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Semi-synthetic aristolactams—inhibitors of CDK2 enzyme

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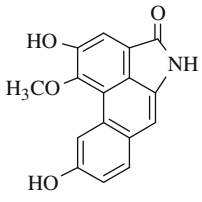
ABSTRACT

Several analogs of aristolochic acids were isolated and derivatized into their lactam derivatives to study their inhibition in CDK2 assay. The study helped to derive some conclusions about the structure–activity relation around the phenanthrin moiety. Semi-synthetic aristolactam **21** showed good activity with inhibition IC_{50} of 35 nM in CDK2 assay. The activity of this compound was comparable to some of the most potent synthetic compounds reported in the literature.

In the preceding Letter we have reported on the isolation of a potent CDK2 enzyme inhibitor SCH 546909, a natural product aristolactam analog with an inhibition IC_{50} of 140 nM. This prompted us to undertake a semi-synthetic study of different analogs from this class. Many total syntheses of aristolactam analogs have been reported in the literature,[1,2] however sub-structure literature searches revealed that these compounds could be easily prepared from naturally occurring, aristolochic acids.



SCH 546909

Several publications and reviews have been published on the occurrence, synthesis and biological activities of aristolochic acids. Aristolochic acids and aristolactams are classified as aporphinoids because of their basic skeleton which bears a distinct similarity to that of aporphins. Aristolochic acids exhibit tumor inhibitory activity against the adenocarcinoma 755 test system but in mice they induced papiloma.[3] They are also known to form covalent DNA adducts by enzymatic reductive activation of aristolochic acids in the presence of DNA.[4] They are also shown to induce mutagenicity in mice.[5] Aristolochic acid is commercially available from Sigma Chemical Co. and ACROS. The commercially available aristolochic acid is a complex mixture of several analogs, with the major components being aristolochic acids II & I in 1:4 ratio. We have separated commercial aristolochic acid mixtures on a preparative HPLC using YMC ODS-A C-18, 10 μm, 5 × 50 cm HPLC column, eluting with 0.05% trifluoroacetic acid and acetonitrile (60:40) to obtain compounds **1–8**. A typical 600 mg of commercial aristolochic acid afforded 27.7, 4.6, 7.8, 71.6, 5.4, 6.8, 315.8, and 3.2 mg of aristolochic acid C (**5**),[6] aristolochic D (**7**),[7] 7-hydroxy aristolochic A(**6**),[8] aristolochic acid II (**1**), aristolochic acid IV (**4**),[9] 7-methoxy aristolochic acid A (**3**),[10] aristolochic acid I (**2**),[2] and aristolochic acid III (**8**).[11]

In our semi-synthetic modifications to prepare aristolactam analogs, the aristolochic acids were first converted to their lactams. The purified aristolochic acids were hydrogenated in ethanolic solution under 40 psi hydrogen in presence of Pd/C catalyst, overnight at room temperature. The amino compound produced on reduction of nitro group, on further ring closure results in lactam.

After separation and derivatization to the resulting lactam, the aromatic phenol ether derivatives were deprotected with BBr_3 in methylene chloride solution. A typical demethylation[12] involved stirring the aristolochic methyl ethers (15 mg) in CH_2Cl_2 (50 ml) at 0 °C with the dropwise addition of BBr_3 (7.5 ml, 1 M) in CH_2Cl_2 at 0 °C and then continue stirring overnight at room temperature. The reaction mixture was quenched in ice, extracted with ethyl acetate, and dried. The demethylated product was purified by HPLC.

The Methylenedioxy group was removed by stirring aristolactams in CH_2Cl_2 at 0 °C and dropwise addition of a solution of PCl_5 (1:1 ratio). The reaction mixture was slowly allowed to attain room

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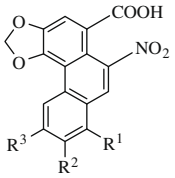
temperature during 2 h and then quenched with ice, extracted with CH_2Cl_2 and dried. The *O*-dihydroxy compound formed was purified by HPLC.

3,4-Dihydroxy-12-chloro aristolactams were prepared from methylenedioxy containing derivatives via treatment with the dropwise addition of PCl_5 (1:2.5 ratio) in CH_2Cl_2 at 0 °C and slowly allowing the reaction mixture to attain room temperature during 3 h. The reaction mixture was quenched in ice, extracted with CH_2Cl_2 and dried. The halogenated product was further purified by HPLC.

ARISTOLOCHIC ACID ANALOGS



1. $R^1 = R^2 = R^3 = -H$
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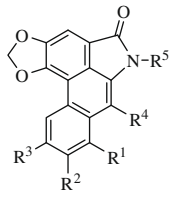
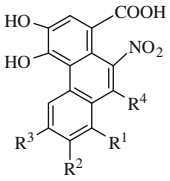
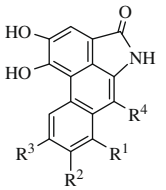
3. $R^1 = R^2 = -OCH_3$, $R^3 = -H$
4. $R^1 = R^3 = -OCH_3$, $R^2 = -H$
5. $R^1 = R^2 = -H$, $R^3 = -OH$
6. $R^1 = -OCH_3$, $R^2 = -OH$, $R^3 = -H$
7. $R^1 = -OCH_3$, $R^2 = -H$, $R^3 = -OH$
8. $R^1 = -OH$, $R^2 = R^3 = -H$
9. $R^1 = R^2 = -OCH_3$, $R^3 = -H$



15. $R^1 = R^2 = R^3 = R^4 = R^5 = -H$
16. $R^1 = -OCH_3$, $R^2 = R^3 = R^4 = R^5 = -H$
17. $R^1 = R^2 = -OCH_3$, $R^3 = R^4 = R^5 = -H$
18. $R^1 = R^3 = -OCH_3$, $R^2 = R^4 = R^5 = -H$
19. $R^1 = -OCH_3$, $R^2 = -OH$, $R^3 = R^4 = R^5 = -H$
20. $R^1 = -OCH_3$, $R^3 = -OH$, $R^2 = R^4 = R^5 = -H$
21. $R^1 = -OH$, $R^2 = R^3 = R^4 = R^5 = -H$
22. $R^1 = R^2 = -OH$, $R^3 = R^4 = R^5 = -H$
23. $R^1 = R^3 = -OH$, $R^2 = R^4 = R^5 = -H$
24. $R^1 = R^2 = R^4 = R^5 = -H$, $R^3 = -OH$,
25. $R^1 = -OCH_3$, $R^2 = R^3 = R^4 = -H$, $R^5 = -CH_3$
26. $R^1 = -OH$, $R^2 = R^3 = R^4 = -H$, $R^5 = -CH_3$



10. $R^1 = R^2 = R^3 = R^4 = -H$
11. $R^1 = R^2 = R^3 = -H$, $R^4 = -Cl$
12. $R^1 = -OCH_3$, $R^2 = R^3 = R^4 = -H$
13. $R^1 = -OH$, $R^2 = R^3 = R^4 = -H$
14. $R^1 = -OCH_3$, $R^2 = R^3 = -H$, $R^4 = -Cl$



27. $R^1 = R^2 = R^3 = R^4 = -H$
28. $R^1 = R^2 = R^3 = -H$, $R^4 = -Cl$
29. $R^1 = -OCH_3$, $R^2 = R^3 = R^4 = -H$
30. $R^1 = -OH$, $R^2 = R^3 = R^4 = -H$
31. $R^1 = -OCH_3$, $R^2 = R^3 = -H$, $R^4 = -Cl$

The aristolochic acid analogs prepared were tested in CDK2 assay[13] with the resulting inhibition IC_{50}s are tabulated in Table 1. Many analogs showed CDK2 activity >10 μM, however compounds **13**, **16**, **19**, **21**, and **24** exhibited CDK2 inhibition under 10 μM. Compound **21** showed a CDK2 inhibition IC_{50} of 35 nM, potency similar to the most potent CDK2 inhibitor reported in the literature[14] Compound **13**, having a hydroxyl group at C-9 also showed activity in the μM range.

Several natural products, like aporphinoids, morphine, and fused berberine classes of compounds, were also tested to evaluate importance of the lactam ring in the CDK2 activity. All these compounds excepting sinomenine, sinoacutine, and tetrahydroberberine, have tetrahydro pyridine ring attached to phenanthrine moiety. Sinomenine and sinoacutine have morphine like ring system but tetrahydroberberine has two tetrahydro-isoquinoline ring system. All these compounds failed to show inhibition in CDK2 assay at 50 μM.

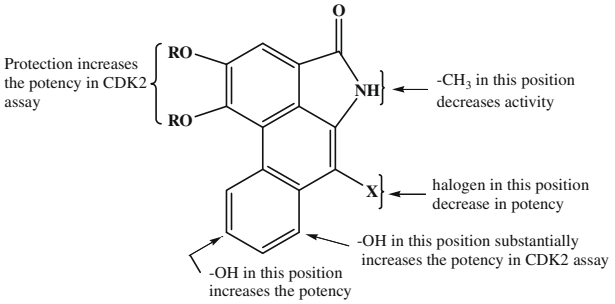
Only compound **21**, displayed strong CDK2 inhibition, about threefold better than the natural product SCH 546909.

Based on the activity profile of the different aristolochic acid and aristolactam analogs, it appears the lactam ring is essential for potent CDK2 inhibition. This has been shown to be true for several potent inhibitors reported in literature.[31,32] Hydroxyl groups

Table 1
CDK2 inhibition IC_{50}s of aristolochic acids and aristolactam analogs

Compound	CDK2 IC_{50} (μM)
1	>20
2	>20
3	>20
4	>20
5	30
6	25
7	25
8	15
9	>20
10	13.4
11	18
12	>30
13	5.7
14	16.5
15[16]	15
16[16]	1.2
17	>15
18[15]	>15
19	2.9
20[18]	>35
21[18]	0.035
22	>30
23[18]	>50
24[20,21]	2.15
25[19,21]	>35
26[17]	>35
27[4]	>35
28	>25
29	>35
30[16]	>35
31	10
Dicentrine[22]	>50
Crebanine[23]	>50
Roemerine-HBr[24]	>50
Isocorydine[25]	>50
Corydine[26]	>50
Corytuberine[27]	>50
Sinoacutine[28]	>50
Sinomenine[29]	>50
Stephanine,[25]	>50
Tetrahydro-berberine[30]	>50

at C-7 or C-9 positions also appear to enhance CDK2 inhibition. Additionally, the protection of the dihydroxy groups at the C-4 and C-5 positions contributes toward the potency. However, protection of amide –NH by a methyl group or substitution by a halogen at C-10 results in reduced activity. The observations are only empirical and a detailed study would be necessary to evaluate a complete structure–activity relationship.

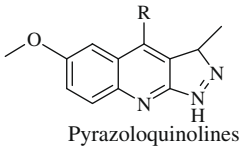


Aristolochic acids and aristolactams have phenanthrin aromatic moiety similar to another class of natural product that includes staurosporine, isolated from fungus. Staurosporins are also potent kinase inhibitors and have been extensively studied as antitumor compounds. Like staurosporine, these compounds are also planar molecules and are sparingly soluble in various solvents including water. Increasing the solubility properties by salt formation or by

forming inclusion compounds with β-cyclodextrin appear to improve cellular activity.

Aristolactam **21** was further tested in a kinase counter screen assays, along with the natural product SCH 546909 and **32**[33], as shown in Table 2. The results indicate that the inhibitors share a similar activity in the CDC2 (cyclin-A dependent kinase, ~90% homology) assay, and a lesser selectivity in other kinases assays like CDK4, AUR2 (Aurora kinase), MAPK (mitogen-activated protein kinase), and AKT (ATP kinase).

Cellular activities: Compound **21**, the most potent and selective CDK2 inhibitor from this series, was evaluated in two cellular proliferation assays: a colony forming assay and a soft agar growth assay. In the soft agar growth assay compound **21** showed comparable activity to **32**, although compound **32** appeared to lose some potency in this assay format compared to the clonogenicity assay (Table 2). In the clonogenicity assay using MCF-7 cells, all three compounds inhibited growth at similar micromolar concentrations. Compound **21** inhibited proliferation of tumor cells, with IC_{50} values consistent with CDK2 inhibitors that are competitive with respect to ATP. The anti-proliferative activity of the compounds was up to eightfold selective for the tumor cells relative to the HFF normal cell line. The anti-proliferative activity of **21** arrests the tumor cells and protects the normal cells from chemotherapy-induced toxicity.[32] These data are consistent with an anti-proliferative mechanism expected for inhibition of CDK2 and revealed that the aristolactam class of compounds have potential for treating proliferative disorders, including chemotherapy-induced alopecia.



Pyrazoloquinolines

32. **R** = -OCH_3 (SCH47089)

Computer based interaction design of **21** *with CDK2 enzyme:* The docking experiments on CDK2 enzyme with **21** (SCH535270) and staurosporine were performed and are shown in Figure 1A and B. The computer docking model suggests the lactam of **21** (SCH 535270) interacts with CDK2 enzyme active sites in a manner analogous to that observed for compounds of staurosporine class of inhibitors bound to fibroblast growth factor receptor kinase.[34]

Two hydrogen bonds were formed between the γ-lactam moiety of **21** and CDK2. Specifically, the amide nitrogen was hydrogen bonded to the backbone carbonyl of glu-81 of the CDK2 enzyme and the lactam carbonyl oxygen was hydrogen bonded with the backbone NH of leu-83 amide. Staurosporin also binds to the CDK2 enzyme in a similar fashion. The C-9 hydroxy group also appears to stabilize the binding at some other sight of enzyme core.[35] SCH 535270, like staurosporin, is a planar molecule and exhibits similar biological properties.

X-ray crystallography: Our attempts to determine the X-ray structure of inhibitor SCH 535270 bound to CDK2 have failed. The crystals were prepared by soaking the compound in presence of CDK2 enzyme and cyclin A. The parameters like compound concentration and duration of soak were screened.

Examination of the electronic density maps did not reveal the binding mode of the compound. In some cases, X-ray crystallography has failed to determine the co-structures of a compound bound to the CDK2 protein even in the case of potent inhibitors. A possible explanation is that inhibitor binding requires the complete CDK2-cyclin-A complex, which is protocol in our screening assay. CDK2 is activated by complexing with cyclin-A that induces conformational changes in the protein that affect the ATP binding site to some degree. The most significant effect involves a rotation of the C-helix, which alters the active-site geometry in the region of the triad of catalytic active-site residues Lys-33, Glu-51, and Asp-145. The amino group of Lys-33 can be potential interaction site for inhibitors. The amino nitrogen appears to hydrogen bond with the oxygen of methylenedioxy group. Efforts to grow crystals

Table 2
Inhibition (IC_{50}) of SCH 546909, **21** and **32** in different kinases

Compound	Activity IC_{50} (nM)	Selectivity (nM)					Cellular activity (μM)	
		CDC2	CDK4	AUR2	MAPK	AKT	SAG MCF7	Clonogenicity
SCH546909	140	214	1420	2140	35,335	—	—	—
32 (SCH47089)	20	200	2000	5000	>50,000	—	>10	3.0
21 (SCH535270)	35	200	9000	3500	12,000	11,400	2–2.5	3.5

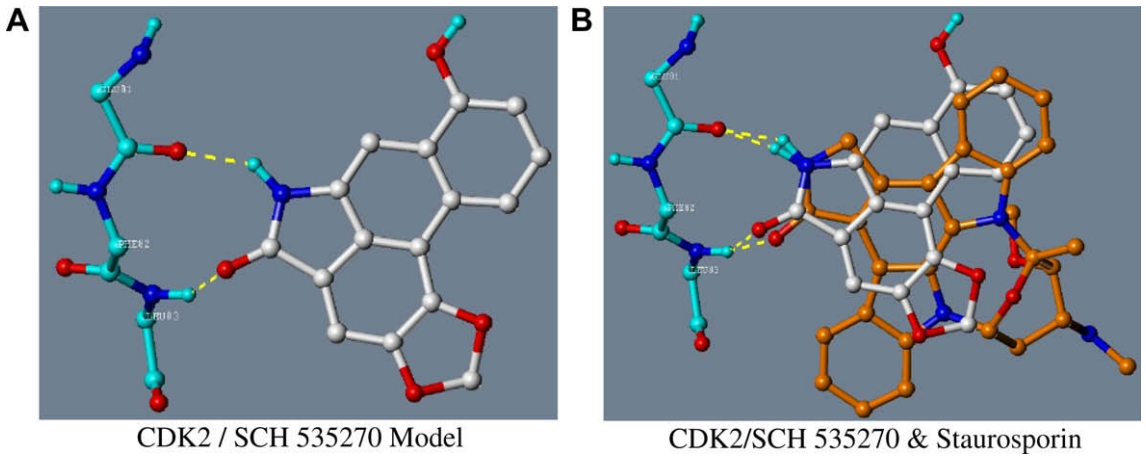


Figure 1. Computer modeling of binding of **21** (SCH 535270) and staurosporin with CDK2 enzyme.

of the activated CDK2-cyclin-A protein complex are in progress and will be reported in future publications.

The aristolactam class of compounds represents a novel class of CDK2 inhibitors. Exploration into semi-synthetic analogs provided a potent CDK2 inhibitor from this class. Binding interactions by docking experiments suggested carbonyl of glu-81 and NH of leu-83 amide of the CDK2 enzyme are involved in hydrogen bonding with the lactam functionality of aristolactams. CDK2 inhibition causes an arrest of the cell cycle and exhibits a selective killing effect on several tumor cell lines.[36]

Acknowledgments

Authors gracefully acknowledge Dr. E. Lees and Dr. R. Doll for their helpful discussion on CDK2 inhibitors.

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    Physico-chemical properties:
    Aristolochic acid C (**5**)*:* UV λ_{max}: 225, 256, 308, 410 nm; FABMS 328 $(M+H)^+$, 350 $(M+Na)^+$, 366 $(M+K)^+$, ^{1}H NMR (DMSO-d_6) δ: 10.63 (COO*H*), 8.48 (9-H), 8.46 (d, J = 4 Hz, 5-H), 8.10 (d, J = 17 Hz, 8-H), 7.75 (s, 2H), 7.29 (dd, J = 17,4 Hz, 7-H), 6.48 (s, 12-H2). ^{13}C NMR (DMSO-d_6) ppm: 168.0 (11-C), 159.8 (6-C), 145.8 (3-C), 145.5 (4-C), 143.1 (10-C), 132.5 (8-C), 131.0 (4b-C), 126.4 (9-C), 123.7 (1-C), 121.5 (8a-C), 118.8 (7-C), 117.2 (10a-C), 116.2 (4a-C), 111.9 (2-C), 111.1 (5-C), 102.8 (12-C).
    7-Hydroxy aristolochic acid A (**6**)*:* UV λ_{max}: 224, 271, 318, 384 nm; ESMS −ve mode, m/z 356$(M-H)^-$.
    Aristolochic acid D (**7**)*:* UV λ_{max}: 224, 243, 333, 408 nm; ESMS m/z 358$(M+H)^+$.
    Compound (**21**)[18] : UV λ_{max}: 214, 242, 258, 294, 328, 398 nm; ESMS: m/z 280 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ: 10.72 (s, NH), 10.2 (s, –OH), 8.03 (d, J = 15 Hz, 5-H), 7.63 (s, 2-H), 7.37 (t, J = 15 Hz, 6-H), 7.36 (s, 9-H), 7.06 (d, J = 15 Hz, 7-H), 6.46 (s, 12-H2). ^{13}C NMR (DMSO-d_6) ppm: 168.1 (11-C), 153.8 (8-C), 148.8 (3-C), 147.1 (4-C), 134.0 (10-C), 125.8 (6-C), 125.3 (4b-C), 125.3 (10a-C), 123.2 (8a-C), 119.3 (1-C), 117.5 (5-C), 112.3 (7-C), 111.3 (4a-C), 105.4 (2-C), 103.2 (12-C), 98.7 (9-C).
    Compound (**16**)[16] : UV λ_{max}: 225, 239, 258, 295, 329, 394 nm; ESMS: m/z 294 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ: 10.67 (s, NH), 8.22 (d, J = 16 Hz, 5-H), 7.70 (s, 2-H), 7.50 (t, J = 16 Hz, 6-H), 7.35 (s, 9-H), 7.20 (d, J = 16 Hz, 7-H), 6.48 (s, 12-H2), 4.0 (s, 13-H3). ^{13}C NMR (DMSO-d_6) ppm: 168.1 (11-C), 155.3 (8-C), 148.8 (3-C), 147.1 (4-C), 134.7 (10-C), 125.7 (6-C), 125.0 (11a-C), 124.8 (4b-C), 124.0 (10a-C), 119.3 (1-C), 118.7 (5-C), 111.0 (4a-C), 108.3 (7-C), 105.7 (2-H), 103.3 (12-C), 97.9 (9-C) add –OCH_3 value.
    Compound (**29**): ESMS: m/z 316 $(M+H)^+$; ^{1}H NMR (DMSO-d_6) δ: 9.12 (d, J = 16 Hz, 5-H), 7.52 (t, J = 16 Hz, 6-H), 7.50 (s, 2-H), 7.22 (d, J = 16 Hz, 7-H), 3.92 (s, –OCH_3), ^{13}C NMR (DMSO-d_6) ppm: NOE from –OCH_3 to proton doublet at δ 7.22 due to 7-H and no NOE from –OCH_3.
    All the new compounds were purified by HPLC and identified by MS.