastoma tumor suppressor protein (RB), which activates E2F transcription factor, and subsequently leads to initiation of DNA synthesis. In addition, CDK2/cyclin-A complex[4] appears to play a critical role in promoting uninterrupted passage through the S phase by preventing unscheduled E2F activity; however, the precise role of CDK2 during the S phase is not defined. Due to the rate-limiting role of CDK2 in the cell cycle, it has been recognized as a potential target for therapeutic intervention in cancer, making it an attractive target for antitumor drug discovery and drug design.[5]

As part of our continuing investigation of natural products as leads for treating cancer, we screened several semi-purified fractions of aqueous methanolic extracts of many plants in our high-throughput CDK2 enzyme inhibition assay. One of these fractions, which were derived from a plant identified as *Aristolochia manshuriensis* sp., was active in the CDK2 enzyme inhibition assay. Bioassay-guided fractionation of this extract led to the isolation of two phenanthrenes **1** and **2**.

Compounds **1** and **2** were isolated in the following manner. The detanninized aqueous methanolic extract (11.2 g) was loaded on a CHP-20 (1.0″ × 12″) column equilibrated with water and chromatographed using a water and methanol gradient. These fractions were screened for their activity in a CDK2 enzyme inhibition assay.



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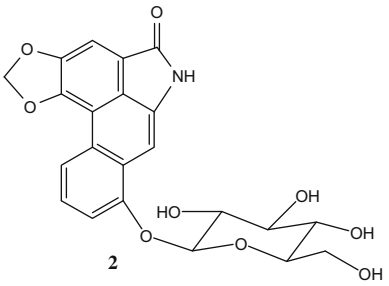

2

The active fractions were then collected and dried to yield 86 mg of an enriched complex. Further separation of the active compounds was achieved by reverse phase preparative HPLC on a Phenomenex Luna C-18 silica column (21.2 × 250 mm), eluting with a mixture of acetonitrile and water (25:75 v/v). Acetonitrile was removed from the active peak eluate and the aqueous solution was freeze-dried to yield 1.0 and 0.5 mg of **1** and **2**, respectively.

Upon isolation, structural characterization of the two natural products was then initiated. SCH 546909 (**1**) showed an *m/z* 282 $(M+H)^+$ and *m/z* 280 $(M-H)^+$ in the +ve and −ve APCI mass spectrum suggesting a molecular weight of 281 Da. The molecular formula of **1** was established as $C_{16}H_{11}NO_4$ by HRMS (High Resolution Mass Spectrum),[6] indicating 12 unsaturations in the molecule. The UV spectrum (MeOH) showed absorption maxima at 207, 230, 251, 260, 277, 290, and 320 nm and the IR spectrum in KBr showed peaks at 1645, 1580, 1472, 1178 and 1078 cm^{-1}, suggesting the presence of an amide functionality. ^1H and ^{13}C NMR chemical shifts of **1** and **2** are listed in Table 1. The ^1H NMR indicated the presence

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Table 1
^{1}H and ^{13}C NMR chemical shifts for **1** and **2**

C#	^{1}H-1	^{13}C-1	^{1}H-2	^{13}C-2
1		121.8		119.0
2	7.58 (s, 1H)	113.2	7.67 (s)	105.6
3		151.7		148.8
4		148.8		146.9
4a		120.0		110.8
4b		127.3		124.7
5	8.53 (d, *J* = 4 Hz, 1H)	111.7	8.25 (d, *J* = 8.0 Hz, 1H)	120.0
6		155.2	7.51 (t, *J* = 8, 8 Hz, 1H)	125.7
7	7.06 (dd, *J* = 14,5 Hz, 1H)	116.9	7.38 (d, *J* = 8.0 Hz, 1H)	112.7
8	7.73 (d, *J* = 14 Hz, 1H)	129.9		153.4
8a		127.6		124.6
9	6.96 (s, 1H)	104.1	7.56 (s)	98.6
10		132.5		134.6
10a		122.5		124.8
11		168.2		168.1
–OMe O–CH_2–O	3.95 (s)	59.4	6.5 (s, 2H)	103.2
–NH	10.6 (s)		10.3(s)	
1[1]			5.07 (d, *J* = 7.8 Hz)	100.9
2[1]			3.42(m)	73.3
3[1]			3.34(m)	76.6
4[1]			3.22 (t, *J* = 9.2 Hz)	69.6
5[1]			3.4(m)	77.0
6[1]			3.50(d, *J* = 12 Hz), 3.71(m)	60.5

*NMR's were run in DMSO-d_6; similar chemical shifts may be interchanged.

of five aromatic protons. D_2O exchange revealed three exchangeable phenolic, aliphatic –OH's, and/or amide protons. The ^{13}C NMR also showed 16 carbon signals in agreement with the number of carbons revealed by HRMS. APT ^{13}C NMR identified them as one >C=O, fourteen olefinic (five =CH–, nine =C<) and one O–CH_3. The UV profile suggested the natural product might be from a phenanthrene class (similarity with staurosporine and Aristolochic acids).

Literature studies on the metabolites produced by this family of plant species suggested this compound might be an aristolactam type structure. Comparison of the UV maxima,[7,8] ^{1}H and ^{13}C NMR chemical shifts of **1** with various known aristolactams further supported **1** as a close analog of aristolactam family of compounds. Using 2D NMR techniques (COSY, HMQC, HMQC-TOCSY, and HMBC) the structure was established as shown in **1**. A literature search indicated two possible known compounds, closely related to this structure—aristolactam AIIIa[9] or doryflavine[10] based on the position of –OMe at 3 or 4. Aristolactam AIIIa was isolated from the plant *Aristolochia argentina*[11] and doryflavine was isolated from the plant *Doryphora sassafras*.[12] The position of –OMe group was unequivocally established by NOE studies. Irradiation of –OMe protons enhanced H-5, suggesting compound **1** as AIIIa. Unfortunately, no comparison could be made with the authentic samples because of the unavailability of the reference compound.

Structural analysis of compound **2** showed a molecular ion of *m*/*z* 442 $(M+H)^+$ in the +ve APCI mass spectrum suggesting the molecular weight of 441 Da. The molecular formula of **2** was established as $C_{22}H_{19}O_9N$ by HRMS data.[6] Compound **2** showed UV maxima (MeOH) 214, 233, 281 327, 389 nm. The ^{1}H and ^{13}C NMR chemical shifts of **1** and **2** are shown in Table 1. Comparing the ^{1}H and ^{13}C NMR chemical shifts revealed a close structural similarity between **1** and **2**. Compound **2** was found to be a closely related glycoside of analog of **1.** The ^{1}H NMR spectrum of **2** showed five olefinic protons, –NH– and a two-proton signal in addition to other sugar protons. The –OCH_3 singlet at δ 3.95 of **1** was absent. The position of the two-proton singlet at δ 6.5 suggested a methylenedioxy functionality at position 3 and 4. The ^{13}C NMR chemical shifts of third ring of **2** suggested a difference compared to **1**. In compound **2**, the C-4a had shifted upfield by 9.2 ppm while C-5 shifted down field by 8.3 ppm and C-6 has shifted upfield by 29.5 ppm while C-8 has shifted down field by 23.5 ppm compared to that of compound **1**. Analysis of these results suggested that for compound **2**, the glycosidic linkage to the aromatic ring must be at C-8. Further confirmation of the structure was derived from HMBC correlation studies (Fig. 1). The HMBC correlation of 9-H to C-8 suggests the position of glycosidic linkage to be at C-8, and the correlations of the O–CH_2–O to C-3 and C-4 establishes the position of methylene dioxy group. The position of –OH at C-8 was further confirmed by comparison of ^{13}C chemical shifts with that of aristolactam Ia.[13] The 9-H is shifted downfield by 0.4 ppm consistent with sugar attached to C-9. The sugar in **2** appears to be glucopyranose based on the relative stereochemistry established from NMR chemical shifts and ^{1}H–^{1}H coupling studies. However the absolute structure has not been established. But since L-glucose is not known in nature it should be D-glucosyl compound. Other aristolactams with glycosidic linkage with glucose at C-7, aristoloside[14] and glucose linked at lactam nitrogen aristolactam-C *N*-β-D-glucose[15] and aristolactam *N*-β-D-glucose[17] are known in literature.

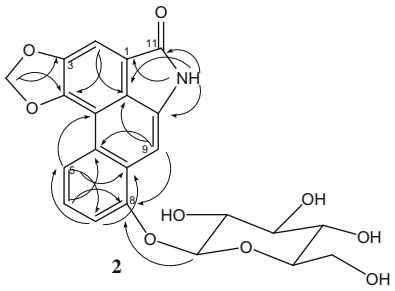

Figure 1. HMBC correlations in **2**.

Table 2
Inhibition IC_{50} for compound **1**

Compound	Activity IC_{50} (nM)				
	CDK2	CDC2	CDK4	AUR2	MAPK
SCH 546909	140	214	1420	2140	35,335

No hydrolysis experiments were preformed to identify the sugars separately, due to the lack of material. Their structures were deduced from the 2D NMR experiments.

Finally, Compounds **1** and **2** were found to be active in the CDK2 enzyme inhibition assay, with IC_{50} values of 140 nM and >10 μM, respectively. Compound **1** was further tested with other closely related cyclin-dependent kinase enzymes to evaluate the selectivity. Table 2 shows inhibition IC_{50} of **1** in other cyclin-dependent kinase enzymes. Compound **1** shows good selectivity for CDK2 compared to other related kinases. Cyclin-dependent (CDC2) has a close homology with CDK2 and compound **1** shows similar inhibitory activity. However **1** showed more selectivity against other kinases like CDK4, aurora-2 kinase and MAP-kinase.

Other analogs of aristolactams have been previously been isolated from the plant *A. manshuriensis* sp.[14,16] Recently another plant natural product, flavopiridol, is the first CDK inhibitor to progress into clinical trials and is being evaluated as an anticancer agent.[18] Other recent CDK2 inhibitors are reported in recent reviews.[19]

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6. *Spectral data for SCH 546909* **1**: FABMS *m*/*z* 282 $(M+H)^+$; HRFABMS for $C_{16}H_{11}NO_4$ (obsd 282.0770 $(M+H)^+$, calcd: 282.0760); **2**: FABMS *m*/*z* 442 $(M+H)^+$; HRFABMS for $C_{22}H_{19}O_9N$ (obsd 442.1117 $(M+H)^+$, calcd: 442.1132).
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