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Synthesis and cytotoxic activity of pyrazino[1,2-*b*]-isoquinolines, 1-(3-isoquinolyl)isoquinolines, and 6,15-iminoisoquino-[3,2-*b*]-3-benzazocines

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Abstract—A series of pyrazino[2,1-*b*]isoquinoline and 6,15-iminoisoquino[3,2-*b*]-3-benzazocine compounds related to renieramycins, cribrostatin 4, and phthalascidin was synthesized and their in vitro cytotoxic activities were evaluated against three human cancer cell lines. Pyrazino[2,1-*b*]isoquinolines, 6,15-iminoisoquino[3,2-*b*]-3-benzazocines, and other more complex octacyclic compounds have been obtained and derived to precursors of iminium ion species. Hydrogenolysis of the lactam function in pentacyclic compounds gave 1-(3-isoquinolyl)isoquinolines. The micromolar cytotoxic activity of representative structures was apparently uninfluenced by the ability to generate intermediates which would permit covalent bonding to DNA.

1. Introduction

Many tetrahydroisoquinoline antitumor antibiotics in which an isoquinoline ring is fused to an iminobenzazocine moiety are widely studied cytotoxic agents.[1] Development of saframycin A (**1**), jorumycin (**2**), and ecteinascidin 743 (E-743, **3**), which is currently in phase II/III clinical trials,[2] has been limited by their natural scarcity and the complexity of their synthesis or semisynthesis.[3–8] A preliminary structure–activity relationship (SAR) study of E-743 simplified analogues showed that most of the biological activity of the natural compound is maintained in phthalascidin (Pt 650, **4**).[9,10] However, structure–activity correlations within these compounds are relatively unexplored,[11–14] and most of the synthetic work carried out so far had focused on total syntheses. In the four compounds above mentioned, the C-ring harbors hydroxyl or cyano leaving groups on position C-21[15] that permit the formation of potent, electrophilic iminium ion species, which has been implicated in the formation of covalent bonds to DNA guanine residues explaining their high cytotoxicity[16] (Fig. 1).

On the other hand, cribrostatin 4 (**5**), which bears a double bond at C-3, and renieramycin G (**6**) are examples of natural amide-containing tetrahydroisoquiniline

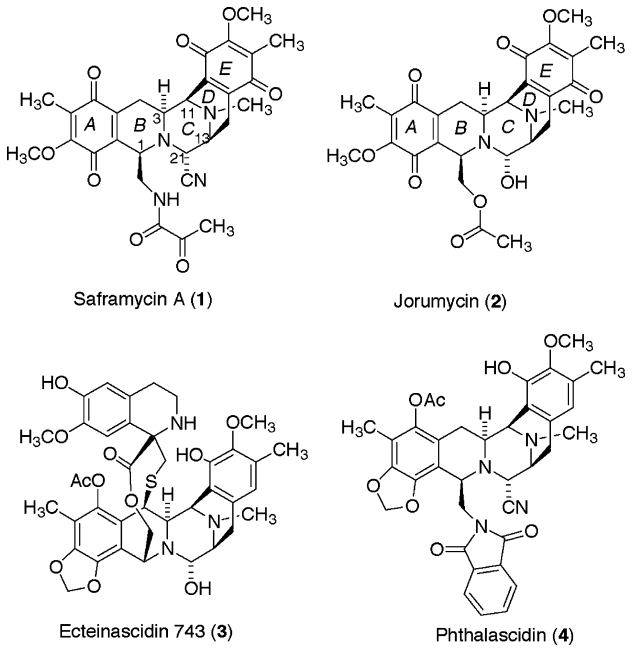

Figure 1.

Keywords: Antitumor agents; Tetrahydroisoquinolines; Cribrostatin 4; Phthalascidin; Acyliminium cations.
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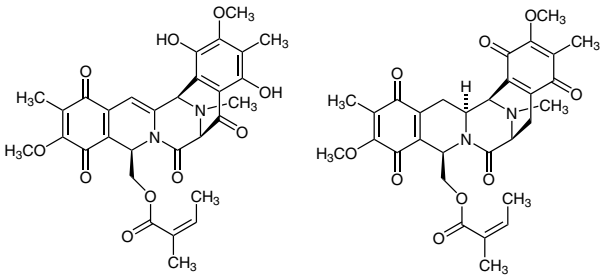


Figure 2.

alkaloids in which the apparent absence of a viable iminium ion precursor subverts the capacity to alkylate DNA. However, both compounds showed significant cancer cell line inhibitory activities, with GI_{50} averaged values of 5.01 μM for cribrostratin 4,[17] and 8.38 μM for renieramycin G.[18] Recent SAR studies have confirmed the importance of the acyloxymethyl substituent at C-1 in simpler analogues of **1**[19] and have shown the relevance of the stereochemical configuration at C-3 in analogues of **6**[18] (Fig. 2).

We have developed a synthetic approach to fully saturated or 11,11a-dehydro derivatives of 6-substituted pyrazino[2,1-*b*]isoquinolines such as **7** through a one-pot alkykation/cyclization process between 1-acetyl-3-arylmethylpiperazine-2,5-diones and acetals.[20] Catalytic hydrogenation of the exocyclic double bond in products obtained by a subsequent aldol-type condensation with aromatic aldehydes gave 3-arylmethyl derivatives (i.e., **9** from **8**,[21] Fig. 3). These compounds cyclize to iminobenzazocines through chemoselective reduction of the activated C(1)-carbonyl group in *N*-isopropyloxycarbonyl derivatives followed by acid treatment. Additionally, the presence in the tricyclic system of a nucleophilic function in the side chain at C-6 and a C-11 double bond permits formation of new octacyclic compounds close to E-743 structure.[21]

The aim of the present work was to synthesize *N*-methyl-21-cyano derivatives of the polycyclic systems and study the in vitro antitumor activity of representative compounds.

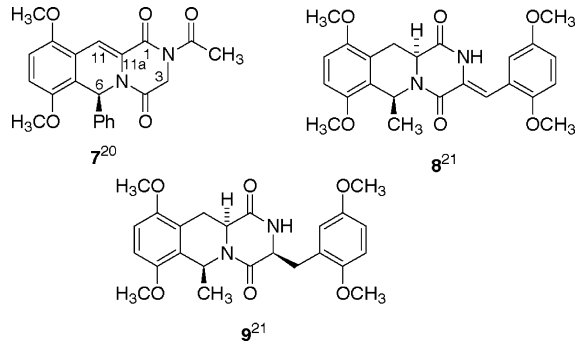


Figure 3.

2. Results and discussion

2.1. Chemistry

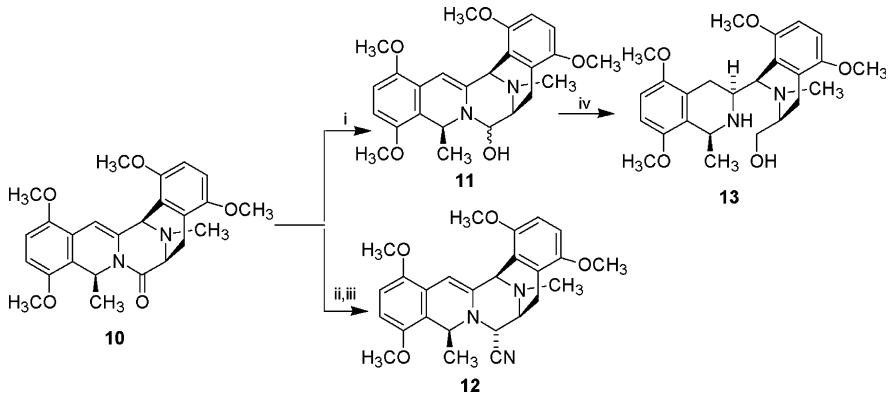
Hydrolysis of the carbamate function in the pentacyclic compounds[21] was followed by reductive methylation of the free imino group with $HCHO/HCO_2H$, which afforded the corresponding *N*-methyl derivatives **10**, **16**, and **20**. These compounds were transformed into acyliminium precursors by reduction with $LiAlH_2(OEt)_2$ or by one-pot reduction/cyanation. Reduction of compounds **10** gave a 75:25 mixture of *cis*- and *trans*-diastereomers **11**, while the reductive cyanation was diastereoselective giving the *trans*-cyano derivative **12**. Catalytic hydrogenation of compound **11** under pressure gave the 1-(3-isoquinolyl)isoquinoline **13**, which is probably formed by reduction of the double bond and the formyl group in the tautomer of hemiaminal **11**. The stereochemical assignments were mainly based on NOESY correlations (Scheme 1).

Carbamate **14** which contains the same phthalimidomethyl substituent that compound **4** was hydrolyzed to **15**,[21] and was similarly *N*-methylated to give compound **16**. As it was expected, one of the phthalimide carbonyl groups of this compound could be selectively reduced to give **17**, while excess of reductive reagent and subsequent cyanation gave compound **18** (Scheme 2).

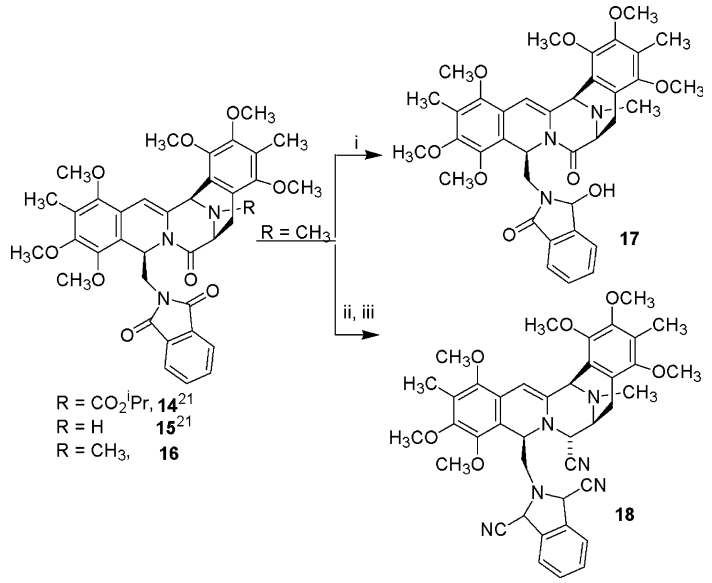
Finally, the previously described octacyclic compound **19**[21] was similarly hydrolyzed and *N*-methylated to give **20** which was reductively cyanated to **21** (Scheme 3).

2.2. Antiproliferative activity

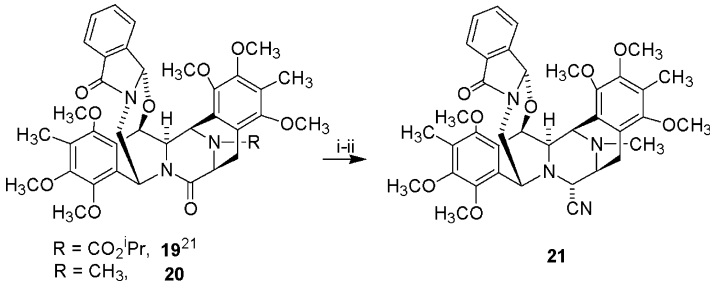
The antiproliferative activity of the studied compounds was evaluated using a panel of three human cell lines. The results are shown in Table 1. Data for E-743 are included for comparison. The GI_{50} values (the drug concentration inhibiting the growth value of cell lines by 50%)[22] show that the cytotoxicity of tricyclic compounds **7** and **8** is about 10-fold higher in the pentacyclic compound **10**, which is selective for the human lung carcinoma cell line A-549. The found activity for the acyliminium precursors derived from **10** was surprisingly lower in compound **11** which was moderately active for the human colon carcinoma cell line HT-29 while cytotoxicity was lost in compound **12**. The activity of the 1-(3-isoquinolyl)isoquinoline **13** was almost identical to that of its parent compound **11** in the same colon cell line being also active for the lung cell line. These data show that the pentacyclic core appears to be not relevant for activity. Compound **16** was 10-fold less active than its C-21 methyl analogue **10** for the same colon cell line showing a moderate activity for the lung cell line. These results contrast with the improving effect of the phthalimidomethyl group in **4**.[10] Cytotoxicity of compounds **17** and **18** was similar to that of their parent compound **16** in spite of the fact that **18** may act as an acyliminium precursor. Finally, in the case of octacyclic derivatives, compound **19** was about 6-fold more active than its pentacyclic analogue **14** and showed the same selectivity for lung and colon cell lines. In this type of compounds the



Scheme 1. Reagents and conditions: (i) $LiAlH_2(OEt)_2$ (4 equiv), rt, 30 min; (ii) $LiAlH(OEt)_2$ (4 equiv), 0 °C, 35 min; (iii) KCN, H_2O, AcOH, rt, 15 h; (iv) H_2/Pd–C, MeOH, 40 °C, 5 atm.



Scheme 2. Reagents and conditions: (i) $LiAlH_2(OEt)_2$ (1 equiv), 0 °C, 1 h; (ii) $LiAlH_2(OEt)_2$ (4 equiv), rt, 45 min; (iii) KCN, H_2O, AcOH, rt, 15 h.



Scheme 3. Reagents and conditions: (i) $LiAlH_2(OEt)_2$ (4 equiv), rt, 45 min; (ii) KCN, H_2O, AcOH, rt, 15 h.

presence of cyanoimine groups in **21** is apparently responsible for the observed 10-fold higher activity.

3. Conclusion

In conclusion, some of the synthesized compounds revealed in vitro cytotoxic activities against some human cancer cell lines with micromolar growth inhibition profiles similar to those found in cribrostratin 4 or renieramycin G. The higher activity observed in the amide compound **10** is relevant taking into account the absence of acyloxymethyl or acylamido methyl side chains at C-1, which are present in the natural products improving their cytotoxicity. The cytotoxic profile of **10** also contrasts with the loss of activity found in derivatives **11** and **12** bearing a leaving group at C-21. Finally, this study constitutes the first cytotoxic evaluation of the

Table 1. In vitro cytotoxicity (GI_{50}, μM) of tetrahydroisoquinoline derivatives against human cancer cell lines

Compound	In vitro cytotoxicity		
	GI_{50} (μM) MDA-MB 231	GI_{50} (μM) A-549	GI_{50} (μM) HT-29
E-743	0.019	0.046	0.022
7	>100	15.80	15.30
8	12.50	10.00	8.89
9	>100	>100	>100
10	>100	1.42	>100
11	>100	>100	21.10
12	>100	>100	>100
13	>100	21.20	18.80
14	>100	75.50	64.40
16	>100	13.10	29.90
17	13.20	12.50	14.00
18	12.70	12.80	11.80
19	>100	11.60	11.70
21	7.03	6.15	5.86

MDA-MB 231, human breast carcinoma; A-549, human lung carcinoma; HT-29, human colon carcinoma.

recently obtained octacyclic compound **21**. Further studies on this class of antitumor compounds are in progress in our laboratories.

4. Experimental

The reagents used were of commercial origin (Aldrich, Fluka) and were employed without further purification. Solvents (SDS, Scharlau) were purified and dried by standard procedures. Reactions were monitored by thin-layer chromatography, using Macherey-Nagel or Merck plates with fluorescent indicator. Separations by flash liquid chromatography were performed using silica gel from SDS 60 ACC (230–400 mesh) or Merck (60, 40–63 μm).

Melting points are uncorrected and were determined using a Hoffler hot stage microscope. Spectroscopic data were obtained with the following instruments: IR, Perkin-Elmer Paragon 1000 FT-IR; NMR, Bruker AC-250 (250 MHz for ^{1}H and 63 MHz for ^{13}C), Varian Unity 300 (300 MHz for ^{1}H and 75 MHz for ^{13}C) or Varian Unity Inova 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C). Combustion elemental analyses were obtained by the Servicio de Microanálisis Elemental, Universidad Complutense, using Perkin-Elmer 2400 CHN and a Leco CHNS 932 microanalyzer.

4.1. *N*-methylation: general procedure

Formaldehyde (37%) solution in methanol (0.8 mL) was added to a stirred solution of the corresponding *N*-unsubstituted derivative[21] (0.30 mmol) in formic acid (1 mL). The mixture was heated for 1 h at 70 °C, then poured into water (200 mL) and extracted with DCM (3× 100 mL). The combined extracts were washed with 10% $NaHCO_3$, H_2O, and brine, dried over anhydrous Na_2SO_4, filtered, and concentrated.

4.1.1. (6*S,9*S**,15*R**)-1,4,10,13-Tetramethoxy-9,16-dimethyl-5,6,9,15-tetrahydro-6,15-iminoisoquino[3,2-*b*]-3-benzazocin-7-one (10).** The crude product was purified by recrystallization in ethyl acetate/hexane (1:8) to give **10** (114 mg, 87% yield). Off-white solid. Mp 201–202 °C. IR (NaCl) ν_{max} 2942, 2832, 1668, 1637, 1598, 1486 cm^{-1}. ^{1}H NMR ($CDCl_3$, 250 MHz) δ ppm 6.68 (d, 1H, J = 8.0 Hz), 6.67 (s, 2H), 6.62 (d, 1H, J = 8.0 Hz), 6.35 (s, 1H), 5.97 (t, 1H, J = 6.5 Hz), 3.84 (s, 3H), 3.81 (s, 3H), 3.76 (s, 6H), 3.10 (m, 2H), 2.54 (s, 3H), 0.75 (d, 3H, J = 6.5 Hz). ^{13}C NMR ($CDCl_3$, 63 MHz) δ ppm 167.0, 151.1, 150.3, 148.8, 148.1, 132.2, 124.1, 123.2, 122.3, 119.7, 109.2, 109.9, 108.4, 108.2, 101.9, 50.1, 55.9, 55.5, 43.2, 41.5, 28.9, 17.8. Anal. Calcd for $C_{25}H_{28}N_2O_5$: C, 68.79; H, 6.47; N, 6.42. Found: C, 68.56; H, 6.12; N, 6.08.

4.1.2. (6*S,9*R**,15*R**)-1,2,4,10,11,13-Hexamethoxy-3,12,16-trimethyl-9-phthalimidomethyl-5,6,9,15-tetrahydro-6,15-iminoisoquino[3,2-*b*]-3-benzazocin-7-one (16).** The crude product was purified by flash chromatography on silica gel with hexane/ethyl acetate (2:8) as eluant to give an off-white solid (92% yield). Mp 97–98 °C. IR (NaCl) ν_{max} 2938, 1776, 1722, 1675, 1642, 1468 cm^{-1}. ^{1}H NMR ($CDCl_3$, 250 MHz) δ ppm 7.61 (m, 4H), 6.32 (s, 1H), 6.32 (t, 1H, J = 8.2 Hz), 4.69 (s, 1H), 3.91 (s, 3H), 3.79 (s, 3H), 3.77 (s, 3H), 3.75 (s, 3H), 3.65 (s, 3H), 3.65 (m, 1H), 3.30 (d, 1H, J = 7.5 Hz), 3.28 (s, 3H), 3.14 (dd, 1H, J = 17.0 and 6.2 Hz), 3.03 (dd, 1H, J = 17.0 and 1.8 Hz), 2.50 (s, 3H), 2.23 (s, 3H), 2.14 (s, 3H). ^{13}C NMR ($CDCl_3$, 63 MHz) δ ppm 167.7, 167.2, 152.4, 150.4, 150.0, 146.6, 146.3, 145.9, 133.3, 132.7, 132.0, 126.0, 125.4, 124.6, 122.8, 121.2, 120.3, 119.5, 102.7, 61.4, 60.4, 60.2, 60.0, 59.8, 59.5, 56.4, 44.9, 41.5, 37.8, 28.9, 9.4, 9.2. Anal. Calcd for $C_{37}H_{39}N_3O_9$: C, 66.36; H, 5.87; N, 6.27. Found: C, 66.21; H, 5.65; N, 6.12.

4.1.3. (6*S,9*R**,14*R**,15*R**)-14,1′-Epoxy-3,12,16-trimethyl-1,2,4,10,11,13-hexamethoxy-9-(3′-oxo-1′,3′-dihydro-2′-isoindolylmethyl)-5,6,9,14,14a,15-hexahydro-6,15-iminoisoquino[3,2-*b*]-3-benzazocin-7-one (20).** The crude product was purified by flash chromatography on silica gel with ethyl acetate as eluant to give an off-white solid (93% yield). Mp >300 °C. IR (NaCl) ν_{max} 2938, 2224, 1693, 1644, 1470 cm^{-1}. ^{1}H NMR ($CDCl_3$, 250 MHz) δ ppm 8.10 (d, 1H, J = 7.4 Hz), 7.56 (m, 2H), 7.31 (d, 1H, J = 7.3 Hz), 6.22 (d, 1H, J = 3.6 Hz), 4.41 (d, 1H, J = 1.0 Hz), 3.83 (s, 3H), 3.76 (s, 3H), 3.74 (m, 1H), 3.70 (s, 3H), 3.54 (s, 3H), 3.17 (dd, 1H, J = 17.1 and 6.9 Hz), 3.14 (d, 1H, J = 6.9 Hz), 3.04 (d, 1H, J = 17.1 Hz), 2.59 (s, 3H), 2.15 (s, 3H), 1.98 (s, 3H). ^{13}C NMR ($CDCl_3$, 63 MHz) δ ppm 169.9, 168.0, 153.3, 151.2, 150.3, 150.1, 147.1, 145.6, 144.6, 132.1, 131.3, 128.2, 126.1, 125.8, 124.9, 124.8, 124.0, 123.5, 123.0, 122.7, 61.5, 61.3, 60.9, 60.8, 60.7, 60.2, 60.0, 58.6, 58.2, 54.8, 46.6, 41.5, 39.7, 24.0, 10.1, 9.6. Anal. Calcd for $C_{37}H_{41}N_3O_9$: C, 66.16; H, 6.15; N, 6.26. Found: C, 65.96; H, 5.95; N, 6.10.

4.1.4. (6*S,9*S**,15*R**)-1,4,10,13-Tetramethoxy-9,16-dimethyl-6,7,9,15-tetrahydro-5*H*-6,15-iminoisoquino[3,2-*b*]-3-benzazocin-7-ol (11).** $LiAlH_2(OEt)_2$ was prepared

by the addition of dry ethyl acetate (0.45 mL, 4.6 mmol) to a 1.0 M solution of $LiAlH_4$ (4.6 mL, 4.6 mmol) in dry THF at 0 °C and stirring at 0 °C for 2 h. Then it was added dropwise to a solution of **10** (1.15 mmol) in 14 mL of dry THF and was stirred for 35 min at room temperature. The reaction mixture was quenched by addition of 20 mg of ice and extracted with ethyl acetate (3× 100 mL). The extracts were washed with H_2O and brine, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to give 450 mg of crude product that was purified by recrystallization in MeOH to give **11** (420 mg) as a 75/25 diastereoisomeric mixture. Yield 84%. IR (NaCl) ν_{max} 2938, 2835, 1668, 1600, 1485, 1259 cm^{-1}. ^1H NMR ($CDCl_3$, 250 MHz) δ ppm 6.90–6.30 (m, 5H), 6.62 (s, 2H), 6.58 (d, 1H, J = 8.8 Hz), 6.49 (d, 1H, J = 8.8 Hz), 6.02 (s, 1H), 5.05 (q, 1H, J = 6.6 Hz), 4.72 (s, 1H), 4.68 (q, 1H, J = 6.6 Hz), 4.63 (s, 1H), 4.47 (wide singlet, 1H), 4.34 (wide singlet, 1H), 3.88 (m,1H), 3.84 (s, 3H), 3.82 (s, 3H), 3.80 (s, 3H), 3.79 (s, 3H), 3.76 (s, 3H), 3.73 (s, 3H), 3.72 (s, 3H), 3.71 (s, 3H), 3.66 (m, 1H), 3.37 (m, 1H), 3.31 (d, 1H, J = 8.2 Hz), 3.21 (m, 1H), 2.98 (dd, 1H, J = 18.8 and 8.3 Hz), 2.56 (d, 1H, J = 18.8 Hz), 2.47 (d, 1H, J = 18.8 Hz), 2.34 (s, 3H), 2.18 (s, 3H), 1.59 (d, 3H, J = 6.6 Hz), 0.70 (d, 3H, J = 6.6 Hz). ^{13}C NMR ($CDCl_3$, 63 MHz) δ ppm 151.0, 150.2, 148.6, 147.5, 124.0, 122.3, 121.5, 108.5, 107.6, 106.8, 86.7, 58.9, 55.1, 50.5, 55.9, 55.8, 55.7, 55.4, 41.4, 20.5, 14.2. Anal. Calcd for $C_{25}H_{30}N_2O_5$: C, 68.47; H, 6.90; N, 6.39. Found: C, 68.15; H, 6.52; N, 6.48.

4.1.5. (6*S,7*R**,9*S**,15*R**)-1,4,10,13-Tetramethoxy-9,16-dimethyl-6,7,9,15-tetrahydro-5*H*-6,15-iminoisoquino[3,2-*b*]-3-benzazocin-7-carbonitrile (12).** A solution of **10** (0.14 mmol) in dry THF (3 mL) was added to a solution of $LiAlH_2(OEt)_2$ (0.56 mmol) and the mixture was stirred for 35 min at 0 °C. Then AcOH (3.05 mmol) and a 4.8 M solution of KCN in H_2O (0.84 mmol) were added. After stirring at room temperature for 14 h, the reaction mixture was quenched with aqueous 10% $NaHCO_3$ (12 mL) and extracted with ethyl acetate (3× 10 mL). The combined organic layers were washed with H_2O and brine, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to give 70 mg of a crude product, which was purified by flash chromatography on silica gel column with hexane/ethyl acetate (4:6) as eluant to give **12** (50 mg, 80% yield) as a white solid. Mp 252–253 °C. IR (NaCl): 2936, 2834, 1484, 1257 cm^{-1}. ^1H NMR ($CDCl_3$, 250 MHz) δ ppm 6.57 (s, 2H), 6.54 (d, 1H J = 8.8 Hz), 6.47 (d, 1H, J = 8.8 Hz), 6.13 (s, 1H), 4.60 (s, 1H), 4.41 (q, 1H, J = 6.5 Hz), 3.79 (d, 1H, J = 1.6 Hz), 3.73 (s, 3H), 3.72 (s, 3H), 3.66 (s, 3H), 3.46 (d, 1H, J = 7.8 Hz), 3.04 (dd, 1H, J = 18.6 and 7.8 Hz), 2.51 (d, 1H, J = 18.6 Hz), 2.48 (s, 3H), 0.63 (d, 3H J = 6.5 Hz). ^{13}C NMR ($CDCl_3$, 63 MHz) δ ppm 150.8, 150.0, 148.6, 147.8, 136.5, 124.3. 121.7, 121.4, 120.5, 119.3, 109.1, 107.8, 107.7, 107.7, 97.5, 56.9, 56.1, 56.0, 55.9, 55.6, 55.5, 55.0, 51.9, 41.7, 23.2, 19.4. Anal. Calcd for $C_{26}H_{29}N_3O_4$: C, 69.78; H, 6.53; N, 9.39. Found: C, 69.52; H, 6.22; N, 9.34.

4.1.6. (1*R,3*S**,1′*S**,3′*S**)-2-Methyl-1(1′-methyl-5′8′-dimethoxy-1′,2′,3′,4′-tetrahydro-3′-isoquinolyl)-5,8-dimethoxy-1,2,3,4-tetrahydro-3-isoquinolylmethanol (13).** Compound **11** (140 mg, 0.32 mmol) was dissolved in methanol (50 mL) containing 10% palladium on-carbon (50 mg) and was vigorously stirred under 5 bar of hydrogen and heated at 40 °C for 15 h. The catalyst was removed by filtration through Celite, and the solvent was evaporated to give compound **13** (120 mg) that was purified by recrystallization in MeOH, yield 85%. Off-white solid. Mp 160–162 °C. ^1H NMR ($CDCl_3$, 250 MHz) δ ppm 6.67 (s, 2H), 6.69 (s, 2H), 4.22 (s, 1H), 3.86 (s, 3H), 3.83 (s, 3H), 3.77 (s, 3H), 3.76 (s, 3H), 3.57 (q, 1H, J = 6.2 Hz), 3.19 (d, 1H, J = 7.0 Hz), 3.15 (dd, 1H, J = 7.0 and 2.3 Hz), 3.07 (dd, 1H, J = 10.6 and 2.5 Hz), 2.83 (m, 2H), 2.94 (dd, 1H, J = 18.2 and 7.9 Hz), 2.51 (d, 1H, J = 18.2 Hz), 2.31 (s, 3H), 1.81 (dd, 1H, J = 16.1 and 11.5 Hz). ^{13}C NMR ($CDCl_3$, 63 MHz) δ ppm 151.9, 150.3, 150.1, 150.0, 129.5, 126.5, 126.3, 123.3, 107.8, 107.7, 107.5, 107.2, 60.7, 59.5, 56.7, 53.0, 41.5, 26.3, 22.2, 18.4. Anal. Calcd for $C_{25}H_{34}N_2O_5$: C, 67.85; H, 7.74; N, 6.33. Found: C, 67.43; H, 7.38; N, 6.01.

4.1.7. (6*S,9*R**,15*R**)-9-(1-Hydroxy-3-oxo-1,3-dihydro-2-isoindolylmethyl)-1,2,4,10,11,13-hexamethoxy-3,12,16-trimethyl-6,7,9,15-tetrahydro-5*H*-6,15-iminoisoquino[3,2-*b*]-3-benzazocin-7-one (17).** $LiAlH_2(OEt)_2$ (0.08 mmol), prepared as above, was added dropwise to a solution of **16** (0.08 mmol) in 2 mL of dry THF and was stirred 1 h at 0 °C. The reaction mixture was quenched by addition of 200 mg of ice and extracted with ethyl acetate (3× 10 mL). The extracts were washed with H_2O and brine, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo to give 50 mg of crude compound, which was purified by flash chromatography on a silica gel column with hexane/ethyl acetate (2:8) to give unreacted compound **16** (56%) and **17** (43% yield). IR (NaCl, film) ν_{max} 2936, 1684, 1636, 1466, 1413 cm^{-1}. ^1H NMR (250 MHz, $CDCl_3$) δ ppm 7.60–7.30 (m, 4H), 6.42 (s, 1H), 6.34 (dd, 1H, J = 8.1 and 5.2 Hz), 5.68 (d, 1H, J = 9.5 Hz), 4.72 (s, 1H), 4.58 (d, 1H, J = 9.5 Hz), 4.02 (s, 3H), 3.85 (s, 3H), 3.84 (s, 3H), 3.81 (s, 3H), 3.63 (m, 1H), 3.62 (s, 3H), 3.51 (s, 3H), 3.43 (dd, 1H, J = 14.0 and 8.1 Hz), 3.33 (dd, 1H, J = 14.0 and 5.2 Hz), 3.10 (dd, 1H, J = 17.0 and 6.6 Hz), 2.88 (dd, 1H, J = 17.0 and 1.3 Hz), 2.49 (s, 3H), 2.29 (s, 3H), 2.19 (s, 3H). ^{13}C NMR (63 MHz, $CDCl_3$) δ ppm 169.3, 166.2, 152.3, 150.7, 150.3, 146.8, 145.7, 144.3, 132.3, 131.9, 130.9, 129.1, 125.8, 125.5, 124.8, 123.1, 123.0, 121.2, 120.8, 119.0, 103.7, 82.9, 61.5, 60.7, 60.4, 60.2, 60.0, 59.9, 59.6, 56.6, 46.8, 42.6, 41.4, 28.9, 9.5, 9.2. Anal. Calcd for $C_{37}H_{41}N_3O_9$: C, 66.16; H, 6.15; N, 6.26. Found: C, 66.23; H, 6.03; N, 6.12.

4.2. Reduction and cyanation: general procedure to obtain compounds 18 and 21

A solution of *N*-methyl derivatives (**16** or **20**) (0.08 mmol), in dry THF (2 mL) was added dropwise to a solution of $LiAlH_2(OEt)_2$ (0.56 mmol) and the mixture was stirred for 45 min at room temperature. Then AcOH

(6 μL, 0.11 mmol) and a 4.8 M solution of KCN in H_2O (0.54 mL, 2.6 mmol) were added. After stirring at room temperature for 15 h, the reaction mixture was quenched with aqueous 10% $NaHCO_3$ (9 mL) and extracted with ethyl acetate (3× 10 mL). The combined organic layers were washed with H_2O and brine, dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo.

4.2.1. (6*S,7*R**,9*R**,15*R**)-9-(1,3-Dicyano–1,3-dihydro-2-isoindolylmethyl)-1,2,4,10,11,13-hexamethoxy-3,12,16-trimethyl-6,7,9,15-tetrahydro-5*H*-6,15-iminoisoquino[3,2-*b*]-3-benzazocin-7-carbonitrile (18).** The crude product (52 mg) was purified by flash chromatography on silica gel column with hexane/ethyl acetate (4:6) as eluant to give **18** (62% yield). Off-white solid. Mp 115–116 °C IR (NaCl, film) ν_{max} 2938, 1467, 1408, 1253, 1072 cm^{-1}. ^1H NMR (250 MHz, $CDCl_3$) δ ppm 7.20–6.90 (m, 4H), 6.06 (s, 1H), 4.62 (d, 1H, J = 9.4 Hz), 4.55 (s, 1H,), 4.23 (s, 1H), 3.82 (d, 3H, J = 1.5 Hz), 3.80 (s, 3H), 3.73 (3H, s), 3.70 (d, 3H, J = 1.0 Hz), 3.68 (s, 3H), 3.46 (m, 3H), 3.39 (d, 3H, J = 1.7 Hz), 3.06 (dd, 1H, J = 18.2 and 6.7 Hz), 2.68 (dd, 1H, J = 18.2 and 10.0 Hz), 2.58 (dd, 1H, J = 13.2 and 10.2 Hz), 2.45 (3H, s), 2.26 (m, 1H), 2.13 (s, 3H), 2.09 (s, 3H). ^{13}C NMR (63 MHz, $CDCl_3$) δ ppm 151.4, 149.7, 149.6, 148.5, 146.7, 144.8, 139.5, 136.8, 126.8, 124.5, 123.7, 122.2, 121.6, 121.1, 119.9, 98.0, 61.0, 60.7, 60.5, 60.1, 60.0, 59.6, 59.4, 57.0, 56.3, 56.2, 55.8, 41.5, 24.3, 9.3, 9.2. Anal. Calcd for $C_{40}H_{42}N_6O_6$: C, 68.36; H, 6.02; N, 11.96. Found: C, 68.21; H, 5.87; N, 11.54.

4.2.2. (6*S,7*R**,9*R**,14*R**,15*R**)-14,1′-Epoxy-3,12,16-trimethyl-1, 2,4,10,11,13-hexamethoxy-9-(3′-oxo-1′,3′-dihydro-2′isoindolylmethyl)-6,7,9,14,14a,15-hexahydro-5*H*-6,15-iminoisoquino [3,2-*b*]-3-benzazocin-7-carbonitrile (21).** The crude product was purified by flash chromatography on silica gel column with ethyl acetate/hexane (9:1) as eluant to give **21** (65% yield). Off-white solid. Mp 143–145 °C. IR (NaCl, film) ν_{max} 2929, 1691, 1469, 1409 cm^{-1}. ^1H NMR (250 MHz, $CDCl_3$) δ ppm 8.02 (d, 1H, J = 8.0 Hz), 7.55 (t, 1H, J = 8.0 Hz), 7.54 (d, 1H, J = 7.5 Hz), 7.30 (dd, 1H, J = 8.0 and 7.5 Hz), 4.80 (s, 1H), 4.44 (m, 1H), 4.20 (m, 2H), 4.08 (d, 1H, J = 2.2 Hz), 4.02 (s, 3H), 3.89 (s, 3H), 3.76 (s, 3H), 3.73 (s, 3H), 3.47 (s, 3H), 3.35 (m, 1H), 3.21 (m, 1H), 3.12 (m, 2H), 3.02 (m, 1H), 2.78 (s, 3H), 2.55 (d, 1H, J = 15.8 Hz), 2.25 (s, 3H), 2.15 (s, 3H), 1.99 (s, 3H). ^{13}C NMR (63 MHz, $CDCl_3$) δ ppm 167.2, 152.1, 150.9, 149.8, 149.7, 147.2, 145.4, 145.3, 132.0, 131.2, 128.0, 126.0, 124.9, 124.7, 124.5, 124.4, 123.9, 123.7, 122.8, 117.5, 62.7, 62.2, 60.9, 60.8, 60.7, 60.5, 59.9, 58.2, 56.1, 55.8, 55.3, 53.4, 49.4, 42.0, 41.5, 22.3, 9.9, 9.4. Anal. Calcd for $C_{38}H_{42}N_4O_8$: C, 66.85; H, 6.20; N, 8.21. Found: C, 66.54; H, 6.01; N, 8.13.

4.3. Cell culture

Human-derived established cell lines used in this study were purchased from ATCC (American Type Culture Collection). A-549, human lung carcinoma—ATCC # CCL-185; HT-29, human colorectal adenocarcinoma—ATCC # HTB-38; and MDA-MB 231, human breast adenocarcinoma—ATCC # HTB-26. All cell lines were maintained in DMEM culture medium supplemented with 10% FBS and 100 U/mL penicillin and streptomycin at 37 °C and 5% CO_2.

4.4. Cell proliferation assay

Cells were plated in 96-well microtiter plates at a density of 5×103/well and incubated for 24 h. After that, cells were treated with vehicle alone (control) or compounds at the concentrations indicated. One plate from each different cell line was fixed and stained, and used for Tz reference (see next paragraph). Treated cells were further incubated for 48 h. To quantify the cytotoxic potential of compounds the sulforhodamine B (SRB) protein stain method was used as follows: cells were washed twice with phosphate-buffered saline (PBS), fixed for 15 min in 1% glutaraldehyde solution, rinsed twice in PBS, and stained in 0.4% (SRB) solution for 30 min at room temperature. Cells were then rinsed several times in 1% acetic acid solution and air-dried. SRB was then extracted in 10 mM trizma base solution and the absorbance measured at 490 nm. Cell survival is expressed as percentage of control cell growth. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT; Sigma Chemical Co., St. Louis, MO) dye reduction assay in 96-well microplates was used. The assay is dependent on the reduction of MTT by mitochondrial dehydrogenases of viable cell to a blue formazan product, which come be measured spectrophotometrically. Tumor cells were incubated in each well with serial dilutions (5, 2.5, 1, 0.5, 0.1, 0.05, 0.01, and 0.005 μg/mL) of the tested compounds. After 2 days of incubation (37 °C, 5% CO_2 in a humid atmosphere) 50 μL of MTT (5 mg/mL in PBS) was added to each well and the plate was incubated for a further 2 h (37 °C). The resulting formazan was dissolved in 100 μL DMSO and read at 490 nm. All determinations were carried out in triplicate.

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