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The Total Synthesis of (–)-Indolactam I

Manuel Mendoza,[a] Niveda Rao,[a] UyenPhuong Tran,[a] Catherine Castaneda,[b] and Kelvin L. Billingsley[a,*]

[a]California State University Fullerton, Department of Chemistry and Biochemistry, Fullerton, CA 92831
[b]Department of Chemistry and Biochemistry, San Francisco State University, San Francisco, CA 94132

*Author to whom correspondence should be addressed

Correspondence to:

Prof. Kelvin Billingsley
California State University, Fullerton
Department of Chemistry and Biochemistry
McCarthy Hall 582C
Fullerton, CA 92831

Email: kbillingsley@fullerton.edu
Phone: 657-278-4252
Fax: 657-278-5316

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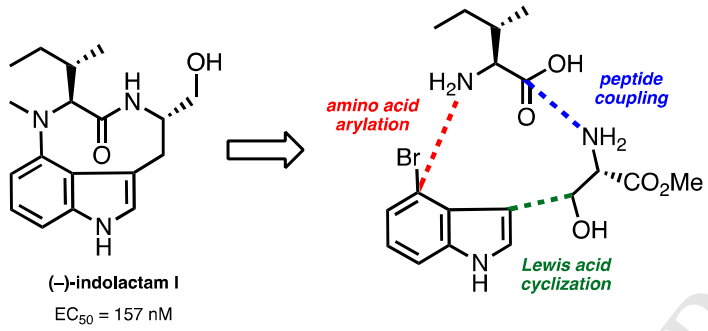
Graphical Abstract



Abstract

The first total synthesis of (–)-indolactam I (**1**) is reported. An efficient synthetic route was established to furnish the natural product in 8 steps. This strategy employed a copper-catalyzed amino acid arylation, peptide coupling, and Lewis acid-mediated macrocyclization to yield **1** from readily available building blocks. In addition, the cell growth inhibition activity of the natural product was examined through assays with the K562 leukemia cell line.

1. Introduction

The teleocidins are a class of indole terpenoids that were first isolated from *Streptomyces mecliocidius* over 50 years ago.[1] Many of these natural products have been discovered to effectively modulate protein kinase C (PKC) activity;[2] therefore, these alkaloids have received considerable attention in biochemical studies related to PKC-mediated cancer regulation.[3] Despite the structural diversity found throughout this teleocidin family (Fig. 1), nearly all members display the core elements represented by (–)-indolactam V (**2**), in which (L)-valine and (L)-tryptophan are assembled into a 9-membered macrocycle. Previous works[4] have demonstrated that **2** maintains the key pharmacophoric elements to promote activation of PKC and correspondingly this natural product has been the focus of several synthetic studies.[5]

(–)-Indolactam I (**1**) was first isolated in 1990 and remains one of the only natural products in this class that possesses a unique amino acid, (L)-isoleucine, in the indolactam macrocycle.[6] Although earlier reports have suggested that increasing hydrophobicity of the teleocidins is advantageous for PKC association,[7] minimal synthetic efforts have been described to access **1**. To date, the only published strategy to prepare **1** required low yielding microbial-based methods.[8] These synthetic limitations prevent suitable biological evaluations of the natural product and thus restrict its therapeutic potential.

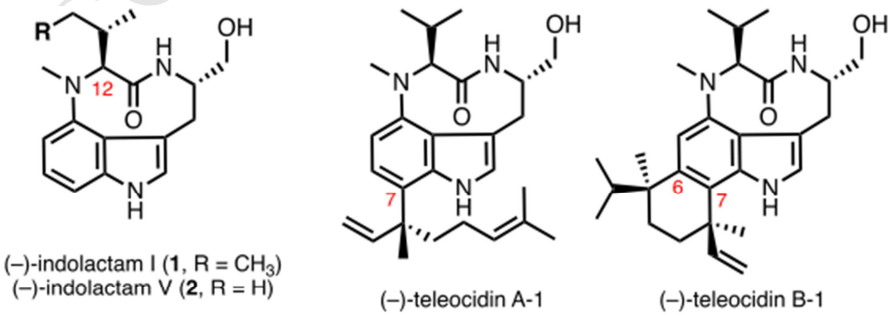


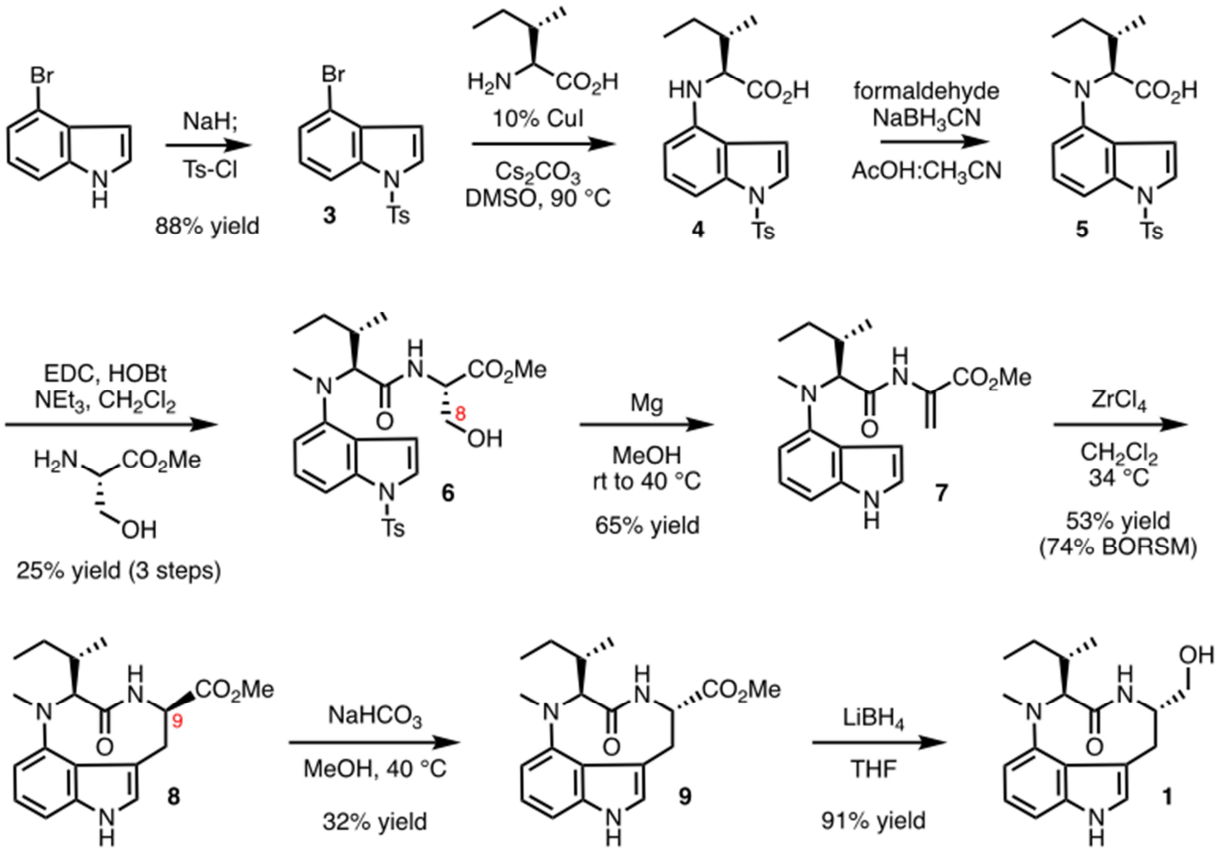
Fig. 1. Representative teleocidin-based natural products.

In this report, we describe the first total synthesis of (–)-indolactam I (**1**). The natural product was prepared from readily available starting materials and assembled through a copper-catalyzed arylation of (L)-isoleucine followed by subsequent zirconium-mediated cyclization. Three natural products in the teleocidin family including **1** were then evaluated for their cell growth inhibitory properties in a human leukemia cell line in order to ascertain the functional effects of increasing hydrophobicity at key positions of the scaffold.

2. Results and Discussion

*2.1 Synthesis of **1***

Several factors establish **1** as an attractive synthetic target for therapeutic applications. The natural product (1) maintains the core structural elements to promote efficient activation of PKC, (2) possesses an increased hydrophobicity relative to **2**, and (3) lacks the C6 and C7 alkyl substitution found in many teleocidins, which have been prepared traditionally by lengthy synthetic routes.[9] In an effort to further elucidate the biological profile of the teleocidin-based natural products, we sought to implement a synthetic route to access **1** that relied upon a modular approach to prepare indolactam alkaloids.[5m] This strategy required efficient C4-nitrogen bond formation with the appropriate amino acid and successful application of methods to establish the (L)-tryptophan subunit of the indolactam macrocycle.



Sceme 1. Synthesis of (–)-Indolactam I.

The synthesis of **1** was initiated from commercially available 4-bromoindole (Scheme 1). Indole protection was performed with tosyl chloride on a multi-gram scale. Recrystallization of the crude product cleanly afforded **3** in an 88% yield. Heteroaryl bromide **3** was then subjected to a copper-catalyzed Ullmann coupling with amino acid (L)-isoleucine to install the C4-nitrogen bond.[10] Although minimal product was observed in the absence of an indole-protecting group, N-tosyl protected **3** smoothly afforded N-aryl amino acid **4** under the standard reaction conditions. However, rapid degradation of **4** was observed upon isolation of the intermediate. To obviate these issues, the crude product from the metal-catalyzed coupling was simply filtered through a pad of silica, concentrated, and immediately transitioned to the next transformation.

Therefore, **4** was directly subjected to reductive amination conditions using formaldehyde and $NaBH_3CN$ to afford **5**. Because air sensitivity was also found for **5**, the material was again advanced to the subsequent step without further isolation. Intermediate **5** underwent peptide coupling with (L)-serine methyl ester to afford **6** as a stable and isolable solid. This protocol for the conversion of **3** to dipeptide **6** resulted in a 25% yield over the 3-step sequence. Although slower reaction rates were observed and decarboxylation[11] of **5** was detected as a major byproduct, this procedure successfully addressed the challenges associated with the instability of intermediates **4** and **5**. In addition, this 3-step protocol has been conducted on a >600 mg scale to yield **6** in short order.

Macrocycle formation requires activation of the C8-alcohol to promote nucleophilic attack by the indole moiety. With dipeptide **6** in hand, we attempted a magnesium-promoted deprotection–dehydration reaction, which would remove the N-tosyl group and simultaneously eliminate the C8-alcohol, to form enoate **7** in one pot. The standard conditions for this transformation were to allow 20 equivalents of magnesium turnings to react with dipeptide **6** for 7 h at room temperature. This procedure, although successful for the production of **7**, afforded the desired product in a modest 21% yield. Through optimization of this process, it was discovered that deprotection occurred efficiently at 0 °C with 10 equivalents of magnesium turnings in 4 h, and no concurrent dehydration was observed. Once complete, the reaction was warmed to 40 °C and elimination of the C8-alcohol was detected over 3 h. This new protocol furnished the desired product **7** in an improved 65% yield.

Enoate **7** was employed in the Lewis acid-mediated closure of the 9-membered indolactam ring.[12] An excess of $ZrCl_4$ and rigorously dried **7** were necessary to promote the transformation. Similar to previous studies[5j,5m] with related indolactam precursors, only one stereoisomer (**8**) was

isolated from the cyclization reaction. Under these conditions, tricycle **8** was produced in a 53% yield with 28% of starting material **7** recovered (74% yield based upon recovered starting material). From **8**, the synthesis of **1** was completed in two final transformations. C9-ester undergoes a relatively slow epimerization when treated with weak base ($NaHCO_3$) at 40 °C for 72 h to produce diastereomer **9** in a 32% isolated yield. Because of a challenging separation of stereoisomers, the mass balance from the purification was a mixture of diastereomers **8** and **9** (33% isolated yield) as well as unreacted starting material **8** (28% yield), which could be isolated and re-subjected to the epimerization conditions. Attempts to further optimize this reaction were unsuccessful, as higher temperatures resulted in decomposition. Fortunately, when ester **9** was treated with $LiBH_4$, natural product **1** was cleanly isolated in a 91% yield. The spectral information for **1** was consistent with the report[6a] of the natural product isolation.[13] This 8-step sequence represents the first total synthesis of alkaloid **1**.

2.2 Biological evaluation of PKC activators

Teleocidin-based natural products have been shown to associate with the C1 regulatory domain of conventional and novel PKCs and thereby induce intracellular PKC signal transduction pathways.[2] Because PKC activation also requires a membrane component, it is believed that hydrophobic groups on the small molecule ligand (e.g., teleocidin) help promote PKC activation by facilitating the interaction of the membrane with the enzyme.[14] Binding affinities of various teleocidins with PKC isozymes provide support for this hypothesis.[7] In this work, we sought to employ cell-based analyses to determine the functional effects of increasing hydrophobicity for the indolactam scaffold.

Cell growth inhibition assays were conducted using the K562 human leukemia cell line. Table 1 provides a summary of the effective concentrations for three natural products: **1, 2** and teleocidin A-1. Nearly all members of the teleocidin family possess the core elements represented by (–)-indolactam V (**2**), and this compound proved to be highly potent *in vitro* (EC_{50} = 143 ± 75 nM). (–)-Indolactam I (**1**) is more hydrophobic than **2** with Log P values[15] of 1.7 and 1.3, respectively. Despite a difference in hydrophobicity, **1** displayed a similar activity as **2**, and an EC_{50} value of 157 ± 56 nM for **1** was found. Concurrent to these studies with **1** and **2**, (–)-teleocidin A-1 was also subjected to the cancer cell-based assay. This natural product possesses a branched geranyl chain at C7, which increases the overall hydrophobicity of the structure (Log P = 4.7). However, a significant decrease in *in vitro* activity was observed for (–)-teleocidin A-1 (EC_{50} = 423 ± 49 nM) versus **2** (ρ = 0.047, t-test) or **1** (ρ = 0.021, t-test). These preliminary results suggest that increasing hydrophobicity for teleocidin analogues may not directly translate to improved *in vitro* activity in specific cancer cell lines.

Table 1
Cell Growth Inhibition Data against K562[a] Cancer Cell Line

Entry	PKC Activator	Cancer cell line EC_{50} (nM)[b]
1	(–)-teleocidin A-1	423 ± 49
2	(–)-indolactam I (**1**)	157 ± 56
3	(–)-indolactam V (**2**)	143 ± 75

[a]Human chronic myelogenous leukemia cell line.
[b]EC_{50} values are the average of at least 6 independent experiments; experimental error indicates standard error of the mean.

3. Conclusions

In summary, we have described a concise synthetic route to access (–)-indolactam I (**1**). This work represents the first total synthesis of the natural product, and we have further demonstrated that **1** exhibits comparable *in vitro* potency to (–)-indolactam V (**2**) in a human leukemia cell line. Both of these indolactam alkaloids displayed lower effective concentrations than the more hydrophobic (–)-teleocidin A-1. These studies will guide our future efforts towards the development of novel therapeutics that have applications in PKC-dependent diseases.

4. Experimental Section

4.1 General

Unless otherwise noted, all reactions were performed under an air atmosphere in oven-dried glassware. (–)-indolactam V (**2**) and (–)-teleocidin A-1 were purchased from Adooq Bioscience and Cayman Chemical, respectively. Commercially available reagents were purchased from Sigma-Aldrich and used without further purification. Reactions were stirred using Teflon-coated magnetic stirrer bars. Reactions were monitored via gas chromatography and/or thin layer silica gel chromatography (TLC) using 0.25 mm silica gel 60F plates with fluorescent indicator. Plates were visualized under UV light without further staining. Products were purified via column chromatography using Silica gel 60, 230-400 mesh and the solvent system(s) indicated.

All new compounds were characterized by ^{1}H NMR, ^{13}C NMR and high-resolution mass spectrometry. Known compounds were characterized by ^{1}H NMR and melting points (for solids) and compared to their literature values. NMR spectra were measured on a Bruker 400 (^{1}H at 400 MHz, ^{13}C at 100 MHz) magnetic resonance spectrometer. ^{1}H chemical shifts are reported relative to the residual solvent peak (chloroform = 7.26 ppm) as follows: chemical shift (δ), (proton ID,

multiplicity (s = singlet, bs = broad singlet, d = doublet, bd = broad doublet, ddd = doublet of doublet of doublets, dddd = doublet of doublet of… etc., ddddd = doublet of doublet of… etc., t = triplet, q = quartet), integration, coupling constant(s) in Hz.). ^{13}C chemical shifts are reported relative to the residual deuterated solvent ^{13}C signals (chloroform = 77 ppm). High-resolution mass spectra were obtained using a high-resolution liquid chromatography mass spectrometer. Melting points were obtained on a Mel-Temp capillary melting point apparatus.

*4.2 4-bromo-1-tosyl-1H-indole (**3**)*

An oven-dried round bottom flask equipped with a magnetic stir bar was charged with 4-bromoindole (2.50 g, 12.8 mmol). The flask was sealed with a septum, and a balloon with needle was inserted through the septum. DMF (25 mL) was added via syringe through a septum and the reaction was cooled to 0 °C with an ice bath. NaH (60%) (245 mg, 15.3 mmol) was added directly to the flask in one portion. The reaction was allowed to warm to room temperature over 45 min followed by re-cooling to 0 °C. Tosyl chloride (3.60 g, 19.1 mmol) in DMF (15 mL) was added via syringe over 2 min. The reaction was allowed to warm to room temperature overnight (15 h). The crude reaction mixture was diluted with H_2O (25 mL). The resulting solution was extracted with EtOAc (100 mL). The organic layer was separated. The aqueous layer was extracted with EtOAc (100 mL, ×2). The combined organic layers were dried with $MgSO_4$, filtered and concentrated to yield a brown oil. Recrystallization (EtOAc/hexanes) provided the desired product **3** as a light brown solid (3.94 g) in 88% yield. Characterization data matched previously reported information.[5m] ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.96 (d, J = 8.27 Hz, 1H), 7.77 (d, J = 8.35 Hz, 2H), 7.64 (d, J = 2.14 Hz, 1H), 7.41 (d, J = 7.80 Hz, 1H), 7.26 (m, 2H), 7.19

(d, J = 8.15 Hz, 1H), 6.75 (d, J = 3.40 Hz, 1H), 2.37 (s, 3H) ppm. ^{13}C-NMR (125 MHz, $CDCl_3$): δ 145.3, 135.1, 131.4, 130.0, 126.9, 126.8, 126.2, 125.9, 125.5, 115.0, 112.6, 108.8, 21.6 ppm.

*4.3 Methyl N-methyl-N-(1-tosyl-1H-indol-4-yl)-L-isoleucyl-L-serinate (**6**)*

An oven-dried schlenk flask equipped with magnetic stir bar was charged with CuI (34.7 mg, 0.18 mmol), (L)-isoleucine (286 mg, 2.18 mmol), **3** (640 mg, 1.82 mmol) and Cs_2CO_3 (889 mg, 2.73 mmol). The flask was sealed with a septum and then evacuated and backfilled with argon (this sequence was carried out three times). DMSO (3.64 mL) was added via syringe through the septum. Under an argon atmosphere, the septum was replaced and sealed with a Teflon screwcap. The reaction was heated to 90 °C for 20 h and then cooled to room temperature. The solution was filtered through a pad of silica eluting with methanol and then concentrated to yield brown oil **4**, which was used directly in the subsequent transformation.

The oil **4** was transferred to a round bottom flask equipped with magnetic stir bar. The flask was sealed with a septum, and a balloon with needle was inserted through the septum. CH_3CN (17.5 mL) followed by formaldehyde (2.2 mL; 38% solution) were added consecutively to the stirring solution via syringe. After 5 min, $NaBH_3CN$ (525 mg, 8.35 mmol) was added directly to the flask followed by the dropwise addition of acetic acid (0.56 mL) over one minute. The reaction was allowed to stir for 1 h at room temperature. The crude reaction mixture was diluted with H_2O (10 mL). The resulting solution was extracted with EtOAc (15 mL). The organic layer was separated. The aqueous layer was extracted with EtOAc (15 mL, ×2). The combined organic layers were dried with $MgSO_4$, filtered and concentrated to yield a light brown oil **5** that was used directly in the subsequent transformation.

L-serine methyl ester hydrochloride (425 mg, 2.73 mmol) was charged into an oven-dried round bottom flask equipped with magnetic stir bar. The flask was sealed with a septum, and a balloon with needle was inserted through the septum. CH_2Cl_2 (14 mL) followed by triethylamine (1.00 mL, 726 mg, 4.21 mmol) were added consecutively to the stirring solution via syringe. The reaction was allowed to stir at room temperature for 30 min and then cooled to 0 °C with an ice bath. Hydroxybenzotriazole hydrate (332 mg, 2.17 mmol) was added directly to flask followed by the addition of **5** dissolved in 4.00 mL of CH_2Cl_2 (an additional 0.50 mL used to ensure all material transferred to reaction flask). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (418 mg, 2.18 mmol) was then added directly to the flask. The reaction was allowed to stir at room temperature overnight (15 h). The crude reaction mixture was diluted with H_2O (10 mL). The resulting solution was extracted with CH_2Cl_2 (10 mL). The organic layer was separated. The aqueous layer was extracted with CH_2Cl_2 (10 mL, ×2). The combined organic layers were dried with $MgSO_4$, filtered and concentrated. Purification by flash column chromatography (55% EtOAc/hexanes) on silica gel afforded the desired product **6** (236 mg) in a 25% yield as a colorless solid. MP: 44-46 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.74 (d, J = 8.44 Hz, 2H), 7.65 (d, J = 8.30 Hz, 1H), 7.55 (d, J = 3.76 Hz, 1H), 7.19-7.23 (m, 3H), 6.80 (d, J = 7.87 Hz, 1H), 6.75 (d, J = 3.77 Hz, 1H), 6.03 (bs, 1H), 4.53 (dt, J = 7.39, 3.55 Hz, 1H), 3.70-3.79 (m, 2H), 3.66 (s, 3H), 3.57 (m, 1H), 2.82 (s, 3H), 2.35 (s, 3H), 2.10 (bs, 1H), 1.94 (bs, 1H), 1.25 (m, 1H), 0.96 (d, J = 6.45 Hz, 3H), 0.93 (t, J = 7.34 Hz, 3H) ppm. ^{13}C-NMR (100 MHz, $CDCl_3$): δ 170.4, 169.6, 145.0, 136.2, 135.2, 129.9, 126.9, 126.8, 125.4, 125.1, 124.0, 113.0, 108.1, 107.0, 71.7, 63.3, 54.0, 52.5, 35.1, 33.6, 25.8, 21.5, 16.1, 11.8 ppm. HRMS-ESI+: calculated for $C_{26}H_{33}N_3O_6S$+H: 516.2168; Found: 516.2159.

*4.4 Methyl 2-((2S,3S)-2-((1H-indol-4-yl)(methyl)amino)-3-methylpentanamido)acrylate (**7**)*

An oven-dried reaction tube equipped with magnetic stir bar was charged with **6** (108 mg, 0.21 mmol). Methanol (5.3 mL) was added directly to tube followed by the addition of magnesium turning (51 mg, 2.10 mmol). The tube was sealed and cooled to 0 °C with an ice bath and allowed to stir for 4 h. After this time, the reaction mixture was warmed to 40 °C and stirred for an additional 3 h. The crude reaction mixture was cooled to room temperature and diluted with aq. NH_4Cl (6 mL). The resulting solution was extracted with EtOAc (10 mL). The organic layer was separated. The aqueous layer was extracted with EtOAc (10 mL, ×2). The combined organic layers were dried with $MgSO_4$, filtered and concentrated. The crude product was further purified by flash column chromatography (25% EtOAc/hexanes) on silica gel to afford the product **7** (46.5 mg) in a 65% yield as a colorless oil. ^{1}H-NMR (400 MHz, $CDCl_3$): 8.21-8.34 (m, 2H), 7.17 (dd, J = 3.26, 2.48 Hz, 1H), 7.07-7.11 (m, 2H), 6.69 (s, 1H), 6.61-6.65 (m, 1H), 6.58 (s, 1H), 5.83 (d, J = 1.48 Hz, 1H), 4.18 (d, J = 7.60 Hz, 1H), 3.71 (s, 3H), 2.89 (s, 3H), 2.07-2.19 (m, 1H), 1.86 (dd, J = 9.47, 7.52 Hz, 1H), 1.22-1.33 (m, 1H), 0.98 (d, J = 6.63 Hz, 3H), 0.94 (t, J = 7.42 Hz, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): 170.3, 164.1, 144.5, 137.4, 130.8, 123.0, 122.7, 121.2, 110.0, 108.6, 106.1, 101.1, 71.5, 52.7, 37.0, 34.4, 26.4, 16.3, 12.1 ppm. HRMS-ESI+: calculated for $C_{19}H_{25}N_3O_3$+H: 344.1974; Found: 344.1964.

*4.5 Methyl (2S,5R)-2-((S)-sec-butyl)-1-methyl-3-oxo-2,3,4,5,6,8-hexahydro-1H-[1,4]diazonino[7,6,5-cd]indole-5-carboxylate (**8**)*

An oven-dried reaction tube equipped with magnetic stir bar was charged with **7** (45.6 mg, 0.13 mmol). The reaction tube was transferred into a glove box. $ZrCl_4$ (465 mg, 2.0 mmol) and then CH_2Cl_2 (1.25 mL) were added. The reaction tube was sealed, transferred out of the glove

box and then heated to 34 °C. After 24 h, the reaction mixture was cooled to room temperature and then added dropwise to saturated aqueous $NaHCO_3$ (10 mL) at 0 °C. The resulting solids were removed by filtration over celite (EtOAc eluent). The layers were separated and the aqueous layer was extracted with EtOAc (10 mL, ×3). The combined organic layers were dried over $MgSO_4$. Evaporation under reduced pressure afforded the crude product, which was further purified by flash chromatography (40% EtOAc/hexanes) to afford the desired product **8** (24.2 mg, 53% yield) as a colorless solid and recovered starting material **7** (12.8 mg, 28% recovered). MP: 122-124 °C. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.08 (s, 1H), 7.06 (t, J = 7.84 Hz, 1H), 7.01 (d, J = 2.45 Hz, 1H), 6.97 (d, J = 8.06 Hz, 1H), 6.77 (d, J = 7.57 Hz, 1H), 6.68 (d, J = 7.51 Hz, 1H), 4.50 (dt, J = 7.15, 3.62 Hz, 1H), 3.94 (d, J = 8.45 Hz, 1H), 3.93 (s, 3H), 3.46 (dd, J = 15.36, 3.25 Hz, 1H), 3.31 (dd, J = 15.48, 3.90 Hz, 1H), 3.13 (s, 3H), 2.52 (m, 1H), 1.51 (m, 1H), 1.26 (m, 1H), 0.71 (d, J = 6.58 Hz, 3H), 0.48 (t, J = 7.39 Hz, 3H) ppm. ^{13}C-NMR (100 MHz, $CDCl_3$): δ 172.5, 172.4, 147.9, 138.4, 122.8, 122.6, 120.3, 113.2, 109.3, 105.2, 67.0, 57.3, 53.1, 35.2, 34.0, 32.5, 24.8, 16.0, 9.5 ppm. HRMS-ESI+: calculated for $C_{19}H_{25}N_3O_3$+H: 344.1974; Found: 344.1967.

*4.6 Methyl (2S,5S)-2-((S)-sec-butyl)-1-methyl-3-oxo-2,3,4,5,6,8-hexahydro-1H-[1,4]diazonino[7,6,5-cd]indole-5-carboxylate (**9**).*

An oven-dried reaction tube equipped with magnetic stir bar was charged with **8** (10.6 mg, 0.031 mmol). Methanol (2.07 mL) was added to the reaction followed by $NaHCO_3$ (71.4 mg, 0.85 mmol). The reaction tube was sealed and heated to 40 °C for 72 h. After this time, the reaction mixture was cooled to room temperature. The solution was concentrated and then partitioned between EtOAc (5.3 mL) and water (5.3 mL). The organic layer was removed, and

the aqueous layer was extracted with EtOAc (6 mL, ×3). The combined organic layers were dried with $MgSO_4$, filtered and concentrated. The crude product was purified by flash column chromatography (10% CH_3CN/benzene) on silica gel to afford the epimer **9** (3.4 mg, 32% yield) as a colorless solid. A mixture of **8** and **9** (3.6 mg, 33% yield) and recovered starting material **8** (3.0 mg, 28% recovered) was also obtained. **9** exists as two rotamers at room temperature. ^{1}H-NMR (400 MHz, $CDCl_3$), signals corresponding to major rotamer shown: δ 8.20 (bs, 1H), 7.15 (t, J = 7.75 Hz, 1H), 7.07 (t, J = 7.91 Hz, 1H), 7.02 (d, J = 7.40 Hz, 1H), 6.91 (d, J = 2.47 Hz, 1H), 5.24 (d, J = 10.54 Hz, 1H), 5.13 (dd, J = 10.57, 4.74 Hz, 1H), 3.69 (s, 3H), 3.62 (dd, J = 17.80, 5.24 Hz, 1H), 3.13-3.35 (m, 2H), 2.72 (s, 3H), 2.43 (m, 1H), 2.19 (m, 1H), 2.04 (m, 1H), 1.07 (t, J = 7.45 Hz, 3H), 0.90 (d, J = 6.46 Hz, 3H) ppm. ^{13}C-NMR (100 MHz, $CDCl_3$), signals corresponding to both major and minor rotamers shown: δ 172.7, 171.5, 171.4, 170.2,147.6, 145.1, 139.4, 139.1, 128.3, 127.9, 124.2, 123.1, 123.0, 122.9, 121.4, 114.5, 110.2, 108.8, 107.0, 104.4, 75.0, 69.8, 58.1, 55.6, 52.9, 52.0, 36.3, 35.8, 34.3, 30.6, 30.3, 29.7, 25.2, 24.6, 17.4, 15.7, 11.1, 10.3 ppm. HRMS-ESI+: calculated for $C_{19}H_{25}N_3O_3$+H: 344.1974; Found: 344.1963.

*4.7 (–)-Indolactam I (**1**).*

9 (3.4 mg, 0.010 mmol) was charged into an oven-dried reaction tube equipped with magnetic stir bar. The reaction tube was transferred into a glove box. $LiBH_4$ (2.2 mg, 0.10 mmol) and THF (100 μL) were added. The reaction tube was sealed, transferred out of the glove box and then allow to stir at room temperature for 5 h. After this time, the reaction was added dropwise to water (0.20 mL) at 0 °C. The resulting solution was extracted with CH_2Cl_2 (0.50 mL). The organic layer was separated. The aqueous layer was extracted with CH_2Cl_2 (0.50 mL, ×2). The combined organic layers were dried with $MgSO_4$, filtered and concentrated.

Evaporation under reduced pressure afforded the crude product, which was further purified by flash chromatography (70% EtOAc/hexanes) to afford the **1** (2.8 mg, 91% yield) as a colorless solid. As previously reported,[6a] **1** exists as two rotamers at room temperature, and the characterization data matched the previous report. ^{1}H NMR (400 MHz, $CDCl_3$), signals corresponding to major rotamer shown: δ 7.99 (bs, 1H), 7.08 (t, J = 7.94 Hz, 1H), 6.84-6.98 (m, 3H), 6.51 (d, J = 7.68 Hz, 1H), 4.52 (d, J = 10.30 Hz, 1H), 4.30 (m, 1H), 3.76 (d, J = 11.16 Hz, 1H), 3.56 (m, 1H), 3.21 (d, J = 17.57 Hz, 1H), 3.03 (dd, J = 17.37, 3.74 Hz, 1H), 2.93 (s, 3H), 2.42 (m, 1H), 2.36 (m, 1H), 1.34 (m, 1H), 0.91 (d, J = 6.41 Hz, 3H), 0.58-0.61 (m, 4H) ppm. ^{13}C-NMR (100 MHz, $CDCl_3$), signals corresponding to major rotamer shown: δ 173.6, 147.8, 139.3, 123.0, 121.3, 117.8, 114.4, 106.1, 103.8, 69.4, 65.2, 55.5, 34.2, 33.1, 29.7, 24.6, 17.2, 10.3 ppm. HRMS-ESI+: calculated for $C_{18}H_{25}N_3O_2$+H: 316.2025; Found: 316.2015.

4.6 Cell growth inhibition assay: MTT viability readout

K562 cells (ATCC) were cultured in RPMI medium 1640 (Gibco) supplemented with FBS (10% v/v), penicillin (100 units/ml) and streptomycin (50 units/mL) at 37 °C, in 5% CO_2 in air in a humidified incubator. Cells were subcultured every 72-96 h (once cellular concentration reaches >1,000,000 cells/mL) to a starting concentration of 200,000 cells/mL. Cell concentration was determined with a cell counter.

To assay compounds, cells were plated in plasma-treated polystyrene 96-well plates at a density of 10,000-15,000 cells/well in 50 μL of culture media, and allowed to incubate for 30 min while compound dilutions were prepared. Compounds were diluted from a 10 mM DMSO stock solution with culture media to a concentration of 20 μM. Colchicine was used as a positive control in all experiments. Triplicate serial dilutions were then prepared in culture media from

40 μM to 160 pM across 10 wells of a 96 well plate (diluted by factors of 4). Plates containing cells were dosed with compound (50 uL/well) to afford final assay concentrations of 20 μM to 80 pM. The maximum DMSO concentration was 0.25%. Cells were then incubated at 37 °C in 5% CO_2 in a humidified incubator for 48 h, at which time 10 μL of a 5 mg/mL solution of thiazolyl blue tetrazolium bromide (Aldrich) in cell culture media was added to each well. The cells were allowed to incubate an additional 2.5 h, at which time they were lysed with 100 μL of a detergent solvent (20 mL Triton-X 100 (Aldrich) in 180 mL of 0.1 N HCl in isopropanol solution).

Plates were analyzed with a VERSAmax tunable microplate reader (Molecular Devices) using SOFTmax Pro® version 3.1.1, reading at 570 nm and subtracting at 690 nm. The sigmoidal dose response curves generated by plotting corrected signal vs. log (drug concentration) were analyzed (least squares regression) using Prism® by GraphPad to generate EC_{50} values. An average of several experiments is reported as indicated; experimental error indicates standard error of the mean.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/.

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