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Cytotoxic Indolocarbazoles from *Actinomadura melliaura* ATCC 39691

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Abstract

Actinomadura melliaura ATCC 39691, a strain isolated from a soil sample collected in Bristol Cove, California, is a known producer of the disaccharide-substituted AT2433 indolocarbazoles (**6**–**9**). Reinvestigation of this strain using new media conditions led to >40-fold improvement in the production of previously reported AT2433 metabolites and the isolation and structure elucidation of the four new analogues, AT2433-A3, A4, A5, and B3 (**1**–**4**). The availability of this broader set of compounds enabled a subsequent small antibacterial/fungal/cancer SAR study that revealed disaccharyl substitution, N-6 methylation, and C-11 chlorination as key modulators of bioactivity. The slightly improved anticancer potency of the newly reported *N*-6-desmethyl **1** (compared to **6**) contrasts extensive SAR of monoglycosylated rebeccamycin-type topoisomerase I inhibitors where N-6 alkylation has contributed to improved potency and ADME. Complete 2D NMR assignments for the known metabolite BMY-41219 (**5**) and ^{13}C NMR spectroscopic data for the known analogue AT2433-B1 (**7**) are also provided for the first time.

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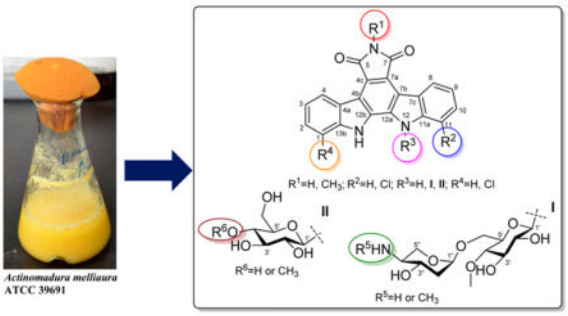
Notes

The authors declare the following competing financial interest(s): The authors report competing interests. J.S.T. is a co-founder of Centrose (Madison, WI, USA).

ASSOCIATED CONTENT

Supporting Information

UV, HPLC, HPLC-MS, HRESIMS, and NMR (1D and 2D) spectra of compounds **1**–**7** and harman. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00429.



Indolocarbazoles (exemplified by staurosporine, rebeccamycin, and AT2433; Figure 1) are actinomycete-derived alkaloids and potent inhibitors of topoisomerase I and kinases relevant to anticancer, antitubercular, antimalarial, and antiviral lead development.[1–4] Of the indolocarbazoles that have advanced to the clinic, midostaurin (PKC412) recently completed a successful phase II trial for AML (Acute Myelogenous Leukemia) and is currently in phase II evaluation for metastatic melanoma, lestauritinib (CEP-701) is currently in clinical phase II/III evaluation for treating FLT3-ITD AML, and CT327 (a PEGylated formulation of the natural product K252a) is currently in phase IIb for treating psoriasis.[5,6] While many indolocarbazole analogues have been synthesized,[2,7–13] it is noteworthy that those that have advanced furthest clinically are either a known natural product (CT327) or subtle variations thereof (including the *N*-alkylated becatecarin or *N*-acylated PKC412). The vast majority of the >90 microbial indolocarbazole natural products exist as glycosides.[14,15] Yet, of these, only four are disaccharide-substituted indolocarbazoles (AT2433 A1, B1, A2, and B2; Figure 1, 6–**9**, respectively).[16,17] While the fundamental mechanism of the disaccharide-substituted indolocarbazole cytotoxicity is poorly understood, the terminal amino sugar has been implicated in both their unique mode of DNA binding and putative topisomerase-independent mechanism of action.[18–20] As part of an effort to identify unique reagents to advance indolocarbazole biosynthetic studies,[3,8,21–36] herein we describe the isolation, structure elucidation, and biological activity of four new naturally occurring indolocarbazoles (AT2433-A3, A4, A5, and B3; **1**–**4**) along with three known counterparts, BMY-41219 (**5**), AT2433-A1 (**6**), and AT2433-B1 (**7**), from *Actinomadura melliaura* ATCC 39691. A comparison of cancer cell line cytotoxicities revealed the attached sugars as critical to bioactivity, where the disaccharide-substituted metabolites (**1**, **6**, and **7**) were found to be more potent than their monosaccharide-substituted congeners (**2**, **4**, and **5**). Chlorination of the indolocarbazole core was also found to be important to bioactivity, particularly in the context of antitubercular, antifungal, and Gram-positive antibacterial assays.

RESULTS AND DISCUSSION

Disaccharide-substituted **6** and **7** represent the major metabolites of *A. melliaura* ATCC 39691, which also produces other related minor metabolites including the aminopentose *N*-desmethyl analogues **8** and **9** (Figure 1).[16,17] In an effort to access these, and potentially additional indolocarbazole analogues as reagents for biosynthetic studies, the strain was fermented in A-medium and the extracts were analyzed by LC-ESIMS. Consistent with

previous studies, this preliminary analysis revealed **6** and **7** as the predominate products along with several additional metabolites that displayed indolopyrrolocarbazole UV and MS-fragmentation signatures. Subsequent purification of compounds from a large-scale fermentation extract using progressive chromatography led to the isolation of four new naturally occurring indolocarbazoles [AT2433-A3 (**1**; 4.3 mg), AT2433-A4 (**2**; 3.0 mg), AT2433-A5 (**3**; 1.7 mg), and AT2433-B3 (**4**; 3.3 mg)] along with the three known previously reported indolocarbazoles [BMY-41219 (**5**; 5.9 mg),[37] AT2433-A1 (**6**, 1.75 g),[16,17] and AT2433-B1 (**7**, 1.2 g)[16,17]] (Table 1, 5Figures 1–3 and S76). Of this latter set, the full NMR assignments for BMY-41219 (; Table S2, Figures S2 and S80) and ^{13}C NMR spectroscopic data for AT2433-B1 (**7**; Table S2) are reported here for the first time. It is also noteworthy that the production levels in A-medium reported herein of compounds AT2433-A1 (**6**) and AT2433-B1 (**7**) were substantially higher than from previously reported conditions (41-fold and 92-fold, respectively).[17] Additional compounds isolated included harman (also known as 1-methyl-*β*-carboline, 2.0 mg),[38–43] 5′-methylthioadenosine (2.0 mg),[44,45] riboflavin (4.3 mg), and lumichrome (1.7 mg)[44,46] (see Experimental Section and Supporting Information Figures S76–S78).

Structure Elucidation

The physicochemical properties of compounds **1**–**7** are summarized in the Experimental Section. Compound **1** was isolated as a yellow solid (4.3 mg) using progressive chromatography (Figure S76). The UV spectrum of **1** was similar to that of AT2433-A1 (**6**) with indolocarbazole UV absorption signatures at 396, 316, 290, and 236 nm (Figure S1). The molecular formula of **1** was deduced as $C_{33}H_{33}ClN_4O_9$ from HRESIMS and ^{1}H and ^{13}C NMR. Compared to **6**, the $\Delta m/z$ = 14 difference observed in **1** implicated the loss of a methyl group. The ^{1}H and ^{13}C NMR spectra of **1** (Table 1) and **6** (Table S2, Figure S81) in CD_3OD revealed both to share a common disaccharide-substituted indolopyrrolocarbazole core, where, compared to **6**, compound **1** lacked the *N*-6 methyl. Confirmation of the common N-12 4″-amino-4″-*N*-methyl-2″,4″-dideoxy-α-L-xylose-(1″→6′)-4′-*O*-methyl-*β*-D-*N*-glucosyl moiety was confirmed through the cumulative analyses of ^{1}H–^{1}H-COSY and HMBC spectroscopic data (Figure 2). Specifically, the ^3J HMBC cross-peaks observed from H-1″ to CH_2-6′ (δ 67.5) and from H_2-6′ to C-1 (δ 100.4) were consistent with the attachment of the 4″-amino-4″-*N*-methyl-2″,4″-dideoxy-α-L-xylose moiety at the 6′-position of indole 4′-*O*-methyl-*β*-D-*N*-glucoside. The corresponding chemical shift of the C-1′ anomeric carbon (δ_C 86.5) and the ^3J HMBC correlation observed from H-1′ to the quaternary carbons at δ 139.9 (C-11a) and δ 131.8 (C-12a) confirmed the *N*-12 glycosyl attachment. All of the remaining HMBC correlations (Figure 2) and NMR data (Table 1) were in full agreement with structure **1**. The relative configurations at the sterocenters of disaccharide sugar residue were indirectly established through the analyses of NOESY correlations (Figure 3), coupling constants, and comparison to those of **6** (Figure S81, Table S2). Thus, HRMS and cumulative ^{1}H, ^{13}C, ^{1}H–^{1}H-COSY, TOCSY (Supporting Information, Figure S2), HSQC, HMBC, and NOESY spectral data revealed compound **1** as a new analogue of the monochlorinated AT2433-A series, and **1** was thereby designated as AT2433-A3.

Compound **2** was obtained as a yellow solid (3.0 mg, Figure S76) and displayed common indolocarbazole UV–vis (Figure S1) and physicochemical properties. The molecular

formula of **2** was deduced as $C_{28}H_{24}ClN_3O_7$ based on the molecular ion peak observed at *m*/*z* 550.1381 in the HRESIMS spectrum, where the 129 amu difference from **6** implicated the absence of the terminal pentose. Consistent with this, no pentosyl proton or carbon signals in the $^1H/^{13}C$ NMR/HSQC spectra of **2** (Table 1) were found. Further COSY, TOCSY (Figure S2), HMBC, and NOESY correlations were in full agreement with compound **2** (Figures 2 and 3) as a new analogue of the monochlorinated AT2433-A series, and **2** was thereby designated as AT2433-A4. Importantly, **2** differs from the prototype dichlorinated monosaccharide-substituted rebeccamycins (Figure 1, 10) via the additional N-6 methyl and lack of the second C-1 chlorine.

Compound **3** was obtained as a yellow solid (1.7 mg, Figure S76) and also displayed common indolocarbazole UV–vis (Figure S1) and physicochemical properties. The molecular formula of **3** was confirmed as $C_{21}H_{12}ClN_3O_2$, where the 176 amu difference from **2** suggested the absence of the N-12 4′-*O*-methyl-*β*-D-*N*-glucosyl moiety. Consistent with this, the ^1H NMR spectrum of **3** in DMSO-d_6 (Table 1) displayed two broad indole-NH signals at δ 11.95 and 11.64. In addition, no corresponding glucosyl proton or carbon signals in the $^1H/^{13}C$ NMR/HSQC spectra of **3** (Table 1) were observed. Further COSY, TOCSY (Figure S2), HMBC, and NOESY correlations were in full agreement with compound **3** (Figures 2 and 3) as a new analogue of the monochlorinated AT2433-A series, and **3** was thereby designated as AT2433-A5.

Compound **4** was also obtained as a yellow solid (3.3 mg, Figure S76) and displayed common indolocarbazole UV–vis (Figure S1) and physicochemical properties. The molecular formula of **4** was confirmed as $C_{28}H_{25}N_3O_7$ on the basis of HRESIMS, where the 35 amu difference from **2** suggested the absence of the C-11 chlorine. The observed additional C-11 proton signal at δ 7.81 (d, J = 8.5 Hz), along with full 1D and 2D NMR (Table 1, 4Figures 2, 3, and S2), provided further support for this distinguishing feature. Thus, compound , as a new analogue of the deschlorinated AT2433-B series, was designated as AT2433-B3. It should be noted that while synthetic **4** was previously reported as a selective topoisomerase I inhibitor,[10,47–50] the discovery of **4** as a natural product and the corresponding full NMR assignments for **4** (Figures 2 and 3; Table 1) are reported here for the first time.

Including AT2433-A3 (**1**), -A4 (**2**), -A5 (**3**), and -B3 (**4**) reported herein, the indolopyrrolocarbazoles make up 74 of the 94 naturally occurring microbial indolocarbazoles, only five of which contain disaccharyl substitutions (the new **1** along with previously reported **6**–**9**).[14,15] Indolocarbazoles including staurosporines,[51–60] K-252 derivatives,[61–63] rebeccamycins,[64,65] RK-1409B,[66] RK-286 C and D,[67,68] tjipanazoles,[69] TAN-999S and TAN-1030A analogues,[54,70] fradcarbazoles,[71] indocarba-zostatins,[72–75] ZHD-0501,[76] fluoroindolocarbazoles,[77] holy-rines,[78] MLR-52,[79] and BE-13793C[80,81] have been reported to have promising antibacterial, antifungal, antitumor, and neuroprotective activities. Thus, compounds **1**–**7** were tested against five bacterial strains (*Staphylococcus aureus* ATCC 6538, *Micrococcus luteus* NRRL B-287, *Mycobacterium smegmatis* ATCC 14468, *Salmonella enterica* ATCC 10708, and *Escherichia coli* NRRL B-3708), one fungal strain (*Saccharomyces cerevisiae* ATCC 204508), and three human cancer cell lines (PC-3,

prostate; A549, lung; and U118, brain). As highlighted in Table 2, the disaccharyl-substituted indolocarbazoles were the most active, where AT2433-A1 (**6**) displayed the best overall antibacterial/fungal activities ranging from 1 μM (0.7 μg/mL, *Staph. aureus*) to 60 μM (40 μg/mL, *M. smegmatis*). Within this context, the removal of the N-6 methyl (**1**) or the C-11 chlorine (**7**) led to similar reductions in overall potency, where the differences in activity against *S cerevisiae* and *M. smegmatis* were the most dramatic. Similar trends were observed in the single-dose *in vitro* anticancer study (Figure 4) with a disaccharyl (**1**, **6**, and **7**) > mono (**2**, **4**, and **5**) > indolocarbazole aglycon (**3**) (Table 3 and Supporting Information, Table S1 and Figure S79). To more accurately assess potency differences, the most active new analogue from the single-dose study (**1**) was compared to the corresponding most active known comparator AT2433-A1 (**6**) in a full-dose study (Table 3). Both compounds displayed notable potencies (IC_{50}'s of 46–106 nM), where **1** was found to be slightly more active than **6** (2-fold) against the PC3 prostate cancer cell line. While **3** appears to stimulate growth in this assessment, this dose–response is consistent with hormesis, a biphasic response with apparent stimulation and inhibition zones observed common to a variety of different drugs, antibodies, and radiation.[82]

In summary, *A. melliaura* ATCC 39691 is a known producer of the indolocarbazole AT2433 series (**6**–**9**). Reinvestigation of this strain using new growth conditions led to a dramatic improvement in production of three known indolocarbazoles (**5**–**7**) and the discovery of four new naturally occurring indolopyrrolocarbazoles (**1**–**4**). The availability of this broader set of compounds enabled a subsequent small antibacterial/ fungal/cancer structure–activity relationship (SAR), which revealed disaccharyl substitution, *N*-6 methylation, and C-11 cholorination as key modulators of activity. The slightly improved anticancer potency of *N*-6-desmethyl **1** (compared to **6**) contrasts the extensive SAR of the rebeccamycin-type topoisomerase I inhibitors that have advanced to clinical evaluation (as exemplified by becatecarin,[83,84] edotecarin,[85] or NB-506[86]), where *N*-6 alkylation has been a key to improvements in potency and ADME (absorption, distribution, metabolism, and excretion).[87] While this subtle distinction could be consistent with the putative uncharacterized top-isomerase-independent mechanism of action for the disacchar-yl-substituted AT2433 metabolites, further SAR and mechanistic study is required.

EXPERIMENTAL SECTION

General Experimental Procedures

UV spectra were recorded on an Ultrospec 8000 spectrometer (GE, Pittsburgh, PA, USA). NMR spectra were measured using Varian Vnmr 500 (^{1}H, 500 MHz; ^{13}C, 125.7 MHz) and Vnmr 400 (^{1}H, 399.8 MHz; ^{13}C, 100.5 MHz) spectrometers, where δ-values were referenced to respective solvent signals [CD_3OD, δ_H 3.31 ppm, δ_C 49.15 ppm; DMSO-d_6, δ_H 2.50 ppm, δ_C 39.51 ppm]. High-resolution electrospray ionization (ESI) mass spectra were recorded on a Thermo Scientific (Rockford, IL, USA) Q Exactive (Orbitrap mass spectrometer) via direct infusion at 3 μL/min. Full-scan mass spectra were recorded in positive (3.8 kV) and negative (3.8 kV) ion modes (capillary temperature: 225 °C; nominal resolution: 140 000). HPLC-MS analyses were accomplished using a Waters (Waters Corp., Milford, MA, USA) 2695 LC module (Waters Symmetry Anal. C_{18}, 4.6 × 250 mm, 5 μm;

solvent A: H_2O–0.1% formic acid, solvent B: CH_3CN–0.1% formic acid; flow rate: 0.5 mL min^{-1}; 0–4 min, 10% B; 4–22 min, 10%–100% B; 22–27 min, 100% B; 27–29 min, 100%–10% B; 29–35 min, 10% B). HPLC analyses were performed on an Agilent 1260 system equipped with a photodiode array (PDA) detector and a Phenomenex C_{18} column (250 × 4.6 mm, 5 μm; Phenomenex, Torrance, CA, USA) (solvent A: H_2O–0.1% TFA; solvent B: CH_3CN; flow rate: 1.0 mL min^{-1}; 0–35 min, 5%–100% B; 35–40 min, 100% B; 40–41 min, 100%–5% B; 41–45 min, 5% B). Semipreparative HPLC was accomplished using Phenomenex on a Varian (Varian, Palo Alto, CA, USA) ProStar model 210 equipped with a PDA detector and a gradient elution profile (C_{18} column, 10 × 250 mm, 5 μm; solvent A: 0.05% TFA–H_2O, solvent B: CH_3CN; flow rate: 5.0 mL min^{-1}; 0–2 min, 25% B; 2–15 min, 25%–100% B; 15–17 min, 100% B; 17–18 min, 100%–25% B; 18–19 min, 25% B). All solvents used were of ACS grade and purchased from the Pharmco-AAPER (Brookfield, CT, USA). R_f values were measured on Polygram SIL G/UV254 (Macherey-Nagel & Co., Dueren, Germany). C_{18}-functionalized silica gel (40–63 μm) was purchased from Material Harvest Ltd. (Cambridge, United Kingdom). Amberlite XAD16N resin (20–60 mesh) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Size exclusion chromatography was performed on Sephadex LH-20 (25–100 μm, GE Healthcare, Piscataway, NJ, USA). *Staphylococcus aureus*, *Mycobacterium smegmatis*, *Salmonella enterica*, and *Saccharomyces cerevisiae* strains were obtained from ATCC (Manassas, VA, USA); *Micrococcus luteus* and *Escherichia coli* were obtained from NRRL (Peoria, IL, USA). PC3, A549, and U118 cells were obtained from ATCC. All other reagents used were reagent grade and purchased from Sigma-Aldrich.

Media, Fermentation, Extraction, Isolation, and Purification

Actinomadura melliaura ATCC 39691 was cultivated on M_2-agar plates [glucose (4.0 g), malt extract (10.0 g), yeast extract (4.0 g), and agar (15.0 g) dissolved in 1 L of H_2O (pH 7.2) and sterilized by autoclaving for 33 min at 121 °C] at 28 °C for 3 days. To prepare the seed culture, a small piece of grown agar plate was used to inoculate one 250 mL baffled flask, containing 100 mL of A-medium [glucose (10.0 g), yeast extract (5.0 g), soluble starch (20.0 g), peptone (5.0 g), NaCl (4.0 g), K_2HPO_4 (0.5 g), $MgSO_4 \cdot 7H_2O$ (0.5 g), and calcium carbonate (2 g) dissolved in 1 L of H_2O (pH 7.0) and sterilized by autoclaving for 33 min at 121 °C], and the culture was grown at 28 °C with shaking (210 rpm) for 2 days. An aliquot of seed culture (1 mL) was subsequently used to inoculate 80 250 mL baffled flasks, each containing 100 mL of A-medium. Fermentation was continued at 28 °C with shaking (210 rpm) for 6 days. The obtained yellow culture broth was centrifuged and filtered over Celite, and the supernatant was mixed with XAD-16 (4%) resin overnight, followed by filtration. The recovered resin was washed with H_2O (3 × 1200 mL) and then extracted with MeOH (3 × 800 mL). The MeOH extract was subsequently evaporated *in vacuo* at 38 °C to afford 7.10 g of yellow, oily crude extract. The biomass (mycelium) was extracted with MeOH (3 × 1200 mL), which was then evaporated *in vacuo* at 38 °C to yield 44.35 g of yellow, oily crude extract. Both extracts revealed the same sets of metabolites based upon HPLC and TLC analysis and were therefore combined to afford a total of 51.45 g of crude extract that served as the source material for the following workup and isolation procedure (Supporting Information, Figure S76).

The obtained crude extract (51.45 g) was dissolved in 50% MeOH–H_2O (100 mL) and fractionated with a gradient of H_2O/0–100% CH_3CN, followed by 100% MeOH using RP-18 column chromatography (column 5 × 45 cm, 150 g) to provide the following fractions: 1.2 L 0% CH_3CN, fractions F1A–F1F (200 mL each); 0.6 L 10% CH_3CN, fractions F2A–F2C (200 mL each); 0.6 L 20% CH_3CN, fractions F3A–F3C (200 mL each); 0.6 L 30% CH_3CN, fractions F4A–F4C (200 mL each); 0.6 L 40% CH_3CN, fractions F5A–F5C (200 mL each); 0.6 L 50% CH_3CN, fractions F6A–F6C (200 mL each); 0.6 L 60% CH_3CN, fractions F7A–F7C (200 mL each); 0.6 L 70% CH_3CN, fractions F8A–F8C (200 mL each); 0.6 L 80% CH_3CN, fractions F9A–F9C (200 mL each); 0.6 L 90% CH_3CN, fractions F10A–F10C (200 mL each); 1.5 L 100% CH_3CN, fractions F11A–F11E (300 mL each); 1.5 L 100% CH_3OH, fractions F11F–F11K (250 mL each); 0.8 L 0.1% TFA CH_3OH, fraction F12A. All fractions were analyzed by TLC and HPLC as a basis for the subsequent final purification steps described in the next paragraph.

As highlighted in Figure S76, fractions F4B–F5A (0.6 g) were combined and subsequently purified by preparative TLC (CH_2Cl_2–MeOH, 9:1), Sephadex LH-20 (CH_2Cl_2–MeOH, 6:4; column 1 × 40 cm), and semipreparative HPLC to afford harman (1.3 mg), riboflavin (4.3 mg), and 5′-methylthioadenosine (2.0 mg). Similarly, fractions F5C–F6C (0.4 g) were combined and purified by Sephadex LH-20 (CH_2Cl_2–MeOH, 6:4; 2.5 × 50 cm) and semipreparative HPLC to yield lumichrome (1.7 mg). Treatment of the combined fractions F7A–F7C (70 mg) using preparative TLC (CH_2Cl_2–MeOH, 9:1), followed by Sephadex LH-20 (CH_2Cl_2–MeOH, 6:4; column 1 × 40 cm), afforded BMY-41219 (**5**; 5.9 mg). Purification of combined fractions F9B–F10C (0.45 g) by preparative TLC and semipreparative HPLC gave AT2433-A4 (**2**, 3.0 mg). Semipreparative HPLC purification of fraction F11A (150 mg) afforded AT2433-A3 (**1**, 2.6 mg) and AT2433-B1 (**7**, 45.0 mg), while resolution of combined fractions F11B–F11E (0.4 g) via Sephadex LH-20 (CH_2Cl_2–MeOH, 6:4; column 2.5 × 50 cm) and semipreparative HPLC provided AT2433-A3 (**1**, 1.7 mg), AT2433-A5 (**3**, 1.7 mg), AT2433-B3 (**4**, 3.3 mg), AT2433-B1 (**7**, 51.9 mg), and harman (0.7 mg). Finally, purification of combined fractions F11H–11K (0.7 g) using Sephadex LH-20 (CH_2Cl_2–MeOH, 6:4; column 2.5 × 50 cm) gave AT2433-A1 (**6**, 450 mg), while the major fractions F11F (1.1 g) and F11G (1.3 g) were composed of AT2433-B1 (**7**) and AT2433-A1 (**6**), respectively (each ≥95% purity), based on HPLC/MS analysis.

AT2433-A3 (1): yellow solid; UV/vis (MeOH) λ_{max} (log ε) 236 (4.17), 290 (3.99), 316 (4.26), 396 (3.25) nm; ^{1}H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 100 MHz), see Table 1; (+)-APCI-MS *m*/*z* 665 $[M + H]^+$; (+)-HRESIMS *m*/*z* 665.2008 $[M + H]^+$ (calcd for $C_{33}H_{34}ClN_4O_9$, 665.2008); (−)-HRESIMS *m*/*z* 663.1846 $[M – H]^-$ (calcd for $C_{33}H_{32}ClN_4O_9$, 663.1863).

AT2433-A4 (2): yellow solid; UV/vis (MeOH) λ_{max} (log ε) 238 (3.97), 290 (3.84), 319 (3.96), 402 (2.93) nm; ^{1}H NMR (DMSO-d_6, 500 MHz) and ^{13}C NMR (DMSO-d_6, 100 MHz), see Table 1; (+)-HRESIMS *m*/*z* 550.1381 $[M + H]^+$ (calcd for $C_{28}H_{25}ClN_3O_7$, 550.1376); (−)-HRESIMS *m*/*z* 548.1222 $[M – H]^-$ (calcd for $C_{28}H_{23}ClN_3O_7$, 548.1230).

AT2433-A5 (3): yellow solid; R_f 0.58 (silica gel, CH_2Cl_2); UV/vis (MeOH) λ_{max} (log ε) 236 (3.79), 285 (3.65), 316 (3.74), 400 (2.73) nm; ^{1}H NMR (DMSO-d_6, 500 MHz) and ^{13}C

NMR (DMSO-d_6, 100 MHz), see Table 1; (−)-HRESIMS *m*/*z* 372.0536 $[M - H]^-$ (calcd for $C_{21}H_{11}ClN_3O_2$, 372.0545).

AT2433-B3 (4): yellow solid; UV/vis (MeOH) λ_{max} (log ε) 237 (3.56), 286 (3.38), 319 (3.53), 406 (2.57) nm; ^1H NMR (CD_3OD, 500 MHz) and ^{13}C NMR (CD_3OD, 100 MHz), see Table 1; (+)-APCI-MS *m*/*z* 516 $[M + H]^+$; (−)-APCI-MS *m*/*z* 514 $[M - H]^-$; (+)-HRESIMS *m*/*z* 516.1769 $[M + H]^+$ (calcd for $C_{28}H_{26}N_3O_7$, 516.1769); (−)-HRESIMS *m*/*z* 514.1611 $[M - H]^-$ (calcd for $C_{28}H_{24}N_3O_7$, 514.1620).

Cancer Cell Line Viability Assays

Cytotoxicity screening was accomplished using previously reported methods for both the single-dose (10 μM) screen and full IC_{50} determinations (0.03 to 100 000 nM) in triplicate.[88]

Antimicrobial Assay

Antimicrobial assays were accomplished in triplicate following previously reported methods.[88] Antibacterial MIC values were obtained after 16–48 h of incubation, while antifungal MIC values were obtained after 24 h of incubation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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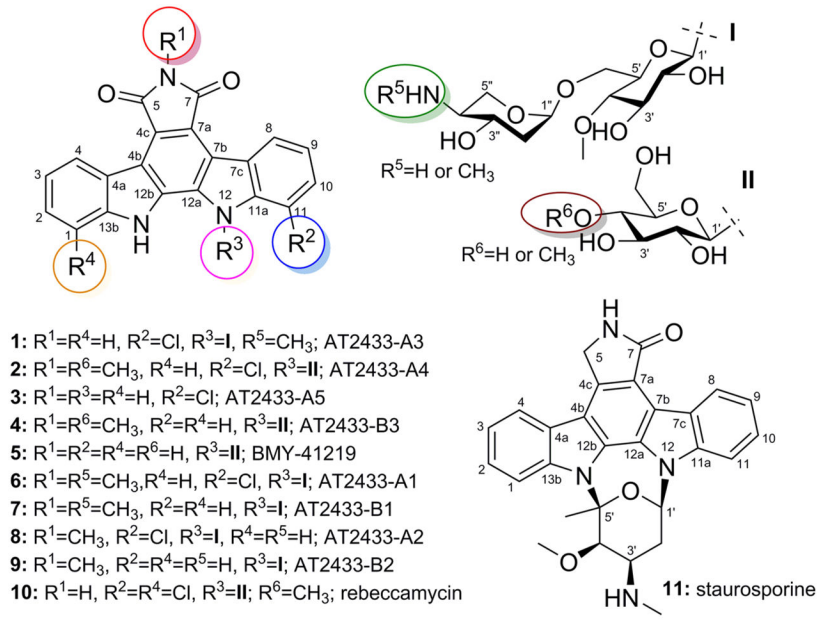

Figure 1.
Chemical structures of indolopyrrolocarbazoles **1**–**11**.

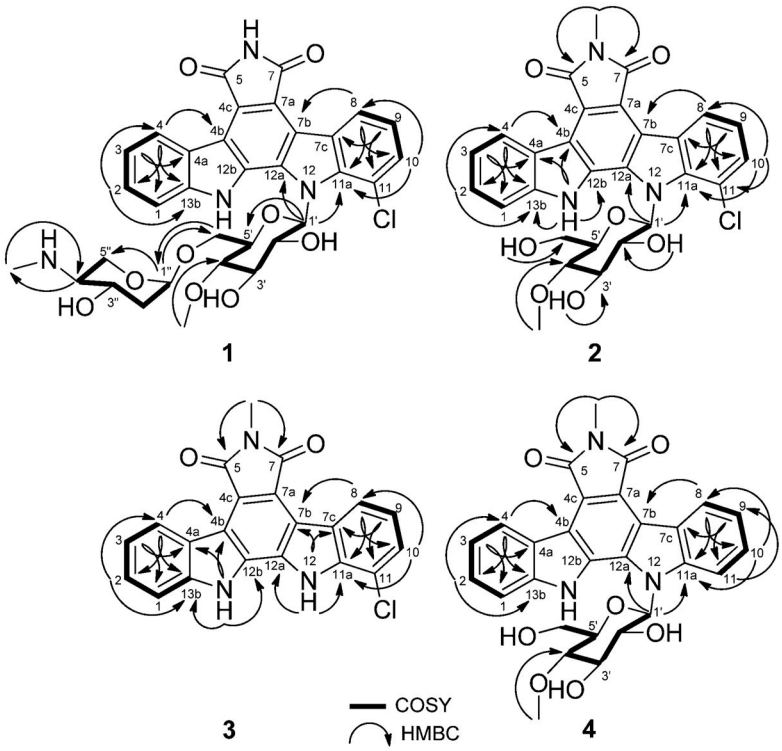

Figure 2.
^{1}H–^{1}H–COSY and selected HMBC correlations for indolocarbazoles **1**–**4**.

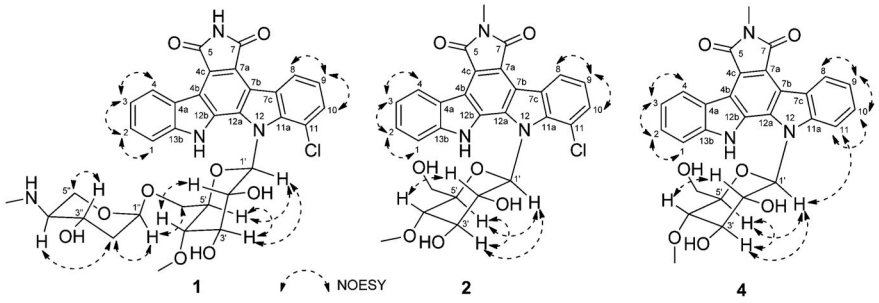

Figure 3.
Key NOESY correlations for indolocarbazoles **1**, **2**, and **4**.

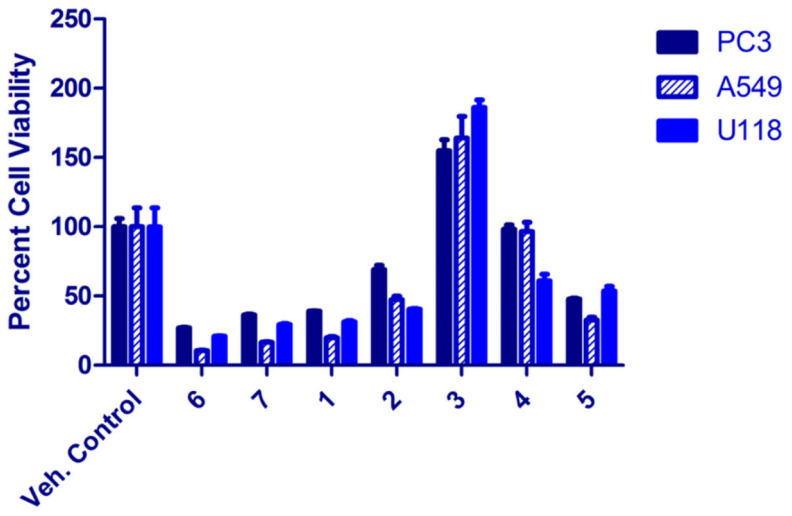

Figure 4.

Viability of PC3 (prostate), A549 (lung), and U118 (brain) human cancer cell lines at 10 μM treatment for compounds **1**–**7** after 72 h (see Supporting Information, Table S1).

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Table 1

^{13}C (100 MHz) and ^{1}H (500 MHz) NMR Data of Compounds 1–4[a]

	AT2433-A3 (1)[b,c]		AT2433-A4 (2)[b,d]		AT2433-A5 (3)[b,d]		AT2433-B3 (4)[b,c]	
position	**δ_C, type**	**δ_H, m (*J* in Hz)**	**δ_C, type**	**δ_H, m (*J* in Hz)**	**δ_C, type**	**δ_H, m, (*J* in Hz)**	**δ_C, type**	**δ_H, m (*J* in Hz)**
1	112.7, CH	7.72, d (8.0)	112.2, CH	7.75, d (8.0)	112.2, CH	7.82, dd (8.0, 1.0)	113.1, CH	7.69, d (7.5)
2	129.2, CH	7.64, t (7.0)	127.6, CH	7.61, td (7.5, 1.5)	127.1, CH	7.56, td (8.0, 1.0)	128.0, CH	7.53, td (8.0, 1.0)
3	122.4, CH	7.32, t (7.0)	120.7, CH	7.40, t (7.0)	121.3, CH	7.36, td (8.0, 1.0)	121.6, CH	7.32, td (8.0, 1.0)
4	126.8, CH	8.99, d (8.0)	124.5, CH	9.12, d (8.0)	124.2, CH	8.95, d (8.0)	126.2, CH	9.13, d (8.0)
4a	121.4, C		121.0, C		119.8, C		123.5, C*	
4b	119.8, C		117.5, C		115.9, C		119.6, C*	
4c	124.4, C		118.2, C		118.9, C		119.7, C	
5	172.2, C		169.3, C		169.7, C		171.3, C*	
6-$\underline{CH_3}$			23.9, CH_3	3.20, s	23.6, CH_3	3.11, s	24.0, CH_3	3.24, s
7	172.2, C		169.5, C		169.7, C		171.3, C*	
7a	123.4, C		121.9, C		120.5, C		119.7, C	
7b	121.4, C		119.4, C		115.5, C		119.6, C	
7c	127.5, C		125.5, C		123.1, C		123.5, C*	
8	125.9, CH	9.20, d (8.0)	123.8, CH	9.27, dd (8.0, 1.0)	123.3, CH	8.87, d (8.0)	126.4, CH	9.23, d (8.0)
9	123.8, CH	7.30, t (8.0)	122.5, CH	7.42, t (8.0)	121.3, CH	7.34, t (7.5)	122.0, CH	7.34, t (8.0)
10	131.0, CH	7.52, d (7.5)	129.6, CH	7.65, dd (7.5, 1.0)	126.0, CH	7.62, dd (7.5, 1.0)	128.3, CH	7.56, td (8.5, 1.5)
11	118.1, C		116.5, C		115.8, C		112.1, CH	7.81, d (8.5)
11a	139.9, C		138.2, C		137.1, C		142.9, C	
12						11.95, brs		
12a	131.8, C		129.7, C		128.8, C		130.8, C	
12b	131.4, C		130.3, C		129.0, C		131.6, C	
13				11.81, brs		11.64, brs		
13a	141.9, C		140.8, C		140.1, C		141.9, C	
1′	86.5, CH	7.01, d (9.5)	83.8, CH	6.90, d (9.0)			86.3, CH	6.18, d (8.5)
2′	73.8, CH	3.82, m	72.1, CH	3.41, m			73.7, CH	3.84, t (8.0)
2′-OH				4.94, d (5.5)				

	AT2433-A3 (1)[b,c]		AT2433-A4 (2)[b,d]		AT2433-A5 (3)[b,d]		AT2433-B3 (4)[b,c]	
position	**δ_C, type**	**δ_H, m (*J* in Hz)**	**δ_C, type**	**δ_H, m (*J* in Hz)**	**δ_C, type**	**δ_H, m, (*J* in Hz)**	**δ_C, type**	**δ_H, m (*J* in Hz)**
3′	79.5, CH	3.68, m	76.6, CH	3.51, m			79.1, CH	3.98, t (9.5)
3′-OH				5.28, d (6.5)				
4′	80.3, CH	3.68, m	77.0, CH	3.67, t (9.5)			79.6, CH	3.85, t (9.5)
4′-O$\underline{CH_3}$	61.5, CH_3	3.74, s	60.1, CH_3	3.61, s			61.4, CH_3	3.80, s
5′	79.5, CH	4.06, m	77.6, CH	3.90, d (10.0)			75.0, CH	4.06, m
6′	67.5, CH_2	4.41, d (10.0) 4.08, m	58.7, CH_2	4.00, brd (2.0)			60.4, CH_2	4.12, d (11.5) 4.08, dd (11.5, 2.0)
6′-OH				6.34, t (4.0)				
1″	100.4, CH	5.28, brs						
2″	38.7, CH_2	2.54, m 2.03, m						
3″	65.4, CH	4.02, m						
4″	61.5, CH	3.05, m						
4″-NH$\underline{CH_3}$	31.6, CH_3	2.51, s						
5″	59.0, CH_2	4.04, m 3.84, m						

[a] Assignments supported by 2D HSQC and HMBC experiments.

[b] See Supporting Information for the NMR spectra.

[c] CD_3OD.

[d] DMSO-d_6.

[*] Obtained from the HMBC spectrum.

Table 2

In Vitro Antimicrobial Activities of AT2433-A3 (1), AT2433-B3 (4), AT2433-A1 (6), and AT2433-B1 (7)

	compound[a]			
organism	**1**	**4**	**6**	**7**
Staphylococcus aureus ATCC 6538	7.5 (5)	7.5 (4)	1 (0.7)	7.5 (5)
Micrococcus luteus NRRL B-287	7.5 (5)	15 (7.7)	7.5 (5)	7.5 (5)
Saccharomyces cerevisiae ATCC 204508	>60 (>40)	>60 (>31)	7.5 (5)	>60 (>40)
Mycobacterium smegmatis ATCC 14468	120 (80)	>120 (>62)	60 (40)	120 (80)

[a] MIC in μM (μg/mL) values are based on three independent replicates. Compounds **2**, **3**, and **5** were inactive against all strains up to 60 μM (120 μm for *M. smegmatis*). Compounds **1**–**7** were inactive against *Salmonella enterica* ATCC 10708 and *Escherichia coli* NRRL B-3708 up to 60 μM. Kanamycin and ampicillin (*S. aureus*, *M. luteus*, *S. enterica*, and *E. coli*), spectinomycin and rifampicin (*M. smegmatis*), and amphotericin B (*S. cerevisiae*) were used as positive controls.

Table 3

Cancer Cell Line Inhibition by AT2433-A3 (1) and AT2433-A1 (6)[a]

	cell line [(IC_{50} (nM)]		
compound	**PC3**	**A549**	**U118**
AT2433-A3 (**1**)	45.8	69.6	47.3
AT2433-A1 (**6**)	106.2	76.4	56.8

[a] See Supporting Information, Figure S79. AT2433-A1, as a known compound, was also considered as a positive control.