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Structurally Rigid 9-Amino-benzo[*c*]cinnoliniums Make Up a Class of Compact and Large Stokes-Shift Fluorescent Dyes for Cell-Based Imaging Applications

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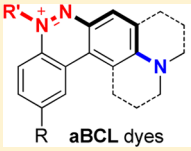
Supporting Information

ABSTRACT: Classic fluorescent dyes, such as coumarin, naphthalimide, fluorescein, BODIPY, rhodamine, and cyanines, are cornerstones of various spectroscopic and microscopic methods, which hold a prominent position in biological studies. We recently found that 9-amino-benzo[*c*]cinnoliniums make up a novel group of fluorophores that can be used in biological studies. They are featured with a succinct conjugative push−pull backbone, a broad absorption band, and a large Stokes shift. They are potentially useful as a small-molecule alternative to R-phycoerythrin to pair with fluorescein in multiplexing applications.



Advantages include:
√ Broad Absorption from 488 to 533 nm;
√ Efficient excitation by multiple laser lines;
√ Large Stokes shift > 60 nm;
√ Promising for one-/two-color experiments.

A multicolor experiment produces a multitude of information through two or more optical channels and prevails in flow cytometry,[1] DNA sequencing,[2] and fluorescence *in situ* hybridization.[3] Technically, monoexcitation dual emission is the least demanding type of multicolor experiment, which employs two fluorophores with overlapping absorptions and well-separated emissions.[4] In other words, their Stokes shifts should be different enough to facilitate individual acquisition of their emissions. Also, considering the availability of 488 nm laser line in different imaging instruments, the capability of efficient excitation at 488 nm is keenly desired from a practical standpoint. Fluorescein and many of its analogues are bright fluorophores with 488 nm excitation capability and exhibit a small Stokes shift (∼25 nm). Therefore, a fluorophore with the same excitation, but a larger Stokes shift (ideally approximately ≥60 nm), is needed to pair with fluorescein. Regardless of recent intensive efforts by the synthetic dye community and flourishing novel fluorescent scaffolds, such a small-molecule organic fluorophore remains unavailable.[5] A fluorescent protein, R-phycoerythrin, is routinely employed in such applications as an alternative. However, its size (250 kDa) could represent a limitation.[6] Fluorophores with a large Stokes shift are also highly regarded for regular imaging applications as well for their low background signal, as a result of reduced cross-talk from biological autofluorescence and Raleigh scattering.[7]

The key to a large Stokes shift is a significant change in the electron-density distribution and/or geometry of the molecule between ground and excited states, e.g., by formation of the excited state charge-transfer complex (exciplex),[8] excited state energy transfer (EET),[9] twisted internal charge transfer (TICT),[10] excited state proton transfer (ESPT),[11] local excitation (LE),[12] and solvent-cage relaxation.[13] Utilization of exciplex formation or energy transfer is not trivial because the use of two structural components, i.e., a donor and an acceptor, is warranted. Additionally, the relative spatial location and orientation of the donor and acceptor need to be optimized through complex molecular engineering to maximize the intended photophysical interaction. TICT often leads to complete quenching and consequently poor fluorescence quantum yield. Inter- or intramolecular ESPT leads to a large Stoke shift by exciting the shorter-wavelength-absorbing conjugate acid and collecting the longer-wavelength emission from the conjugate base.[11] However, inevitable pH sensitivity of ESPT type fluorophores could be a liability in many applications. Fluorescent dyes with a large ground state dipole moment typically exhibits a large Stokes shift, especially in polar medium, as a result of solvent-cage relaxation. Coumarins, nitrobenzoxadiazoles (NBDs), naphthalimides are such fluorophores and have found widespread applications, albeit with short-wavelength excitation in the range of the UV or blue region. Dicyanomethylene-benzopyran[14] or seminaphthoxanthenes[15] are attractive candidates for multiplexing with fluorescein. However, they display chemo-instability toward nucleophiles and endogenous oxidants to various extents.

Benzo[*c*]cinnolinium is a short-wavelength dye with a maximal absorption at 416 nm in aqueous media.[16] Introduction of an electron-donating amino group at C-9 allows efficient electronic delocalization and hence is expected to induce a bathochromic shift. A number of 9-dialkylamino-benzo[*c*]cinnoliniums were patented as dyestuffs for polyacrylonitrile, for different shades of red.[17] Herein, we show that

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further rigidification of the dialkylamino group at C-9 of the benzo[*c*]cinnolinium backbone leads to a class of structurally compact fluorophores (**aBCL2**−**5**), with efficient Ar^+ laser line excitation capability, pH insensitive fluorescence, and large Stokes shifts (Figure 1).

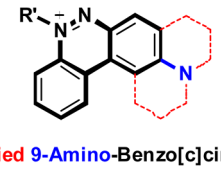


Figure 1. Structures of **aBCL** species.

The dialkylamino groups within the structures of **aBCL2** and **aBCL3** are rigidified to eliminate the rotational quenching pathway, and **aBCL1** is synthesized as a negative control to manifest the necessity of such rigidification (Scheme 1). Structures of **aBCL4** and **aBCL5** were derived from **aBCL3** by changing the mildly electron-donating methyl group into a H or an electron-withdrawing F.

The **aBCL**s were readily prepared via a three-step cascade (Scheme 1). The Suzuki−Miyaura coupling of a 2-bromoaniline (**1a**−**c**) and a 3-dialkylaminophenylboronic acid (**2a**−**c**) catalyzed by $Pd(PPh_3)_4$ furnished a biphenyl (**3a**−**e**), diazotization of which with $NaNO_2$ in acidic aqueous medium at 0 °C generated the corresponding benzo[*c*]cinnoline derivative (**4a**−**e**). Alkylation of compounds **4a**−**e** occurred regioselectively to afford **aBCL1**−**5** by refluxing in CH_3CN with excess EtI. This high regioselectivity is apparently a result of polarization of the nitrogen−nitrogen double bond (-N═N-) due to the presence of a strong electron-donating dialkylamino group at the para position and further verified via 2D NOESY with **aBCL3** as an example (Figure S2 of the Supporting Information).

The spectral properties of **aBCL1** were studied in neutral phosphate buffer (50 mM, at pH 7.4) containing 1% DMSO as a cosolvent, to examine its potentials for biological applications (Figure 2 and Table 1). Compound **aBCL1** exhibited a broad

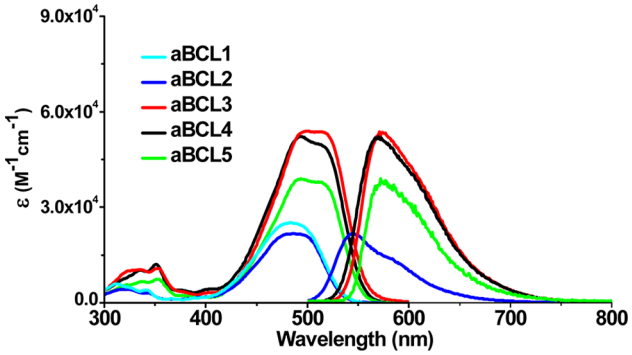
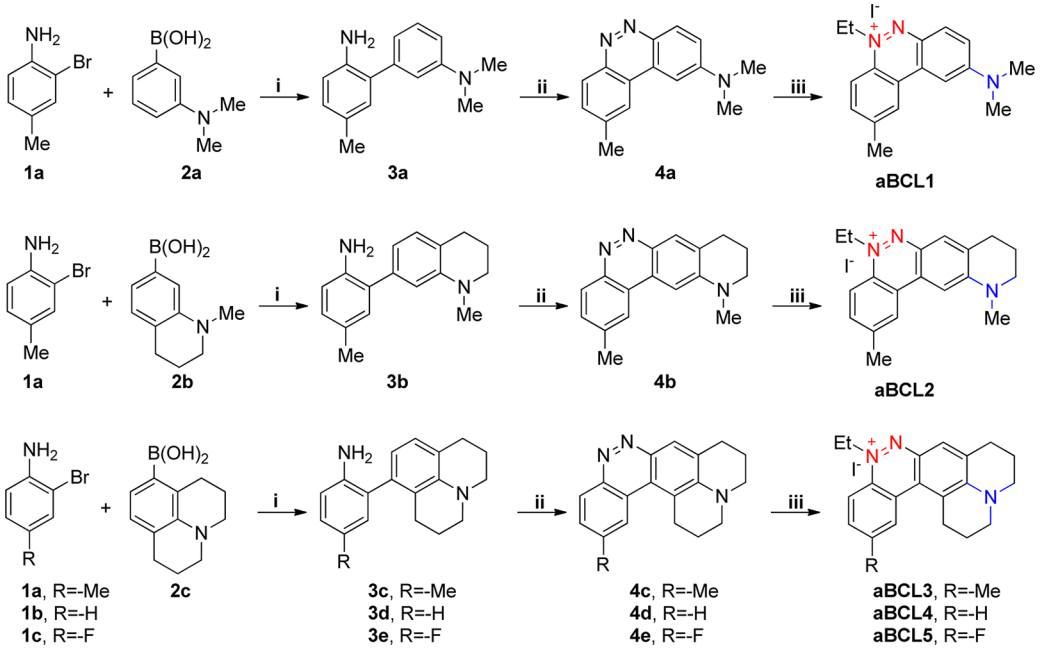


Figure 2. Absorption and fluorescence emission spectra (λ_{ex} = 488 nm) of **aBCL1**−**5** in phosphate buffer (pH 7.4) containing 1% DMSO.

absorbance band ranging from 420 to 570 nm, with an absorption maximum located at 483 nm. **aBCL1** is indeed fluorescent at 542 nm, though with a diminishing fluorescence quantum yield of 0.003.

Rigidification of the dimethylamino group in **aBCL1** dramatically promoted the fluorescence brightness (Figure 2 and Table 1). Rigidification with one propylene unit, as exemplified by **aBCL2**, did not induce noticeable changes in the absorption wavelength or molar absorptivity compared with those of **aBCL1**, but the quantum yield of **aBCL2** was as high as 0.54. All julolidine analogues, i.e., **aBCL3**−**5**, displayed a red-shifted absorption and emission spectrum, compared to those of **aBCL1** and **aBCL2**, by ∼10 and ∼25 nm, respectively. Their quantum yields are much higher than that of **aBCL1** but lower

Scheme 1. Chemical Structures and Syntheses of aBCL1−5[a]



[a]Reaction conditions: (i) $Pd(PPh_3)_4$ or $PdCl_2(dppf)$, Na_2CO_3, THF, reflux; (ii) $NaNO_2$, H^+, H_2O, 0 °C; (iii) EtI, CH_3CN, reflux.

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Table 1. Spectral Properties of aBCL1−5 in Neutral Phosphate Buffer (pH 7.4) Containing 1% DMSO[a]

	λ_{abs} (nm)	λ_{em} (nm)	Stokes shift[b]		ε (cm^{-1} M^{-1})	φ	$\varepsilon\varphi$[c]	τ (ns)
aBCL1	483	542	2253 cm^{-1}	59 nm	2.51×10^4	0.003	67	*d*
aBCL2	487	544	2151 cm^{-1}	57 nm	2.17×10^4	0.54	1.18×10^4	4.3
aBCL3	500	571	2486 cm^{-1}	71 nm	5.37×10^4	0.27	1.47×10^4	4.2
aBCL4	493	570	2740 cm^{-1}	77 nm	5.21×10^4	0.25	1.34×10^4	3.6
aBCL5	494	564	2512 cm^{-1}	70 nm	3.89×10^4	0.34	1.33×10^4	4.4

[a]λ_{abs} is the absorption maximum, λ_{em} the emission maximum, ε the molar absorptivity, φ the fluorescence quantum yield, $\varepsilon\varphi$ a measure of the fluorescence brightness, and τ the fluorescence lifetime. [b]Stokes shifts are listed in units of both inverse centimeters and nanometers. [c]The unit of fluorescence brightness (cm^{-1} M^{-1}) has been omitted. [d]The lifetime of **aBCL1** could not be accurately measured because of its poor fluorescence intensity.

than that of **aBCL2**. The presence of allylic strain[xx] in **aBCL3−5** likely accounts for the reduced fluorescence quantum yields. Minor structural variation from -Me, -H, and -F on **aBCL3**, **aBCL4**, and **aBCL5**, respectively, seems to have a noticeable influence on their spectral and photophysical properties. The stronger electron donating capability of the substituent has led to a higher molar absorptivity and, at the same time, a lower quantum yield. The fluorescence lifetime of all these scaffolds was measured to be ∼4 ns. The broad nature of the absorption bands of **aBCL**s is not due to aggregation (Figure S3 of the Supporting Information).

Compound **aBCL3** is chosen as an example to showcase the potentials of this class of dyes for real applications, because of its higher fluorescence brightness, estimated by the product ($\varepsilon\varphi$) of the molar absorptivity (ε) and the fluorescence quantum yield (φ).

We tested the chemostability and photostability of **aBCL3** (10 μM) in pure H_2O with 1% DMF (Figure S1 of the Supporting Information). The absorption and emission spectra of **aBCL3** remained unchanged within a broad range of pH values from 2 to 12. Also, spectral properties were not affected by addition of a large excess of strong nucleophiles, such as methylsulfide (MeS^-). **aBCL3** was also stable to oxidants, including H_2O_2 and $OONO^-$, within the tested range up to 100 equiv. In comparison, bolus addition of hypochlorite (100 equiv) caused ∼30% bleaching of the fluorescence intensity. However, such a high concentration of hypochlorite is unlikely physiological, and therefore, **aBCL3** is suitable for most *in vitro* applications except perhaps activated immune cells. **aBCL3** also displayed photostability superior to that of fluorescein (Figure S4 of the Supporting Information). Continuous illumination of an **aBCl3** solution (λ_{abs} = 0.3 at 450 nm) with a laser at 450 nm for 300 min was <5% bleached. Under the same condition, 40% of fluorescein was bleached.

The potentials of **aBCL3** to pair with fluorescein in multiplexing experiments were assessed spectroscopically (Figure 3). Fluorescein displays a narrow absorption band with a maximum at 490 nm and an emission band with a maximum at 514 nm. **aBCL3** absorbs strongly at ∼490 nm and therefore can also be excited with a 488 nm laser line. The emission band of **aBCL3** peaks at 571 nm, which is well-resolved from the emission of fluorescein. Therefore, emissions of fluorescein and **aBCL3** can be individually collected with a dual-emission filter band-pass, e.g., 500−530 nm for fluorescein and 570−610 nm for **aBCL3**. Such dual-emission band-pass filters are commercial. Minor emission leak-over from fluorescein to **aBCL3** is present and can be corrected mathematically.

The potentials of benzo[*c*]cinnolinium dyes for cell-based imaging applications, with **aBCL3** as an example, were

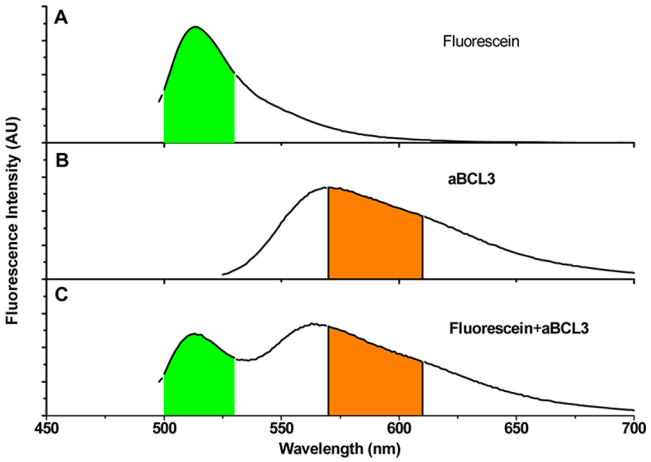

Figure 3. Emission spectra of (A) fluorescein, (B) **aBCL3**, and (C) a mixture of fluorescein and **aBCL3** in a molar ratio of 1:10, with excitation at 488 nm. Positions of band-passes of a suitable dual-channel emission filter by shaded areas are highlighted in green for 500−530 nm and orange for 570−610 nm.

demonstrated with HeLa and human glioma U251 cell lines as *in vitro* biological models. Compound **aBCL3** showed good membrane permeability, and an incubation as short as 15 min was sufficient. Minimal cytotoxicity if any was observed for **aBCL3** as >90% cell viability was observed after incubation of HeLa cells with up to 10 μM **aBCL3**.

Cell images were also collected with a confocal microscope using three different laser lines as excitation (Figure 4). Emissions of 500−550 nm were collected when a laser at 488 nm was used as excitation. Emissions between 570 and 620 nm were collected when 514 or 561 nm laser excitation was applied.

In summary, rigidified 9-aminobenzo[*c*]cinnoliniums make up a novel class of fluorophores suitable for cell-based applications, with good pH stability, chemostability, and photostability. They exhibit a broad absorption band in the cyan−green region and a large Stokes shift of ∼60 nm. They have potentials to be used as a small-molecule alternative to R-phycoerythrin in multiplexing applications with fluorescein.

EXPERIMENTAL SECTION

***N*³′,*N*³′,5-Trimethyl[1,1′-biphenyl]-2,3′-diamine (3a).** Compound **1a** (1.00 g, 5.43 mmol), **2a** (0.98 g, 5.94 mmol), Na_2CO_3 (1.73 g, 16.30 mmol), and 26 mL of a combined solvent of H_2O, EtOH, and benzene [3:3:10 (v/v)] were mixed in a round-bottom flask and deoxygenated by bubbling Ar for 15 min before addition of $Pd(PPh_3)_4$ (6 mg, 0.005 mmol). The resulting mixture was heated to reflux overnight while the mixture was being stirred under an Ar atmosphere before the reaction mixture was cooled to room

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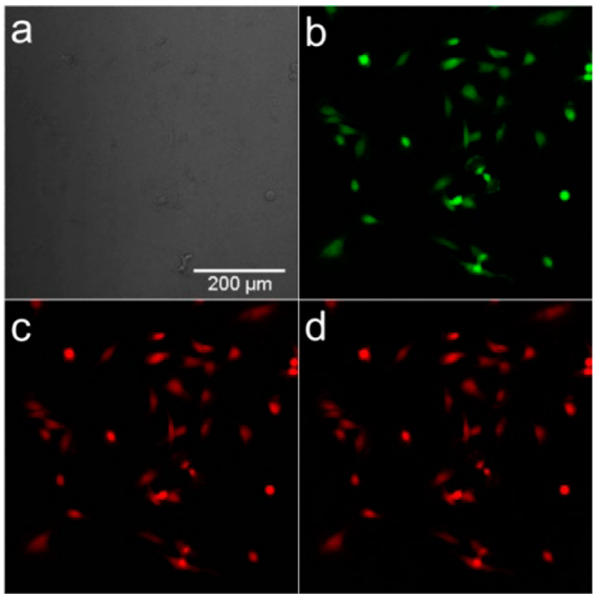

Figure 4. Fluorescence images of U251 cells acquired over a confocal microscope: (a) phase contrast, (b) 488 nm excitation and FITC emission filter, (c) 514 nm excitation and TRITC emission filter, and (d) 561 nm excitation and TRITC emission filter.

temperature and poured into H_2O. The resulting biphasic mixture was extracted with CH_2Cl_2. The organic layer was dried with $MgSO_4$, filtered, and evaporated under reduced pressure to afford the crude product, which was further purified with a flash column with petroleum and EtOAc [5:1 (v/v)] as the eluent to afford the desired biphenyl (**3a**, 1.02 g) as a colorless viscous residue in an 82% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.42 (t, 1H, J = 8.0 Hz), 7.13 (s, 1H), 7.08 (d, 1H, J = 8.0 Hz), 6.93−6.91 (m, 2H), 6.84 (d, 1H, J = 8.0 Hz), 6.77 (d, 1H, J = 8.0 Hz), 3.77 (s, 2H), 3.08 (s, 6H), 2.41 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 150.9, 141.1, 140.5, 130.8, 129.4, 128.8, 128.6, 127.5, 117.3, 115.7, 113.3, 111.3, 40.6, 20.5. ESI-HRMS calcd for $C_{15}H_{19}N_2$ $[M + H]^+$ m/z 227.1543, found m/z 227.1546.

***N*,*N*,9-Trimethylbenzo[*c*]cinnolin-2-amine (4a).** Compound **3a** (110 mg, 0.49 mmol) was dissolved in a dilute HCl solution (1 M) and cooled to 0 °C. A solution of $NaNO_2$ (40 mg, 0.59 mmol in H_2O) was added slowly while the mixture was being constantly stirred. The ice bath was then removed and the reaction mixture allowed to warm to room temperature for an additional 2 h while the mixture was being constantly stirred. A concentrated $NaHCO_3$ solution was added to adjust the solution pH to neutral before the mixture was extracted with CH_2Cl_2. The organic layer was dried with $MgSO_4$, filtered, and evaporated under reduced pressure to afford the crude product, which was purified with a column with CH_2Cl_2 and MeOH [50:1 (v/v)] as the eluent to afford the desired cinnoline derivatives **4a** (60 mg) as an amorphous brownish-yellow solid in a 51% yield. Mp: 193.3−195.2. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.28 (d, 1H, J = 8.4 Hz), 8.20 (d, 1H, J = 9.2 Hz), 7.78 (s, 1H), 7.41 (d, 1H, J = 8.4 Hz), 6.95 (d, 1H, J = 9.2 Hz), 6.81 (s, 1H), 2.92 (s, 6H), 2.45 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 150.9, 143.7, 139.9, 139.9, 131.8, 130.3, 129.9, 122.8, 120.6, 120.3, 115.8, 97.3, 40.0, 22.0. ESI-HRMS calcd for $C_{15}H_{16}N_3$ $[M + H]^+$ m/z 238.1339, found m/z 238.1343.

9-(Dimethylamino)-5-ethyl-2-methylbenzo[*c*]cinnolin-5-ium Iodide (aBCL1). Compound **4a** (40 mg, 0.21 mmol), EtI (98 mg, 0.41 mmol), K_2CO_3 (58 mg, 0.41 mmol), and 5 mL of an anhydrous CH_3CN were added to a round-bottom flask and heated to reflux for 3 h while being stirred. The resulting mixture was then poured into H_2O and extracted with CH_2Cl_2. The organic layer was dried with $MgSO_4$, filtered, and evaporated under reduced pressure to afford the crude product, which was purified with a flash column with CH_2Cl_2 and MeOH [25:1 (v/v)] to afford the desired **aBCL1** (46 mg) as an amorphous reddish-yellow solid in 83% yield that decomposed before melting. ^{1}H NMR (400 MHz, CD_3OD): δ 8.66 (s, 1H), 8.23 (d, 1H, J = 9.2 Hz), 8.21 (d, 1H, J = 9.6 Hz), 7.90 (d, 1H, J = 9.2 Hz), 7.69 (dd, 1H, J = 9.6, 2.4 Hz), 7.65 (d, 1H, J = 2.4 Hz), 5.10 (q, 2H, J = 7.2 Hz), 3.52 (s, 6H), 2.73 (s, 3H), 1.74 (t, 3H, J = 7.2 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ 160.7, 146.6, 141.8, 139.1, 138.3, 137.8, 132.7, 128.4, 127.5, 124.9, 121.8, 103.2, 59.4, 33.4, 25.3, 17.9. ESI-HRMS calcd for $C_{17}H_{20}N_3$ $[M + H]^+$ m/z 266.1652, found m/z 266.1653.

4-Methyl-2-(1-methyl-1,2,3,4-tetrahydroquinolin-7-yl)-aniline (3b). This compound was synthesized in a manner analogous to that of **3a**, from compound **1a** (203 mg, 1.10 mmol), **2b** (233 mg, 1.00 mmol), Na_2CO_3 (318 mg, 3.00 mmol), $PdCl_2$(dppf) (82 mg, 0.1 mmol), and 26 mL of a combined solvent of H_2O, EtOH, and benzene. The desired **3b** (145 mg) was obtained as a crystalline colorless solid in 86% yield. Mp: 72.3−75.1 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.06−7.03 (m, 2H), 6.98 (d, 1H, J = 8.0 Hz), 6.72 (s, 1H), 6.69 (d, 2H, J = 6.4 Hz), 3.75 (s, 2H), 3.29 (t, 2H, J = 5.6 Hz), 2.93 (s, 3H), 2.84 (t, 2H, J = 6.4 Hz), 2.32 (s, 3H), 2.09−2.02 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 147.0, 141.2, 138.5, 130.9, 129.2, 128.8, 128.7, 127.6, 121.8, 116.8, 115.7, 111.7, 51.4, 39.3, 27.7, 22.5, 20.6. ESI-HRMS calcd for $C_{17}H_{21}N_2$ $[M + H]^+$ m/z 253.1699, found m/z 253.1705.

2,11-Dimethyl-8,9,10,11-tetrahydrobenzo[*c*]pyrido[2,3-*g*]-cinnoline (4b). This compound was synthesized in a manner analogous to that of **4a**, from compound **3b** (94 mg, 0.37 mmol) and $NaNO_2$ (31 mg, 0.44 mmol). Compound **4b** (58 mg) was obtained as an amorphous brownish-yellow solid in 62% yield. Mp: 194.9−196.3 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.41 (d, 1H, J = 8.4 Hz), 8.15 (s, 1H), 8.13 (s, 1H), 7.58 (d, 1H, J = 8.4 Hz), 7.18 (s, 1H), 3.50 (t, 2H, J = 5.6 Hz), 3.18 (s, 3H), 3.03 (t, 2H, J = 6.4 Hz), 2.64 (s, 3H), 2.10−2.04 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 148.9, 144.1, 140.8, 140.0, 130.4, 130.0, 127.8, 122.6, 121.0, 120.6, 96.0, 51.5, 39.4, 28.5, 22.4, 21.9. ESI-HRMS calcd for $C_{17}H_{18}N_3$ $[M + H]^+$ m/z 264.1495, found m/z 264.1499.

5-Ethyl-2,11-dimethyl-8,9,10,11-tetrahydrobenzo[*c*]pyrido-[2,3-*g*]cinnolin-5-ium Iodide (aBCL2). This compound was synthesized in a manner analogous to that of **aBCL1**, from compound **4b** (30 mg, 0.11 mmol), EtI (32 mg, 0.21 mmol), K_2CO_3 (29 mg, 0.21 mmol), and 5 mL CH_3CN. Compound **aBCL2** (22 mg) was obtained as an amorphous brownish-red solid in 75% yield that decomposed before melting. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.86 (s, 1H), 8.17 (d, 1H, J = 8.8 Hz), 7.75 (d, 1H, J = 8.8 Hz), 7.71 (s, 1H), 7.63 (s, 1H), 5.04 (q, 2H, J = 7.2 Hz), 3.77 (t, 2H, J = 5.6 Hz), 3.63 (s, 3H), 3.03 (t, 2H, J = 6.0 Hz), 2.70 (s, 3H), 2.14−2.08 (m, 2H), 1.68 (t, 3H, J = 7.2 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 154.7, 142.3, 138.1, 135.2, 133.5, 132.5, 130.2, 128.3, 124.6, 123.7, 117.6, 99.2, 55.5, 52.9, 42.6, 27.9, 21.9, 20.4, 14.5. ESI-HRMS calcd for $C_{19}H_{22}N_3$ $[M + H]^+$ m/z 292.1808, found m/z 292.1815.

2-(1,2,3,5,6,7-Hexahydropyrido[3,2,1-*ij*]quinolin-8-yl)-4-methylaniline (3c). This compound was synthesized in a manner analogous to that of **3a**, from compound **1a** (899 mg, 3.00 mmol), **2c** (500 mg, 2.70 mmol), Na_2CO_3 (859 mg, 8.10 mmol), $PdCl_2$(dppf) (220 mg, 0.27 mmol), and 20 mL of a combined solvent of H_2O, EtOH, and benzene. The desired **3c** (270 mg) was obtained as a yellowish crystalline solid in 73% yield. Mp: 64.4−64.9 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.95 (dd, 1H, J = 8.0, 1.6 Hz), 6.86−6.84 (m, 2H), 6.66 (d, 1H, J = 8.0 Hz), 6.41 (d, 1H, J = 8.0 Hz), 3.44 (s, 2H), 3.18 (t, 2H, J = 5.6 Hz), 3.13 (t, 2H, J = 5.6 Hz), 2.83−2.79 (m, 2H), 2.49 (t, 2H, J = 6.8 Hz), 2.25 (s, 3H), 2.05−1.98 (m, 2H), 1.92−1.86 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 143.3, 141.2, 136.6, 130.6, 128.4, 128.3, 127.2, 127.0, 120.9, 120.4, 117.5, 115.0, 50.4, 50.0, 27.9, 25.3, 22.2, 22.1, 20.5. ESI-HRMS calcd for $C_{19}H_{23}N_2$ $[M + H]^+$ m/z 279.1856, found m/z 279.1858.

13-Methyl-1,2,3,5,6,7-hexahydrobenzo[*c*]quinolizino[1,9-*fg*]cinnoline (4c). This compound was synthesized in a manner analogous to that of **4a**, from compound **3c** (215 mg, 0.77 mmol) and $NaNO_2$ (64 mg, 0.92 mmol). Compound **4c** (160 mg) was obtained as a red amorphous solid in 72% yield. Mp: 161.4−161.8 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.41 (d, 1H, J = 8.4 Hz), 8.26 (s, 1H), 8.01 (s, 1H), 7.53 (d, 1H, J = 8.4 Hz), 3.40−3.37 (m, 6H), 2.98 (t, 2H, J = 6.4 Hz), 2.58 (s, 3H), 2.05−1.98 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$):

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δ 145.7, 145.3, 140.7, 138.4, 130.4, 129.4, 129.3, 126.3, 125.8, 122.0, 120.8, 111.7, 51.1, 49.7, 29.4, 28.8, 22.7, 21.8, 21.3. ESI-HRMS calcd for $C_{19}H_{20}N_3$ $[M + H]^+$ *m/z* 290.1652, found *m/z* 290.1657.

10-Ethyl-13-methyl-1,2,3,5,6,7-hexahydrobenzo[*c*]quinolizino[1,9-*fg*]cinnolin-10-ium Iodide (aBCL3). This compound was synthesized in a manner analogous to that of **aBCL1**, from compound **4c** (120 mg, 0.41 mmol), EtI (128 mg, 0.82 mmol), K_2CO_3 (113 mg, 0.82 mmol), and 20 mL of CH_3CN. Compound **aBCL3** (109 mg) was obtained as a red amorphous solid in 84% yield that decomposed before melting. ^1H NMR (400 MHz, $CDCl_3$): δ 8.38 (s, 1H), 8.09 (d, 1H, *J* = 8.8 Hz), 7.75 (d, 1H, *J* = 8.8 Hz), 7.66 (s, 1H), 4.96 (q, 2H, *J* = 7.2 Hz), 3.87−3.79 (m, 4H), 3.48 (t, 2H, *J* = 5.6 Hz), 3.06 (t, 2H, *J* = 5.6 Hz), 2.64 (s, 3H), 2.16−2.13 (m, 4H), 1.69 (t, 3H, *J* = 7.2 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 153.7, 139.6, 138.0, 134.5, 133.8, 131.3, 130.5, 128.4, 125.1, 124.0, 116.9, 114.3, 54.8, 53.0, 51.7, 28.4, 28.3, 22.4, 20.8, 20.2, 14.3. ESI-HRMS calcd for $C_{21}H_{24}N_3$ $[M + H]^+$ *m/z* 318.1965, found *m/z* 318.1977.

2-(1,2,3,5,6,7-Hexahydropyrido[3,2,1-*ij*]quinolin-8-yl)aniline (3d). This compound was synthesized in a manner analogous to that of **3a**, from compound **1b** (500 mg, 2.92 mmol), **2c** (960 mg, 3.21 mmol), Na_2CO_3 (929 mg, 8.76 mmol), $Pd(PPh_3)_4$ (35 mg, 0.03 mmol), and 30 mL of a combined solvent of H_2O, EtOH, and benzene. The desired **3d** (686 mg) was obtained as a yellowish crystalline solid in 89% yield. Mp: 67.7−70.3 °C. ^1H NMR (400 MHz, $CDCl_3$): δ 7.16 (t, 1H, *J* = 7.6 Hz), 7.06 (d, 1H, *J* = 7.6 Hz), 6.89 (d, 1H, *J* = 7.6 Hz), 6.81 (t, 1H, *J* = 7.6 Hz), 6.76 (d, 1H, *J* = 7.6 Hz), 6.46 (d, 1H, *J* = 7.6 Hz), 3.59 (s, 2H), 3.22 (t, 2H, *J* = 5.6 Hz), 3.15 (t, 2H, *J* = 5.6 Hz), 2.91−2.79 (m, 2H), 2.52 (t, 2H, *J* = 6.4 Hz), 2.08−2.02 (m, 2H), 1.95−1.89 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 143.8, 143.4, 136.5, 130.1, 128.1, 128.0, 127.1, 121.0, 120.5, 118.1, 117.5, 114.9, 50.4, 50.1, 27.9, 25.3, 22.2, 22.1. ESI-HRMS calcd for $C_{17}H_{18}N_3$ $[M + H]^+$ *m/z* 265.1699, found *m/z* 265.1708.

1,2,3,5,6,7-Hexahydrobenzo[*c*]quinolizino[1,9-*fg*]cinnoline (4d). The compound was synthesized in a manner analogous to that of **4a**, from compound **3d** (300 mg, 1.14 mmol) and $NaNO_2$ (94 mg, 1.37 mmol). Compound **4d** (226 mg) was obtained as a red amorphous solid in 72% yield. Mp: 158.4−161.2 °C. ^1H NMR (400 MHz, $CDCl_3$): δ 8.56−8.54 (m, 2H), 8.09 (s, 1H), 7.74 (t, 1H, *J* = 7.6 Hz), 7.66 (t, 1H, *J* = 7.6 Hz), 3.47−3.41 (m, 6H), 3.04 (t, 2H, *J* = 6.4 Hz), 2.10−2.02 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 146.6, 146.0, 140.7, 130.7, 129.5, 128.2, 127.5, 126.3, 126.3, 121.8, 120.9, 111.6, 51.1, 49.7, 29.3, 28.8, 21.8, 21.2. ESI-HRMS calcd for $C_{18}H_{18}N_3$ $[M + H]^+$ *m/z* 276.1495, found *m/z* 276.1504.

10-Ethyl-1,2,3,5,6,7-hexahydrobenzo[*c*]quinolizino[1,9-*fg*]cinnolin-10-ium Iodide (aBCL4). This compound was synthesized in a manner analogous to that of **aBCL1**, from compound **4d** (100 mg, 0.36 mmol), EtI (112 mg, 0.72 mmol), K_2CO_3 (99 mg, 0.72 mmol), and 20 mL of CH_3CN. Compound **aBCL4** (88 mg) was obtained as a red amorphous solid in 80% yield that decomposed before melting, ^1H NMR (400 MHz, $CDCl_3$): δ 8.63 (d, 1H, *J* = 8.4 Hz), 8.13 (d, 1H, *J* = 8.4 Hz), 7.93 (t, 1H, *J* = 8.4 Hz), 7.80 (t, 1H, *J* = 8.4 Hz), 7.67 (s, 1H), 4.96 (q, 2H, *J* = 7.2 Hz), 3.86 (t, 4H, *J* = 5.6 Hz), 3.48 (t, 2H, *J* = 6.0 Hz), 3.08 (t, 2H, *J* = 6.0 Hz), 2.18−2.12 (m, 4H), 1.70 (t, 3H, *J* = 7.2 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): δ 154.0, 138.0, 136.0, 132.0, 131.4, 130.6, 129.2, 128.5, 125.2, 123.5, 116.7, 114.6, 54.5, 53.1, 51.7, 28.2, 28.1, 20.6, 20.0, 14.1. ESI-HRMS calcd for $C_{20}H_{22}N_3$ $[M + H]^+$ *m/z* 304.1808, found *m/z* 304.1804.

4-Fluoro-2-(1,2,3,5,6,7-hexahydropyrido[3,2,1-*ij*]quinolin-8-yl)aniline (3e). This compound was synthesized in a manner analogous to that of **3a**, from compound **1c** (300 mg, 1.59 mmol), **2c** (570 mg, 1.91 mmol), Na_2CO_3 (506 mg, 4.77 mmol), $Pd(PPh_3)_4$ (23 mg, 0.02 mmol), and 30 mL of a combined solvent of H_2O, EtOH, and benzene. The desired **3e** (377 mg) was obtained as a yellowish viscous residue in 84% yield. ^1H NMR (400 MHz, $CDCl_3$): δ 6.86−6.81 (m, 2H), 6.76 (dd, 1H, *J* = 9.2, 3.2 Hz), 6.65 (dd, 1H, *J* = 8.8, 4.8 Hz), 6.38 (d, 1H, *J* = 7.6 Hz), 3.41 (s, 2H), 3.18 (t, 2H, *J* = 5.6 Hz), 3.13 (t, 2H, *J* = 5.6 Hz), 2.82−2.75 (m, 2H), 2.47 (t, 2H, *J* = 6.4 Hz), 2.04−1.98 (m, 2H), 1.92−1.86 (m, 2H). ^{19}F NMR (376 MHz, $CDCl_3$): δ −127.36 to −127.40 (m). ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.0 (d, *J* = 234 Hz), 143.3, 139.9, 135.4, 129.3 (d, *J* = 7 Hz), 127.1, 121.3, 120.1, 117.0, 116.4 (d, *J* = 22 Hz), 115.6 (d, *J* = 7 Hz), 114.3 (d, *J* = 22 Hz), 50.3, 49.9, 27.9, 25.2, 22.1, 22.0. EI-HRMS calcd for $C_{18}H_{19}FN_2$ $[M - e^-]^+$ *m/z* 282.1532, found *m/z* 282.1530.

13-Fluoro-1,2,3,5,6,7-hexahydrobenzo[*c*]quinolizino[1,9-*fg*]cinnoline (4e). This compound was synthesized in a manner analogous to that of **4a**, from compound **3e** (158 mg, 0.56 mmol) and $NaNO_2$ (46 mg, 0.67 mmol). Compound **4e** (144 mg) was obtained as a red amorphous solid in 88% yield. Mp: 137.1−138.3 °C. ^1H NMR (400 MHz, $CDCl_3$): δ 8.56−8.53 (dd, 1H, *J* = 8.8, 6.4 Hz), 8.18 (dd, 1H, *J* = 11.6, 2.0 Hz), 8.07 (s, 1H), 7.48 (td, 1H, *J* = 8.4, 2.4 Hz), 3.45−3.37 (m, 6H), 3.04 (t, 2H, *J* = 6.0 Hz), 2.10−2.03 (m, 4H). ^{19}F NMR (376 MHz, $CDCl_3$) δ −108.60 to −108.67 (m). ^{13}C NMR (100 MHz, $CDCl_3$): δ 161.4 (d, *J* = 247 Hz), 145.9, 143.9, 140.1, 133.2 (d, *J* = 10 Hz), 129.5, 127.0, 123.2 (d, *J* = 10 Hz), 120.5 (d, *J* = 4 Hz), 116.8 (d, *J* = 25 Hz), 111.5, 111.0 (d, *J* = 25 Hz), 51.1, 49.6, 29.0, 28.8, 21.7, 21.1. EI-HRMS calcd for $C_{18}H_{16}FN_3$ $[M - e^-]^+$ *m/z* 293.1328, found *m/z* 293.1329.

10-Ethyl-13-fluoro-1,2,3,5,6,7-hexahydrobenzo[*c*]quinolizino[1,9-*fg*]cinnolin-10-ium Iodide (aBCL5). This compound was synthesized in a manner analogous to that of **aBCL1**, from compound **4e** (80 mg, 0.27 mmol), EtI (84 mg, 0.54 mmol), K_2CO_3 (75 mg, 0.54 mmol), and 20 mL of CH_3CN. Compound **aBCL5** (66 mg) was obtained as a red amorphous solid in 76% yield that decomposed before melting. ^1H NMR (400 MHz, CD_3OD): δ 8.34−8.31 (m, 2H), 7.80 (s, 1H), 7.77 (td, 1H, *J* = 8.6, 2.4 Hz), 4.98 (q, 2H, *J* = 7.2 Hz), 3.78−3.73 (m, 4H), 3.43 (t, 2H, *J* = 6.4 Hz), 3.08 (t, 2H, *J* = 6.0 Hz), 2.16−2.10 (m, 4H), 1.69 (t, 3H, *J* = 7.2 Hz). ^{19}F NMR (376 MHz, $CDCl_3$): δ −105.95 to −106.01 (m). ^{13}C NMR (100 MHz, CD_3OD): δ 164.8 (d, *J* = 248 Hz), 157.6, 141.4, 137.2, 135.6, 134.8, 129.5 (d, *J* = 9 Hz), 128.7 (d, *J* = 1 Hz), 124.8 (d, *J* = 25 Hz), 124.0 (d, *J* = 9 Hz), 118.4, 117.8 (d, *J* = 25 Hz), 58.7, 56.6, 55.0, 31.8, 31.7, 24.2, 23.7, 17.5. ESI-HRMS calcd for $C_{20}H_{21}FN_3$ $[M + H]^+$ *m/z* 322.1714, found *m/z* 322.1722.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

General methods, additional spectral studies, and cell cytotoxic studies. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.5b00242.

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The authors declare no competing financial interest.

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