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Synthesis and antiproliferative activity of clausine E, mukonine, and koenoline bioisosteres

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Abstract—Aza-analogues of clausine E, mukonine and koenoline were prepared from 1-(benzenesulfonyl)-1*H*-pyrrolo[2,3-*b*]pyridine-3-carboxaldehyde and their antiproliferative activity was evaluated against miscellaneous cancer cell lines and compared to those obtained with clausine E and mukonine.

1. Introduction

The 1-oxygenated carbazole alkaloids like clausine E, mukonine, and koenoline (Fig. 1) have been isolated from higher plants of the *Rutaceae* family.[1] The cytotoxic activity of this alkaloid class has been roughly reported in the literature.[2,3] Nevertheless, koenoline was screened in the NCI in vitro anticancer drug discovery (NCS-654286) on sixty cancer cell lines.[4] The compound showed low antiproliferative activity (mean log GI_{50} value over all cell lines = −4.63). The broad range of useful biological activities exhibited by many carbazole alkaloids prompted several research groups to develop chemical strategies (Fischer indolization, oxidative cyclization of diarylamine, transition metal-mediated and -catalyzed processes) for their total synthesis.[1] Amongst them, two close and practical syntheses were reported by Bringmann and Brenna to prepare 1-oxygenated carbazoles.[5,6]

Pyrrolo[2,3-*b*]pyridine is an indole surrogate of interest in medicinal chemistry. It has been used as a new scaffold to prepare new drug candidates with improved biological activities, physicochemical and pharmacokinetic properties.[7]

With regard to the pharmacological potential of these 1-oxygenated carbazole alkaloids, we have designed aza-analogues **1**–**3** (Fig. 1) in order to evaluate their antiproliferative activities against miscellaneous tumor cell lines and compare it with the natural products clausine E and mukonine prepared in our Laboratory (in

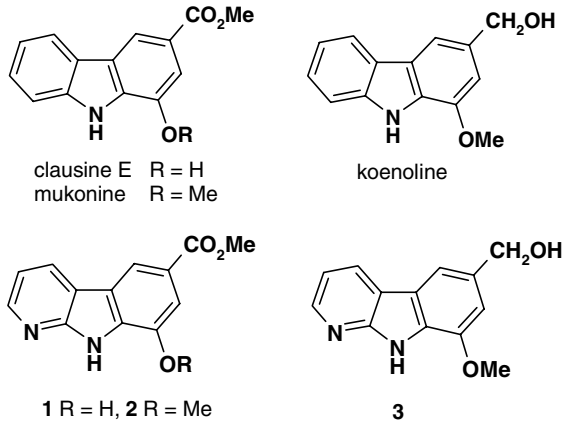

Figure 1. Clausine E, mukonine, koenoline, and bioisosteres **1**–**3**.

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our hands, koenoline was found particularly labile as already reported in the literature[8] and was not tested).

2. Results and discussion

2.1. Chemistry

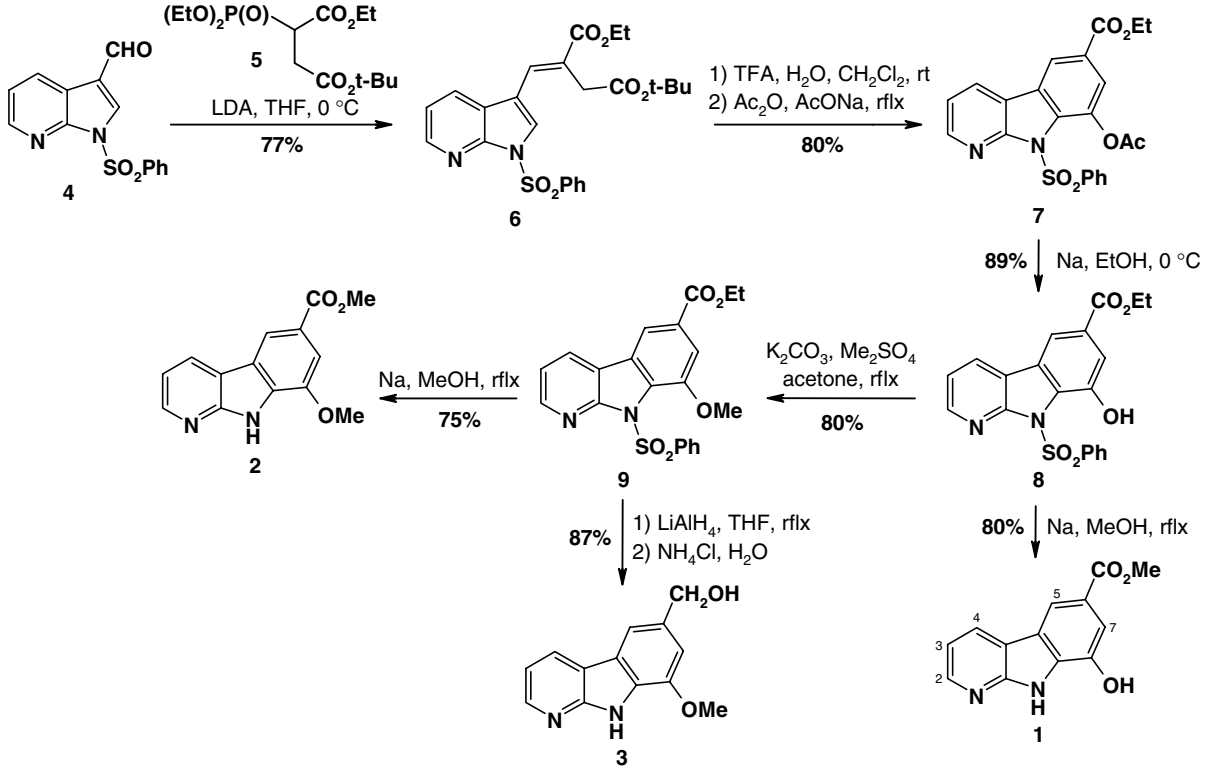
The preparation of derivatives **1**–**3** is summarized in Scheme 1. Following the synthetic pathway of clausine E disclosed by Bringmann,[5] 1-(benzenesulfonyl)-1*H*-pyrrolo[2,3-*b*]pyridine-3-carboxaldehyde **4**[9] was engaged in a Horner–Wadsworth–Emmons reaction in the presence of diester phosphonate **5**[10] to afford alkene **6**. *Tert*-butyl ester hydrolysis of **6** in acidic medium followed by intramolecular cyclization of monoester acid led to the desired aza-carbazole **7** in 80% yield (two steps). Deacetylation of **7** occurred in the presence of EtONa/EtOH at 0 °C to give alcohol **8** in 89% yield. Benzenesulfonyl group of **8** was removed in MeONa/MeOH to provide aza-clausine E **1** in 80% yield. *O*-Methylation (K_2CO_3, Me_2SO_4, acetone) of **7** led to the intermediate **9** which was then treated by MeONa/MeOH to afford aza-mukonine **2**. Finally, the reduction of the ester group was performed on compound **9** to give aza-koenoline **3** in 87% yield.

2.2. Antiproliferative activity

Clausine E, mukonine, and compounds **1**–**3** were tested for their antiproliferative activities against MCF-7 breast adenocarcinoma cell line, Mes-sa sarcoma cell line, and three colorectal carcinoma cell lines (HCT-116, SW-48, and SW-480). IC_{50} values for **1**–**3** and 5-FU (for comparison) are reported in Table 1. Clausine E inhibited the proliferation of MCF-7 breast cancer line at high nanomolar concentrations, and of Mes-sa sarcoma and colon cancer cell lines at low micromolar concentrations. Mukonine was much less potent than clausine E in all the cancer cell lines with antiproliferative activity obtained at high micromolar concentrations. Compound **1** displayed almost the same antiproliferative activity in all the cancer cell lines used here. Derivative **2** showed a preferential growth inhibitory activity in the breast and colorectal cancer cell lines (concentration range 1–10 μM). The pattern of activities of compounds **1** and **2** looked similar to that of clausine E. However, compound **1** was more active than clausine E in the Mes-sa sarcoma line, while both compounds showed higher antiproliferative activity in the SW-48 colorectal cancer line. More importantly, both compounds showed similar activity profiles to that of the clinically used 5-FU in colorectal cancer models.

Finally, compound **3** showed a weak antiproliferative activity in all cellular models compared to clausine E and compounds **1** and **2**. This weak antiproliferative activity was similar to that observed for mukonine.

The cell cycle effects of clausine E, mukonine, **1**, **2**, and **3** are shown in Table 2. Clausine E induced a moderate G_2/M arrest in all colorectal cell lines. Differently, treatment of HCT-116 and SW-48 with mukonine induced a slight increase of cells in the G_1 phase; however, this drug showed no major perturbations of the cell cycle in the SW-480 cells. Incubation of HCT-116, SW-48,



Scheme 1. Synthetic pathway to bioisosteres **1**, **2**, and **3**.

Table 1. In vitro cytotoxicity of clausine E, mukonine, **1**, **2**, **3**, and 5-FU against human cancer cell lines

Compound	IC_{50} [a] (μM)				
	MCF-7	Mes-sa	HCT-116	SW-48	SW-480
Clausine E	0.6 ± 0.3	35 ± 1	1.9 ± 0.2	21 ± 7	4.8 ± 3
Mukonine	30 ± 9	80 ± 38	43 ± 5	62 ± 21	72 ± 26
1	11 ± 4	16 ± 8	7.1 ± 1.4	9.0 ± 1.3	8.8 ± 1.8
2	4.4 ± 3.4	144 ± 32	8.6 ± 1.8	5.6 ± 2.6	4.7 ± 2.4
3	58 ± 14	65 ± 26	36.6 ± 11.6	21.1 ± 2.2	63.1 ± 9.5
5-FU	0.3 ± 0.1	ND	2.8 ± 1.8	5.3 ± 2.8	3.5 ± 3.6

[a] Values are expressed as means ± standard deviation (SD) of five different experiments (ND, not determined).

Table 2. Percentage of cells (%) in the different phases of the cell cycle

Compound	HCT-116			SW-48			SW-480		
	G_1 [a]	S	G_2/M	G_1	S	G_2/M	G_1	S	G_2/M
Control	61.8 ± 8.7	27.2 ± 6.9	11 ± 1.9	66.1 ± 2.9	21 ± 1.3	12.9 ± 1.9	61.8 ± 2.8	27.5 ± 2.3	10.7 ± 2.8
Clausine E	60 ± 10	12.1 ± 0.1	28 ± 6.2	65.6 ± 7.9	12.5 ± 8	21.9 ± 1.6	59.9 ± 14.1	17.5 ± 10.5	25.7 ± 3.6
Mukonine	70.8 ± 7.1	17.4 ± 6.8	11.7 ± 0.4	76.7 ± 2	14.1 ± 1.4	9 ± 3.4	55.8 ± 1.5	33.8 ± 4.8	10.3 ± 3.3
1	56.6 ± 5.5	29.1 ± 5.4	14.3 ± 4.2	62.4 ± 3.1	18.9 ± 1.4	18.7 ± 2	58.7 ± 2.9	26.5 ± 2.1	14.7 ± 1.3
2	52.6 ± 5.5	31 ± 7.2	16.4 ± 2.6	59 ± 2	23.0 ± 1.4	18.1 ± 2.5	60.7 ± 5.6	27.3 ± 6	11.9 ± 1.2
3	53.3 ± 1.4	17.4 ± 1.1	29.3 ± 1.5	57.4 ± 0.9	9.7 ± 2.6	32.4 ± 3	52.3 ± 0.5	33 ± 2	14.7 ± 2.5

[a] Values are expressed as means ± SD of three different experiments.

and SW-480 colorectal cancer cells with **1** and **2** induced a slight increase in G_2/M phases suggesting induction of a modest mitotic arrest. For compound **3**, a higher G_2/M arrest pattern was observed in HCT-116 and SW-48 cells that may be ascribed to a moderate mitotic arrest. This mitotic arrest was not so evident in the SW-480 cells.

3. Conclusion

In summary, we have developed an efficient and straightforward route to 1-oxygenated aza-carbazoles. Aza-clausine E **1** and aza-mukonine **2** exhibited significant human colorectal cancer cell growth inhibitory activity in the micromolar range. These compounds represent promising new anticancer agents and structure–activity relationship studies are in progress to reach more potent derivatives.

4. Experimental

Melting points were measured with a Büchi Tottoli SMP-20 heating unit and are uncorrected. IR spectra were recorded with a Perkin-Elmer spectrum one spectrophotometer. NMR spectra were recorded at 300 K with an AVANCE 300 Bruker spectrometer at 300 MHz for ^{1}H and 75 MHz for ^{13}C. Chemical shifts are expressed in parts per million (ppm) relative to TMS. Mass spectra were recorded with a Thermo Finnigan Mat 95 XL. Elemental analyses were performed on a Thermoquest Flash 1112 series EA analyser. TLC was conducted on precoated silica gel plates (Merck $60F_{254}$) and the spots were visualized under UV light. Flash chromatography was carried out on column using flash silica gel 60 Merck (40–63 mm) using the indicated solvents (petroleum ether (PE): bp 40–60 °C). All reactions requiring anhydrous conditions were conducted in flame-dried apparatus. Clausine E (mp 207–209 °C, lit. mp 203°C), mukonine (mp 195–197 °C, lit. mp 201 °C) were synthesized according to the procedure described by Bringmann et al.[5] IR, NMR, and MS data were identical with those reported in the literature.[5]

4.1. *Tert*-butyl 4-(1-benzenesulfonyl-1*H*-pyrrolo[2,3-*b*]-pyridin-3-yl)-3-(ethoxycarbonyl)but-3-enoate (6)

At 0 °C and under argon atmosphere, a solution of 2 M lithium diisopropylamide (3.49 mL, 6.98 mmol) in THF was added dropwise to a solution of diester phosphonate **5** (2.36 g, 6.98 mmol) in THF (30 mL). The solution was stirred at 0 °C for 20 min. A solution of 1-(benzenesulfonyl)-1*H*-pyrrolo[2,3-*b*]pyridine-3-carboxaldehyde **4** (1.33 g, 4.65 mmol) was added dropwise at 0 °C. The final mixture was stirred at room temperature for 4 h. After evaporation of solvent and addition of water, the aqueous phase was extracted with EtOAc (2× 10 mL). The organic phases were combined, dried over $MgSO_4$, and evaporated in vacuo. The residue was purified by flash chromatography (PE/EtOAc 85:15) to give **6** (1.68 g, 77%) as a solid. Mp 102–104 °C (EtOH); IR (KBr) ν 3063, 2980, 1712, 1640, 1537, 1450, 1252, 1179 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.39 (t, 3H, J = 7.2 Hz, CH_3), 1.56 (s, 9H, CH_3), 3.61 (s, 2H, CH_2), 4.34 (q, 2H, J = 7.2 Hz, CH_2), 7.29 (dd, 1H, J = 4.6, 7.9 Hz, H_{Ar}), 7.53 (t, 2H, J = 7.5 Hz, H_{Ar}), 7.63 (t, 1H, J = 7.5 Hz, H_{Ar}), 7.88 (s, 1H, H_{Ar}), 8.00 (dd, 1H, J = 1.3, 7.9 Hz, H_{Ar}), 8.02 (s, 1H, =CH), 8.25 (d, 2H, J = 7.5 Hz, H_{Ar}), 8.51 (dd, 1H, J = 1.3, 4.6 Hz, H_{Ar}); ^{13}C NMR ($CDCl_3$) δ 14.4 (CH_3), 28.1 (3 CH_3), 35.9 (CH_2), 61.4 (C), 81.8 (CH_2), 114.1 (C), 119.4 (CH), 122.6 (C), 125.7 (CH), 127.4 (C), 128.2 (2 CH), 128.3 (CH), 129.2 (2 CH), 129.9 (CH), 134.4 (CH), 138.0 (C), 145.9 (CH), 147.0 (C), 167.0 (CO), 169.8 (CO); MS (ESI) m/z 471 $(M+H)^+$; Anal. Calcd for $C_{24}H_{26}N_2O_6S$: C, 61.26; H, 5.57; N, 5.95. Found: C, 60.88; H, 5.33; N, 6.05.

4.2. Ethyl 8-acetoxy-9-benzenesulfonyl-8-hydroxy-9*H*-pyrido[2,3-*b*]indole-6-carboxylate (7)

A solution of **6** (570 mg, 1.21 mmol), TFA/H_2O (8.2 mL, 8/0.2) in CH_2Cl_2 (8 mL) was stirred at room temperature for 2 h. The solvents were evaporated and the residue was dissolved in acetic anhydride (10 mL) and sodium acetate (220 mg, 2.68 mmol) was added. The mixture was heated at reflux for 3 h. After cooling, the solvent was evaporated. The crude solid was purified by flash chromatography (PE/EtOAc 8:2) to afford **7** (340 mg, 80%) as a solid. Mp = 178–180 °C (EtOH); IR (KBr) ν 3070, 2987, 1776, 1728, 1590, 1370, 1260, 1202 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.43 (t, 3H, J = 7.2 Hz, CH_3), 2.47 (s, 3H, CH_3), 4.43 (q, 2H, J = 7.2 Hz, CH_2), 7.32 (dd, 1H, J = 4.9, 7.7 Hz, H_3), 7.39 (t, 2H, J = 7.4 Hz, H_{Ar}), 7.51 (t, 1H, J = 7.4 Hz, H_{Ar}), 7.94 (d, 1H, J = 1.5 Hz, H_7), 7.99 (br d, 2H, J = 7.4 Hz, H_{Ar}), 8.22 (dd, 1H, J = 1.5, 7.7 Hz, H_4), 8.49 (d, 1H, J = 1.5 Hz, H_5), 8.56 (dd, 1H, J = 1.5, 4.9 Hz, H_2); ^{13}C NMR ($CDCl_3$) δ 14.4 (CH_3), 21.4 (CH_3), 61.6 (CH_2), 118.9 (C), 119.8 (CH), 120.2 (CH), 125.6 (CH), 127.3 (C), 127.7 (C), 127.7 (2 CH), 128.8 (CH), 128.9 (2 CH), 132.9 (C), 134.0 (CH), 138.6 (C), 138.7 (C), 148.1 (CH), 152.9 (C), 165.3 (CO), 169.7 (CO); MS (ESI) m/z 439 $(M+H)^+$; Anal. Calcd for $C_{22}H_{18}N_2O_6S$: C, 60.27; H, 4.14; N, 6.39. Found: C, 60.01; H, 4.05; N, 6.27.

4.3. Ethyl 9-benzenesulfonyl-8-hydroxy-9*H*-pyrido[2,3-*b*]indole-6-carboxylate (8)

At 0 °C and under argon atmosphere, sodium (18 mg, 0.78 mmol) was added portionwise to a solution of **7** (340 mg, 0.77 mmol) in EtOH/THF (15 mL, 1/2). The solution was stirred at 0 °C for 2 h. At 0 °C, the reaction was hydrolyzed by addition of H_2O and the solvents were evaporated in vacuo. The residue was taken up in H_2O and extracted with EtOAc (2× 5 mL). The organic phases were combined, dried over $MgSO_4$, and evaporated in vacuo. The crude solid was recrystallized from EtOH to give **8** (275 mg, 89%) as a white solid. Mp = 198–200 °C (EtOH); IR (KBr) ν 3301, 2963, 1716, 1585, 1377, 1261 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.44 (t, 3H, J = 7.2 Hz, CH_3), 4.43 (q, 2H, J = 7.2 Hz, CH_2), 7.32 (dd, 1H, J = 4.9, 7.7 Hz, H_3), 7.41 (t, 2H, J = 7.4 Hz, H_{Ar}), 7.54 (t, 1H, J = 7.4 Hz, H_{Ar}), 7.87 (d, 1H, J = 1.5 Hz, H_7), 7.98 (br d, 2H, J = 7.4 Hz, H_{Ar}), 8.15 (d, 1H, J = 1.5 Hz, H_5), 8.19 (dd, 1H, J = 1.5, 7.7 Hz, H_4), 8.55 (dd, 1H, J = 1.5, 4.9 Hz, H_2), 9.51 (s, 1H, OH); ^{13}C NMR ($CDCl_3$) δ 14.4 (CH_3), 61.4 (CH_2), 113.9 (CH), 119.4 (CH), 119.6 (C), 120.5 (CH), 126.7 (C), 127.9 (2 CH), 128.0 (C), 128.7 (C), 129.0 (CH), 129.2 (2 CH), 134.6 (CH), 136.9 (C), 145.2 (C), 147.8 (CH), 152.0 (C), 165.9 (CO); MS (ESI) m/z 397 $(M+H)^+$; Anal. Calcd for $C_{20}H_{16}N_2O_5S$: C, 60.60; H, 4.07; N, 7.07. Found: C, 60.55; H, 3.98; N, 6.99.

4.4. Methyl 8-hydroxy-9*H*-pyrido[2,3-*b*]indole-6-carboxylate (1)

A solution of **8** (100 mg, 0.25 mmol) and catalytic amount of sodium in MeOH (6 mL) was heated at reflux for 12 h. After addition of H_2O and evaporation of solvent, the residue was taken up in H_2O and extracted with EtOAc (2× 5 mL). The organic phases were combined, dried over $MgSO_4$, and evaporated in vacuo. The crude solid was washed with MeOH to afford **1** (49 mg, 80%) as a solid. Mp > 210 °C (washing MeOH); IR (KBr) ν 3300–3100, 2851, 1677, 1638, 1586, 1353, 1255, 1089 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.87 (s, 3H, CH_3), 7.23 (dd, 1H, J = 4.9, 7.7 Hz, H_3), 7.51 (d, 1H, J = 1.3 Hz, H_5), 8.35 (d, 1H, J = 1.3 Hz, H_7), 8.45 (dd, 1H, J = 1.5, 4.9 Hz, H_2), 8.60 (dd, 1H, J = 1.5, 7.7 Hz, H_4), 10.27 (s, 1H, OH), 12.08 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 51.8 (CH_3), 111.2 (CH), 114.7 (CH), 115.6 (CH), 115.8 (C), 121.4 (C), 121.5 (C), 129.1 (CH), 131.8 (C), 143.1 (C), 146.7 (CH), 152.2 (C), 166.9 (CO); MS (ESI) m/z 243 $(M+H)^+$; Anal. Calcd for $C_{13}H_{10}N_2O_3$: C, 64.46; H, 4.16; N, 11.56. Found: C, 64.76; H, 3.99; N, 11.40.

4.5. Ethyl 9-benzenesulfonyl-8-methoxy-9*H*-pyrido[2,3-*b*]indole-6-carboxylate (9)

A solution of **8** (160 mg, 0.40 mmol), potassium carbonate (56 mg, 0.41 mmol), and dimethylsulfate (80 mL, 0.82 mmol) in acetone (10 mL) was stirred at reflux for 7 h. After cooling, the solvent was evaporated. The residue was taken up in H_2O and extracted with EtOAc (2× 10 mL). The organic phases were combined, dried over $MgSO_4$, and evaporated in vacuo. The crude solid was recrystallized from EtOH to give **9** (132 mg, 80%) as a solid. Mp = 161–162 °C (EtOH); IR (KBr) ν 2979, 1721, 1590, 1499, 1345, 1260 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.44 (t, 3H, J = 7.2 Hz, CH_3), 3.92 (s, 3H, CH_3), 4.43 (q, 2H, J = 7.2 Hz, CH_2), 7.38 (dd, 1H, J = 4.9, 7.7 Hz, H_3), 7.55–7.65 (m, 3H, H_{Ar}), 7.68 (d, 1H, J = 1.5 Hz, H_7), 8.26 (br s, 1H, H_5), 8.28-8.33 (m, 3H, H_{Ar} + H_4), 8.68 (dd, 1H, J = 1.5, 4.9 Hz, H_2); ^{13}C NMR ($CDCl_3$) δ 14.5 (CH_3), 56.1 (CH_3), 62.5 (CH_2), 112.4 (CH), 115.0 (CH), 119.7 (C), 119.9 (CH), 126.0 (C), 127.6 (2 CH), 127.8 (C), 128.8 (2 CH), 128.9 (CH), 131.0 (C), 133.5 (CH), 140.9 (C), 147.7 (CH), 148.7 (C), 153.7 (C), 166.2 (CO); MS (ESI) m/z 411 $(M+H)^+$; Anal. Calcd for $C_{21}H_{18}N_2O_5S$: C, 61.45; H, 4.42; N, 6.83. Found: C, 61.66; H, 4.52; N, 6.97.

4.6. Methyl 8-methoxy-9*H*-pyrido[2,3-*b*]indole-6-carboxylate (2)

A solution of **9** (120 mg, 0.29 mmol) and catalytic amount of sodium in MeOH (6 mL) was heated overnight at reflux. After addition of H_2O and evaporation of solvent, the residue was taken up in H_2O and extracted with EtOAc (2× 5 mL). The organic phases were combined, dried over $MgSO_4$, and evaporated in vacuo. The crude solid was washed with MeOH to afford **2** (56 mg, 75%) as a white solid. Mp > 210 °C (washing MeOH); IR (KBr) ν 3122, 2990, 1698, 1584, 1406, 1227 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.90 (s, 3H, CH_3), 4.04 (s, 3H, CH_3), 7.26 (dd, 1H, J = 4.7, 7.7 Hz, H_3), 7.56 (s, 1H, H_5), 8.47 (d, 1H, J = 4.7 Hz, H_2), 8.48 (s, 1H, H_7), 8.65 (d, 1H, J = 7.7 Hz, H_4), 12.38 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 51.9 (CH_3), 55.7 (CH_3), 107.1 (CH), 115.5 (C), 115.9 (CH), 116.4 (CH),

121.0 (C), 121.5 (C), 129.3 (CH), 132.0 (C), 145.3 (C), 146.9 (CH), 152.2 (C), 166.8 (CO); MS (ESI) *m/z* 257 $(M+H)^+$; Anal. Calcd for $C_{14}H_{12}N_2O_3$: C, 65.62; H, 4.72; N, 10.93. Found: C, 65.44; H, 4.66; N, 11.05.

4.7. (8-Methoxy-9*H*-pyrido[2,3-*b*]indol-6-yl)methanol (3)

A solution of **9** (60 mg, 1.46 mmol), $LiAlH_4$ (14 mg, 0.29 mmol) in THF (3 mL) was heated at reflux for 2 h. After addition of NH_4Cl, the aqueous phase was extracted with EtOAc (2× 10 mL). The organic phases were combined, dried over $MgSO_4$, and evaporated in vacuo. The crude solid was recrystallized from EtOAc to afford **3** (29 mg, 87%) as a white solid. Mp > 210 °C (EtOAc); IR (KBr) *ν* 3400–3100, 2994, 1607, 1586, 1407, 1279 cm^{-1}; ^1H NMR ($CD_3OD + D_2O$) *δ* 4.05 (s, 3 H, CH_3), 4.76 (s, 2H, CH_2), 7.08 (s, 1H, H_5), 7.20 (dd, 1H, $J = 4.7$, 7.7 Hz, H_3), 7.68 (s, 1H, H_7), 8.35 (dd, 1H, $J = 1.5$, 5.1 Hz, H_2), 8.43 (dd, 1H, $J = 1.5$, 7.7 Hz, H_4); ^{13}C NMR (CD_3OD) *δ* 56.1 (CH_3), 66.0 (CH_2), 108.1 (CH), 112.8 (CH), 116.1 (CH), 118.2 (C), 122.7 (C), 129.9 (C), 130.1 (CH), 135.4 (C), 146.2 (CH), 147.4 (C), 152.8 (C); MS (ESI) *m/z* 229 $(M+H)^+$; Anal. Calcd for $C_{13}H_{12}N_2O_2$: C, 68.41; H, 5.30; N, 12.27. Found: C, 68.66; H, 5.15; N, 12.13.

4.8. Cell proliferation assay

In vitro antiproliferative activity was determined in five separate experiments, each of which was performed in triplicate as previously described.[11] Briefly, asynchronously growing cells were transferred into 96-well culture plates (Costar®, Corning Inc., New York) in 100 μL of medium at a final cell concentration of 5×10^3 cells/well and incubated in media for 24 h. Corresponding drug concentrations were then added to each plate. After 72 h of drug exposure, 20 μL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reagent (5 mg/mL) was added to each well. Cell growth was expressed as the percent of absorbance of treated wells relative to the untreated control wells. IC_{50} values were defined as drug doses resulting in 50% cell growth inhibition relative to untreated cells.

4.9. Cell cycle assay

For analysis of DNA content and cell cycle distribution, colorectal cancer cell lines were treated with compounds **1**, **2**, and **3** for 72 h. Based on cytotoxicity assay, a concentration of 100 μM (aprox IC_{80} values for these cell lines) was chosen for drug exposure experiments. After drug exposure, 10^6 cells/mL were resuspended in 2 mL of propidium iodide solution (50 μL/mL), incubated at 4 °C overnight, and then analyzed by flow cytometry. Flow cytometry was performed on a FACScalibur (Becton–Dickinson, San Jose, California). Cell cycle distribution and DNA ploidy status were calculated after exclusion of cell doublets and aggregates on a FL2-area/FL2-width dot plot using Modfit LT 2.0™ software (Verity Software Inc. Topsham. ME).

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