

Synthesis, in vitro and in vivo cytotoxicity of 6,7-diaryl-2,3,8,8a-tetrahydroindolizin-5(1H)-ones

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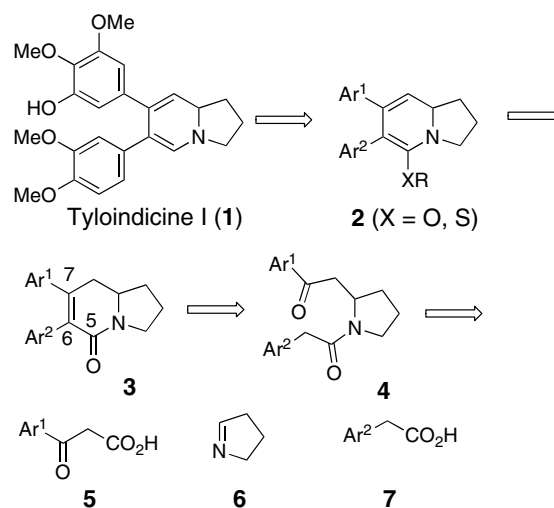
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Abstract—A 6,7-diaryl-2,3,8,8a-tetrahydroindolizin-5(1H)-one library was constructed and tested against the colon cancer cell line HCT-116 as an initial screen for cytotoxic properties. Of this library, the parent compound, in which the southern aromatic ring remains unsubstituted, and the northern aromatic ring carries a 4-methoxy group, exhibited the most potent cytotoxicity with an IC₅₀ value of 0.39 μ M and displayed promising activity in vivo in the NCI's mouse hollow fiber assay.
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As cancer cells adapt to various treatment modalities and subsequently become resistant to modern and conventional chemotherapy, the discovery and exploitation of new chemical classes of compounds that avoid the various cancer resistance mechanisms become an increasingly crucial endeavor. Moreover, the discovery of small molecules that exert cytotoxic properties via novel mechanisms of action would be of paramount importance. Such a discovery could also identify a novel cancer target and thus provide a lead for the development of a new class of anticancer agents. As such, our continuing search for the discovery and development of small molecule anticancer agents that operate through an unknown mechanism of action led us to our studies of the alkaloid tyloindicine family.

Tyloindicine I (**1**, Scheme 1) was isolated in 1991 in minute quantities from the aerial parts of *Tylophora indica* and falls into the family of seco-phenanthroindolizidine alkaloids.^{1,2} Our interest in tyloindicine I is a result of its potent nanomolar and cancer cell-selective cytotoxic properties and therefore, we set out to synthesize **1** and related analogs to pursue additional biological studies.²

Although no total syntheses exist to date for tyloindicine I, synthetic endeavors toward the related alkaloids ipalbidine^{3–13} and septicine^{11,14–24} have been utilizing synthetic methods that have been incorporated into this work.^{20,21} Research focusing on analogs related to tyloindicine I, ipalbidine, or septicine is sparse in the literature with the notable research in this area coming from the laboratories of Sharma et al. and focusing on 6-aryl-7-(4-(methylthio)phenyl)-2,3,8,8a-tetrahydroindolizin-5(1H)-ones.²⁵



Scheme 1.

Keywords: Seco-phenanthroindolizidine alkaloids; COMPARE analysis; Colon cancer; HCT-116; NCI mouse hollow fiber assay.

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In an attempted route to **1**, for which the retrosynthesis is shown in Scheme 1, we devised a synthesis starting from β -keto acid **5**, pyrrole **6**, and aryl acetic acid **7**. These building blocks could be assembled to provide intermediate pyrrolidine **4**, which upon aldol condensation would provide bicyclic lactam **3**. Alkylation of the *O*- or *S*-enol amide of **3**, followed by deoxygenation or desulfurization of intermediate **2**, could lead to the targeted tyloindicine I. A model study was initiated as shown in Scheme 2, and the synthesis of the desired lactam intermediate **12** was accomplished as planned. Saponification of the commercially available β -keto ester **8** furnished the β -keto acid **9**. A subsequent addition of **9** to (di-(3,4-dihydro-2*H*-pyrrole))diiodozinc, followed by decarboxylation, constructed the pyrrolidine **10**.²⁶ The amine in **10** was then acylated with phenylacetyl chloride to provide **11**, which was subjected to KOH in refluxing ethanol to provide the target indolizidine lactam **12**.²⁷ The subsequent steps, the conversion of **12** to the corresponding tyloindicine I analog did not proceed as planned and that route toward tyloindicine I was abandoned.

7-(4-Methoxyphenyl)-7-phenyl-2,3,8*a*-tetrahydroindolizin-5(1*H*)-one (**12**, NSC 707904) was submitted to the NCI for screening in their panel of 60 cancer cell lines and demonstrated selective cytotoxicity at sub-micromolar concentrations toward colon cancer and leukemia cell lines. In one screen the concentration that inhibited growth of the HCT-116 colon tumor cell line was 0.024 μ M. In another screen it was 0.53 μ M.

The NCI's COMPARE analysis of the 60 cell line assay results indicated that **12** acts by a different mechanism of action compared to all other anticancer agents in their database including tyloindicine I.^{28,29}

As a result of these interesting findings, **12** (NSC 707904) was selected by the NCI for in vivo studies in the NCI's mouse hollow fiber assay.^{30,31} In this assay, compounds are tested against a standard panel of 12 human cancer cell lines, including one colon cancer cell line (COLO 205). A point system is used to assess the

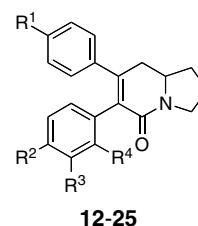
activity of the test compounds. A value of 2 is assigned for each compound dose that results in 50% or greater reduction in viable cell mass either IP or SC. Compounds with a combined IP and SC score of 20, an SC score of 8, or a net cell kill of one or more cell lines are referred to the NCI's Biological Evaluation Committee for Cancer Drugs. Compound **12** received an SC score of 8, demonstrating that **12** has promising activity according to this point system. Considering that only one colon cancer cell line was present in this assay panel, the SC in vivo activity is encouraging.

Because of these promising test results, we set out to prepare analogs of **12** so as to achieve an understanding of the structure–activity relationship for this class of compounds, to improve potency, and to prepare affinity analogs that could be used to identify the biological target or targets of **12**. The analog library was generated utilizing the synthetic sequence shown in Scheme 2 from the appropriately substituted β -keto and aryl acetic acids and are shown in Table 1.

The analog library, along with compound **12**, was tested against the HCT-116 colon cancer cell line as the initial screen to determine cytotoxicity (Table 1).

The results from the toxicity screen revealed that the activity we observed in our laboratory with **12** is

Table 1. Cytotoxicity of 6,7-diaryl-2,3,8*a*-tetrahydroindolizin-5(1*H*)-one analogs **12–25**

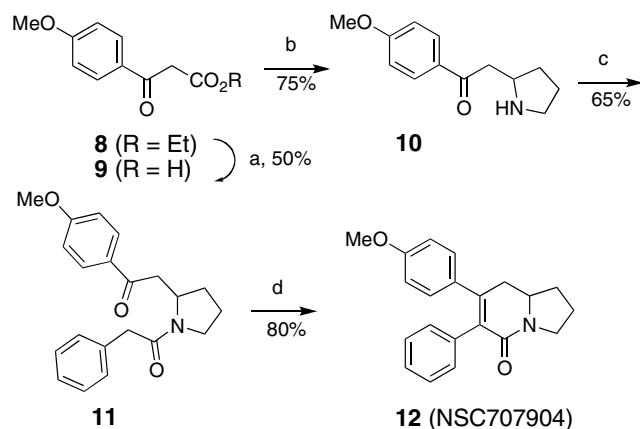


Analog	Northern aryl substitution R ¹	Southern aryl substitution R ² , R ³ , R ⁴	HCT-116 cytotoxicity IC ₅₀ (μ M)
12	OMe	H, H, H	0.39
13	OMe	Cl, H, H	13
14	OMe	OMe, H, H	3.2 ^a
15	OMe	Me, H, H	10
16	OMe	Cl, Cl, H	4
17	OMe	H, Cl, H	8
18	OMe	H, H, Cl	8
19	OMe	H, NH-LCB ^b , H	>25
20	OMe	NH-LCB ^b , H, H	25
21	OMe	N ₃ , H, H	25
22	O-LCB ^b	H, H, H	17
23	OMe	H, NH ₂ , H	>25
24	OH	H, H, H	9
25	OBn	H, H, H	8.9

The cytotoxicity experiments were done as previously described³² except that the cells were incubated for 48 h after the addition of the compounds.

^a Assay conducted by National Cancer Institutes Developmental Therapeutics Program.

^b LCB = (+)-biotinyl-6-aminoheptanoic acid.



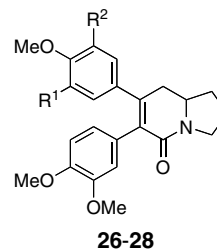
Scheme 2. Reagents and condition: (a) 2.5% KOH_(aq); (b) (di-(3,4-dihydro-2*H*-pyrrole))diiodozinc, pH 7, phosphate buffer, MeOH; (c) phenylacetyl chloride, TEA, DCM; (d) 5% KOH_(EtOH), reflux.

consistent with the higher of the two values obtained in the NCI screen. A Topliss decision tree approach was applied toward the selection of the initial analogs **13–16** with the lead compound **12** being at the top of the schematic Topliss tree.³³ With respect to structural modifications, the 4-chloro analog **13** was less active than **12** suggesting that the southern aryl ring has either stringent steric requirements or is controlled by either $-\sigma$ or $-\pi$ factors. In attempts to further define the southern aryl ring's role, continuing down the Topliss tree to the 4-methoxy analog **14** revealed that it too was less active than the lead compound **12**. This information indicates that the steric requirements of the southern ring may supercede any favorable or negative influences the $-\sigma$ or $-\pi$ factors may have been contributing. The other analogs in the Topliss series, **15–18**, further suggested that regardless of the lipophilicity or the chlorine substitution patterns, overall changes in the southern ring are not well tolerated. Notwithstanding these results, one photoaffinity analog (**21**) and three biotinylated analogs (**19**, **20**, **22**) were generated in attempts to aid in the isolation of biological targets and allow for the determination of the mechanism of action of this class of compounds.³⁴ These efforts were not successful with affinity analogs **19–22** showing poor cytotoxicity. Furthermore, aniline **23** had poor activity. These results further illustrate the steric sensitivity of the southern aromatic ring.

With respect to the northern ring, the phenol **24** as well as the benzyl protected analog **25** were prepared and tested. These compounds also showed reduced cytotoxicity. Phenol **24** was prepared in quantitative yield from **12** by treatment with BCl_3 , *n*-Bu₄NI, CH_2Cl_2 (-78°C to 0°C) for 2 h. Analog **25** was synthesized as shown in Scheme 2, using the *O*-benzyl analog of **8**. Thus, the northern aromatic ring appears to favor the 4-methoxy substitution over the 4-hydroxy substitution thereby suggesting that the electronic or lipophilic character of the northern ring affects the biological activity of these analogs.

Further efforts to investigate the steric and electronic parameters for the southern aryl ring were inspired by the nanomolar cytotoxicity of the related alkaloids tyloindicine I (Scheme 1)^{1,2} and tylophorine,²⁴ that carry two methoxy groups on each aromatic ring. The cytotoxicity of these natural products suggests that electronic factors may serve to override the steric sensitivity of the southern aromatic ring inherent in analogs **13–21** and **23**. As such, the more highly substituted analogs **26–28** were prepared following the synthesis shown in Scheme 2, using the appropriate β -keto esters and acyl chlorides. The IC_{50} values for analogs **26–28** are listed in Table 2. With the introduction of a second methoxy group at the southern ring, a comparison of **27** and **12** further confirms that substitution of the southern ring is detrimental to activity. In addition, the inferior cytotoxicity of the tri- and tetra-substituted northern ring analogs **26** and **28**, relative to the sub-micromolar activity of the parent compound **12**, suggests that increasing the steric encumbrance around the northern aromatic ring has detrimental effects as

Table 2. Cytotoxicity of tylophorine analogs **26–28**



Analog	R ¹ , R ²	HCT-116 cytotoxicity IC_{50} (μM)
26	H, OMe	>10
27	H, H	5.0 ^a
28	OBn, OMe	3.3

^a Assay conducted by the National Cancer Institute's Developmental Therapeutics Program.

well. It would seem that for this series, the electronics of the aryl rings might have a larger influence than previously anticipated.

Overall, our results exhibit trends complementary to those outlined by Sharma et al. in which a 6,7-diaryl-2,3,8,8a-tetrahydroindolizin-5(1*H*)-one scaffold was employed with variously substituted northern and southern aromatic rings.²⁵ Notably, Sharma found that more highly substituted northern rings led to a decrease in activity, whereas the southern ring did retain moderate activity with mono- and di-substituted systems similar to analogs **13–18**, **27**, and **28**. Although a southern ortho- and/or para-substituted methoxy group did not significantly affect activity, replacement with a hydroxyl group led to a significant decrease in activity suggesting H-bonding moieties were detrimental to activity similar to our aniline analog **23**. Activities were tested against 8 cell lines, not including the HCT-116 cell line, thereby making direct comparisons with our results difficult.

In summary, a short synthesis was designed to construct 6,7-diphenyl-2,3,8,8a-tetrahydroindolizin-5(1*H*)-one (**12**) as well as the subsequent analogs **13–28**. The lead compound **12** was shown to exhibit the most potent cytotoxicity toward the HCT-116 colon cancer cell line and showed promising activity in the mouse hollow fiber assay. There appear to be stringent steric requirements controlling the degree of aryl substitution in the southern and northern aromatic rings. Overall, the low molecular weight, potent and selective cytotoxicity toward colon cancer cell lines, and potential of ultimately determining a novel mechanism of action make this collection of compounds an interesting avenue toward the discovery of a new class of anticancer agents worthy of further examination and scientific scrutiny.

Acknowledgments

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and for his support of this project. We also wish to thank the NCI's DTP for carrying out cytotoxicity assays for compounds **12** (NSC 707904), **14** (NSC 707907), and **27** (NSC 707909). The complete cancer screening data for these compounds are available on the DTP website.

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- Synthesis of 9.** To **8** (2.2 g, 10 mmol, neat) was added a freshly prepared KOH solution (2.5% aq 50 mL). The reaction mixture was stirred at room temperature for 40 h. Then the solution was extracted with ether (50 mL) and the ether layer was discarded. The aqueous layer was cooled to 0 °C and acidified to pH ~4 with cold sulfuric acid (1 M). A white precipitate formed, which was taken into ether (50 mL). The aqueous layer was extracted with ether (2 × 20 mL). The combined ether extracts were dried over Na₂SO₄ and concentrated under reduced pressure, keeping the water bath at 20 °C. The crude acid (semi-solid) was used immediately in the next step without any purification (0.970 g, 50%).
- Synthesis of 10.** To a solution of **9** (4.0 g, 20 mmol) in MeOH (10 mL) were added 1 M phosphate buffer (5 mL) to a pH ~7.5 and a freshly prepared solution of (di-(3,4-dihydro-2H-pyrrole)diiodozinc (4.57 g, 10 mmol in water (60 mL). The reaction mixture was stirred for 3 days at room temperature and then cooled to 0 °C and acidified using Congo red as the indicator. The reaction mixture was extracted with ether and the ether layer was discarded. The water layer was cooled to 0 °C and basified carefully to pH ~8 using K₂CO₃. The water layer was extracted with CHCl₃ (3 × 20 mL). The combined extracts were dried over Na₂SO₄ and concentrated to provide **10** as oil. The crude product (3 g, 75%) was used in the next step without additional purification.
- Synthesis of 11.** To a solution of **10** (2.1 g, 10 mmol) in dry CH₂Cl₂ (10 mL), Et₃N (1.5 mL, 15 mmol) was added and stirred for 5 min. To this solution phenylacetyl chloride (1.6 g, 15 mmol) was added slowly and stirred at room temperature for 12 h. The reaction mixture was extracted with CH₂Cl₂ (100 mL), washed with sat. NaHCO₃ (20 mL), brine (10 mL), and water (10 mL), and dried over Na₂SO₄. The combined organic extracts were concentrated under reduced pressure. The crude product was purified twice by column chromatography on silica gel using 100% CH₂Cl₂ for the first column, followed by EtOAc/hexanes (7:3) for the second purification to provide **11** (2 g, 65%) as a semisolid.
- Synthesis of 12.** To **11** (3.3 g, 10 mmol) was added ethanolic KOH (5%, 20 mL). After refluxing this reaction for 2 h, the solvent was removed under reduced pressure. The crude product was washed with 3% HCl and extracted with CH₂Cl₂ (2 × 50 mL), dried over Na₂SO₄, and concentrated under reduced pressure. Column chromatography on silica gel, using EtOAc/hexanes (1:1), provided **12** (2.7 g, 80%, mp = 179–182 °C) as colorless crystals. ¹H NMR (400 MHz, CDCl₃) δ 1.75 (m, 1H), 1.88 (m, 1H), 2.06 (m, 1H), 2.29 (m, 1H), 2.82 (m, 2H), 3.63–3.72 (m, 2H), 3.75 (s, 3H), 3.95 (m, 1H), 6.67 (d, *J* = 7.4 Hz, 2H), 6.98 (d, *J* = 8.6 Hz, 2H), 7.19 (m, 5H); ¹³C NMR (100 MHz, CDCl₃) δ 23.59, 34.22, 37.96, 45.21, 55.54, 56.02, 113.69, 127.04, 127.96, 130.29, 131.54, 132.77, 136.75, 145.10, 159.23, 164.53. HRMS (ES+) *m/z* calcd for [M+H]⁺ C₂₁H₂₂NO₂: 320.1651, found 320.1635.
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- Synthesis of 19.** With gentle heating and stirring, LC-biotin (0.070 g, 0.20 mmol) was solubilized in DMF (2 mL) after which was added EDCI (0.050 g, 0.26 mmol). With the addition of DMAP (0.070 g, 0.57 mmol) the

reaction mixture turned orange in color and the solution was stirred for 30 min. at ambient temperature. In a separate flask was dissolved aniline **23** (0.021 g, 0.063 mmol) in DMF (2 mL) and the LC-biotin mixture was subsequently added. The reaction was allowed to progress over 24 h after which it was quenched with the addition of water. EtOAc was added and used to extract the product into the organic phase. Equal amounts of water and EtOAc were added and the organic product was extracted into the EtOAc layer. The aqueous and organic phases were separated and the aqueous phase was extracted an additional four times with EtOAc. The combined organic fractions were washed four times with

water to remove excess DMF then once with brine. The organic phase was dried over sodium sulfate then condensed under reduced pressure. The biotinylated product **19** was purified via flash column chromatography using silica gel (9% MeOH/CH₂Cl₂) in 29% yield (0.029 g): ¹H NMR (400 MHz, CDCl₃) δ 1.20–2.35 (m, 20H), 2.60–2.95 (m, 4H), 3.10–3.35 (m, 3H), 3.60–3.73 (m, 2H), 3.73 (s, 3H), 4.00 (m, 1H), 4.28 (m, 1H), 4.42 (m, 1H), 6.36 (d, *J* = 19.58 Hz, 1H), 6.51 (d, *J* = 7.55 Hz, 1H), 6.65 (d, *J* = 7.56 Hz, 2H), 6.76 (d, *J* = 9.56 Hz, 1H), 6.96 (m, 4H), 7.39 (d, *J* = 9.73 Hz, 1H), 7.61 (m, 1H), 9.03 (d, *J* = 11.84, 1H); MS (FAB+) [*M*+*H*]⁺ C₃₇H₄₈N₅O₅S: *m/z* 674.