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Design and Synthesis of Fluorescent Sensors for Zinc Ion Derived from 2-Aminobenzamide

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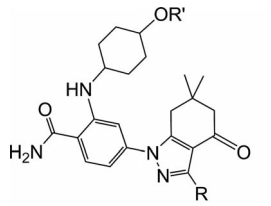
Four novel Zn^{2+} fluorescent sensors have been designed with *N*,*N*-bis(2-pyridylmethyl)ethylenediamine as chelator and 2-aminobenzamide as fluorophore. These sensors were prepared in two or three steps from readily available starting materials. Of the four designed sensors, ZnABA was found to be the most efficient Zn^{2+}-specific fluorescent probe and has good solubility in biological buffer, a large Stokes shift (186 nm), a high off–on fluorescence response (16-fold enhancement), and distinct selectivity towards Zn^{2+} over other metal ions. Our results have demonstrated an excellent linear relationship between the fluorescence intensity of ZnABA and the Zn^{2+} concentration from 0 to 10 μM, which indicates that ZnABA has the potential to be used for the quantitative determination of Zn^{2+} in an aqueous environment.

Introduction

The zinc ion (Zn^{2+}) is considered the second most abundant heavy metal in the human body.[1] It has been found that Zn^{2+} is widely distributed in all organisms, especially in the brain,[2] pancreas,[3] and spermatozoa.[4] Many biological studies have implicated the significance of Zn^{2+} in gene expression, apoptosis, enzyme regulation, and immunological modulation, etc.[5] Zinc deficiency usually leads to several diseases and disorders including anorexia, diabetes, hypogonadism, immune dysfunction, and growth retardation.[6] Therefore considerable effort has been focused on developing highly sensitive and selective fluorescent sensors to detect Zn^{2+} in biological and environmental systems.

In 1987, Frederickson et al.[7] reported the first Zn^{2+}-specific fluorescent probe based on *p*-toluenesulfonamide–quinoline. Over the years, many kinds of fluorescent sensor molecules have been designed and synthesized for measuring Zn^{2+} in vivo or in vitro.[8] In general, the chemical structure of a molecular probe for Zn^{2+} is composed of two components: A chelator and a fluorophore. The most typical chelators for Zn^{2+} include *N*,*N*-bis(2-pyridylmethyl)-amine (BPA),[9] *N*,*N*-bis(2-pyridylmethyl)ethylenediamine (BPEA),[10] *N*,*N*,*N*′-tris(2-pyridylmethyl)ethane-1,2-diamine (TPEA),[11] and 1,4,7,10-tetraazacyclodecane.[12] Various fluorophore structures have been reported, for example, quinoline,[13] fluorescein,[9a,14] anthracene,[12a,15] benzoxazole,[11b,16] phthalimide,[17] and cyanine.[18] Although great progress has been made in the synthesis of novel Zn^{2+} sensors, the fluorophores used are essentially known fluorescent organic molecules. To the best of our knowledge, there have been no reports of bioactive small molecules with fluorescent properties being used for the design of Zn^{2+} sensors. The development of novel multifunctional organic molecules for Zn^{2+}-specific fluorescent probes remains an interesting and important challenge.

In recent years, novel 2-aminobenzamide analogues **1a**–**1c** (Figure 1) have been developed as heat shock protein 90 (Hsp90) inhibitors[19] with **1b** being in multiple phase I clinical trials.[19a] Previously, we reported[20] the molecular mechanism of apoptosis of human chronic myeloid leukemia (CML) K562 cells by **1b** and developed a sensitive and specific reversed-phase high-performance liquid chromatography method for the identification and quantification of **1b** in rat plasma. More recently, **1c** (BJ-B11) was synthe-



R = CH_3, R' = H **1a**
R = CF_3, R' = H **1b**
R = CH_3, R' = OAc **1c**, BJ-B11

Figure 1. Structures of 2-aminobenzamide analogues **1a**–**1c**.

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sized and evaluated for in vitro antiviral activity against several viruses;[21] we found **1c** to be potent against HSV, and it is currently being evaluated for use in anti-HSV clinical trials.

During the synthesis of these 2-aminobenzamides, it came to our notice that compounds **1a**–**1c** possess strong fluorescent properties. As a part of our efforts to develop new 2-aminobenzamide analogues and study their new functions, herein, we report the design and synthesis of novel fluorescent sensors for Zn^{2+} based on 2-aminobenzamide.

Results and Discussion

Design of Zn^{2+} Sensors

A desirable Zn^{2+} sensor for biological and environmental applications should possess unique characteristics and properties, such as sufficient water solubility, high selectivity/sensitivity, long shelf-life, good photostability, and facile preparation. Initially we screened the fluorescence response of the known Hsp90 inhibitor **1a** to different metal ions. Unfortunately, no obvious fluorescence turn-on or quenching was observed for any of the metal ions used. To develop an efficient Zn^{2+} fluorescent probe, it was necessary to modify the chemical structure of **1a**.

Pyridyl-containing ligands such as BPA, BPEA, and TPEA[9–11] have been reported to show high selectivity towards Zn^{2+} over other metal ions. In this study, we introduced BPEA as chelator for Zn^{2+} at the 2-position of 4-indazolone-substituted benzamide. The chemical structure of the first designed Zn^{2+} sensor (ZnABA, **2**) is shown in Figure 2. For comparison, the BPEA and indazolone moieties on the benzamide were interchanged to make the second Zn^{2+} sensor **3**. Moreover, to further understand the influence of the indazolone substituent on the fluorescence, di-indazolone–2-aminobenzamide **4** and **5** with no indazolone substituent were synthesized (Figure 2).

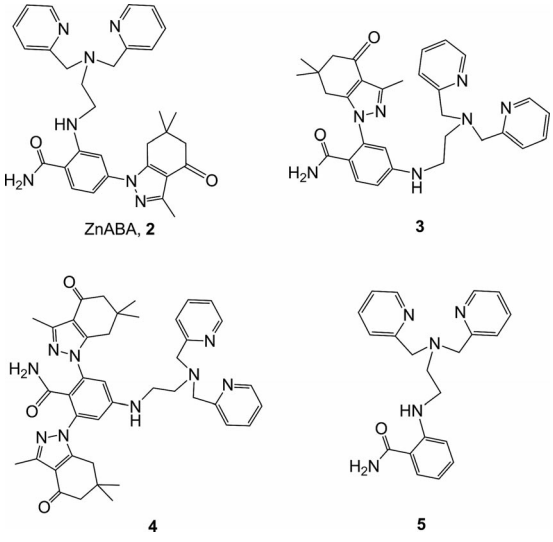
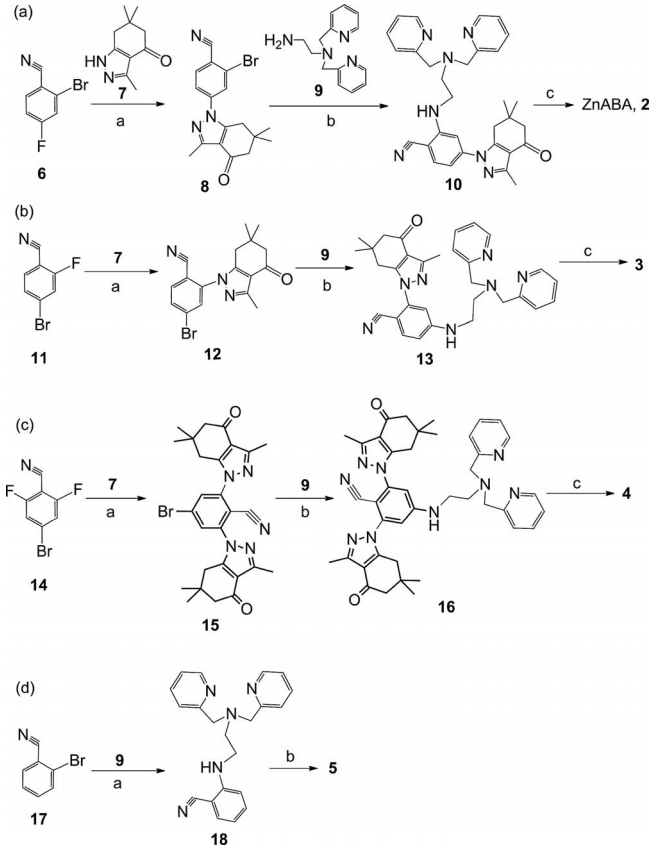


Figure 2. Structures of the newly designed Zn^{2+} sensors **2**–**5** with a 2-aminobenzamide scaffold.

Synthesis of the Zn^{2+} Sensors

The synthetic routes to ZnABA and **3**–**5** are shown in Scheme 1. All of these compounds can be easily prepared in two to three steps with readily available starting materials.



Scheme 1. Synthesis of 2-aminobenzamides. Reagents and conditions: (a) K_2CO_3, DMF or NaH, DMF; (b) $PdCl_2$, DPPF, NaO*t*Bu, toluene, 100 °C; (c) KOH, H_2O_2, EtOH/DMSO.

For the synthesis of ZnABA, initially, 2-bromo-4-fluorobenzonitrile (**6**) was coupled with indazol-4-one **7** in the presence of NaH to afford the desired compound **8** in a moderate yield (60%; Table 1, Entry 1). When the weaker base K_2CO_3 was used instead of NaH for the reaction, a more satisfactory yield (82%) was obtained (Table 1, Entry 2), which indicates that the strong base NaH leads to

Table 1. Coupling of **7** with bromobenzonitrile by using different bases.

Entry	Substrate	Base	Product	Yield [%]
1	**6**	NaH	**8**	60
2	**6**	K_2CO_3	**8**	82
3	**11**	NaH	**12**	40
4	**11**	K_2CO_3	**12**	92
5	**14**	NaH	**15**	n.d.
6	**14**	K_2CO_3	**15**	72

many more complicated byproducts, as detected by TLC during the substitution.

Then BPEA was incorporated into the aromatic ring under Buchwald–Hartwig conditions[22] by using catalytic $PdCl_2$, 1,1′-bis(diphenylphosphanyl)ferrocene (DPPF), and NaO*t*Bu in toluene at 100 °C. Note that the substrate concentration had a significant influence on the Pd-mediated coupling yield (Table 2, Entries 1 and 2). The decrease in the substrate (**8**) concentration from 0.05 to 0.025 M led to a higher yield of the product (from 22 to 50%). This is due to the fact that BPEA is poorly soluble in toluene. Thus, the reaction should be more effective when performed at low concentrations.

Table 2. Effect of substrate concentration on the Buchwald–Hartwig coupling reaction.

Entry	Substrate	[Substrate] [mol L^{-1}]	Product	Yield [%]
1	**8**	0.05	**10**	22
2	**8**	0.025	**10**	50
3	**12**	0.07	**13**	22
4	**12**	0.04	**13**	78
5	**15**	0.05	**16**	<5
6	**15**	0.026	**16**	44
7	**17**	0.066	**18**	33
8	**17**	0.025	**18**	59

Finally, hydration[23] of the resulting benzonitrile **10** with KOH catalyzed by H_2O_2 in a mixture of EtOH/DMSO produced the Zn^{2+} sensor ZnABA (**2**) in high yield (87%). The crystal structure of ZnABA was confirmed by single-crystal X-ray diffraction (Figure 3).[24]

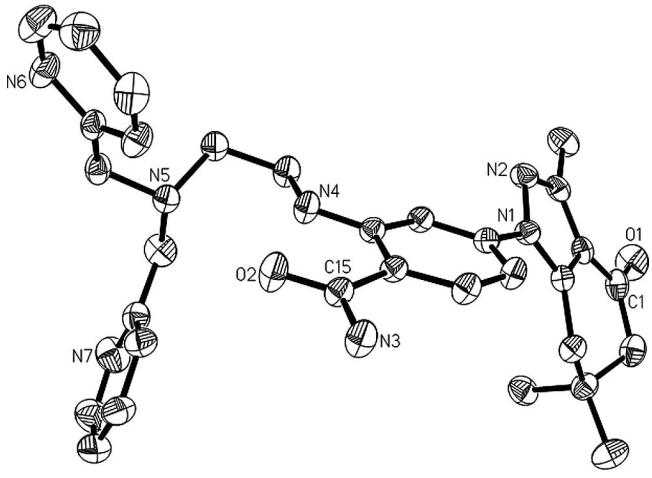


Figure 3. X-ray crystallographic structure of ZnABA.

Similar synthetic methods were used to synthesize 2-aminobenzamides **3**–**5**. It was observed that K_2CO_3 was a better base for the coupling of indazol-4-one **7** with bromobenzonitriles **11** and **14** (Table 1, Entries 3–6). Notably, when the aromatic nucleophilic substitution reaction of 4-bromo-2,6-difluorobenzonitrile (**14**) with **7** was carried out with NaH as the base, the desired product was not obtained (Table 1, Entry 5), which suggests that **14** is very sensitive to strong bases and quickly decomposes under the reaction conditions.

In addition, for Buchwald–Hartwig coupling reactions with **12**, **15**, and **17** as substrates, higher coupling yields were achieved with lower substrate concentrations (Table 2, Entries 3–8). These results are consistent with those obtained with substrate **8**.

Fluorescence Properties

With the synthesized Zn^{2+} sensors **2**–**5** in hand, the fluorescence spectra were first recorded in 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer (pH = 7.4), and the results are shown in Figure 4. Compared with the strong fluorescence emissions of **1a** and **5** at around 440 nm, ZnABA, **3**, and **4** exhibited weak fluorescence emissions, which showed their potential as off–on Zn^{2+} sensors. However, upon addition of Zn^{2+} (1 equiv.), only the emission intensity of ZnABA increased significantly. Therefore, ZnABA was chosen as the Zn^{2+} sensor suitable for further evaluation.

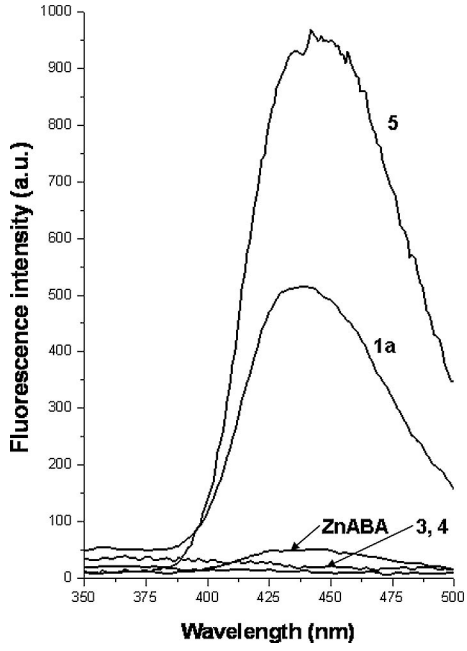


Figure 4. Fluorescence emission spectra of **1a** and **2**–**5** in aqueous solution (10 μM, 25 mM HEPES buffer, 0.1 M $NaClO_4$, pH = 7.4, I = 0.1). All the excitation wavelengths are at 260 nm.

Table 3 shows the absorption and fluorescence properties of all the 2-aminobenzamides used in this study. Compound **5** (without the indazolone moiety in its structure) has the smallest ε value (1.35×10^4 M^{-1} cm^{-1}), whereas compound **4** (with two indazolone moieties) possesses the highest ε value (3.23×10^4 M^{-1} cm^{-1}) among the designed Zn^{2+} sensors (**2**–**5**). ZnABA has the lowest fluorescence quantum yield (Φ_0 = 0.0096). After titration with 1 equiv. of Zn^{2+}, the value of Φ for the complex of ZnABA–Zn^{2+} increased significantly (Φ_{Zn} = 0.077), thus showing an efficient off–on response with Zn^{2+}.

Table 3. Absorption and fluorescence properties of **1a** and **2**–**5**.

Compound	ε[a] [M^{-1} cm^{-1}]	λ_{em}[b] [nm]	Φ[c]
1a	36500	438	0.012
2[d]	28835	–	0.0096
2+Zn^{2+}[e]	21900	446	0.077
3	27900	–	0.014
4	32380	–	0.015
5	13520	441	0.286

[a] Data were evaluated at the maximum λ_{abs} of 260 nm. [b] The excitation wavelength was 260 nm. [c] The fluorescence quantum yields were obtained by using quinine sulfate (in 0.05 M H_2SO_4, Φ = 0.55) as the standard. [d] Data were determined in the absence of Zn^{2+}. [e] Data were determined in the presence of 1.0 equiv. Zn^{2+}.

ZnABA readily dissolves in HEPES buffer (pH = 7.4) and fluoresces weakly with λ_{ex} and λ_{em} at 260 and 446 nm, respectively. Addition of Zn^{2+} (0–1.5 equiv.) revealed that the emission intensity is dependent upon the Zn^{2+} concentration and reaches a maximum at $Zn^{2+} \geq 1.0$ equiv. (Figure 5). Its Stokes shift is 186 nm, which is much larger than the reported values of many Zn^{2+} sensors.[9–18]

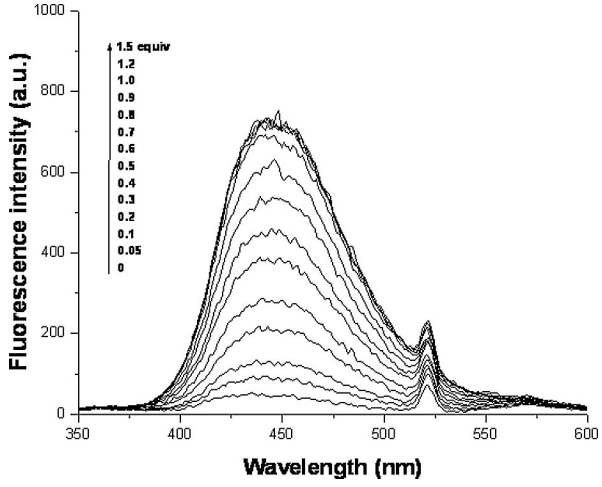


Figure 5. Fluorescence emission spectra (excitation at 260 nm) of ZnABA (10 μM; in 25 mM HEPES buffer, 0.1 M $NaClO_4$, pH = 7.4, I = 0.1) upon addition of Zn^{2+} [added as $Zn(ClO_4)_2$, 0–15 μM]. The small peak at 520 nm arises from second-order scattering.

Note that the fluorescence emission intensity of ZnABA was enhanced 16-fold upon addition of 1.0 equiv. of Zn^{2+}. This enhancement can be explained by the intermolecular charge transfer (ICT) effect of the aromatic plane with BPEA as the conjugated electron donor and tetrahydroindazol-4-one as the receptor. The UV titration spectra show two absorption bands centered at 355 and 260 nm (Figure S1, Supporting Information). On addition of 0–1 equiv. Zn^{2+}, the intensities of both bands decreased, and the original 355 nm band was blueshifted to 350 nm, which suggests that on Zn^{2+} binding (1) the electron-donating ability of the 2-amino group in the BPEA moiety is decreased and (2) the electron-withdrawing ability of the 1-carbamoyl group is increased. The clear isosbestic points at 317 and 275 nm also demonstrate the formation of the ZnABA–Zn^{2+} complex (Figure S1). Furthermore, a Job plot (Figure S2, Supporting Information) shows the 1:1 binding of ZnABA and Zn^{2+}. On the basis of all these results as well as the X-ray crystallographic structure of free ZnABA, a Zn^{2+} binding mode has been proposed (Figure S3).

The selectivity of ZnABA towards Zn^{2+} and other metal ions was examined, and the results are shown in Figure 6. No change in the fluorescence emission intensity was observed upon addition of the metal cations Ba^{2+}, Ca^{2+}, Cr^{3+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Pb^{2+}, the transition-metal ions Co^{2+} and Cu^{2+} quenched the fluorescence to some extent, and both Zn^{2+} and Cd^{2+} induced turn-on fluorescence, however, the fluorescence enhancement (16-fold) of ZnABA towards Zn^{2+} is higher than that towards Cd^{2+} (8-fold).

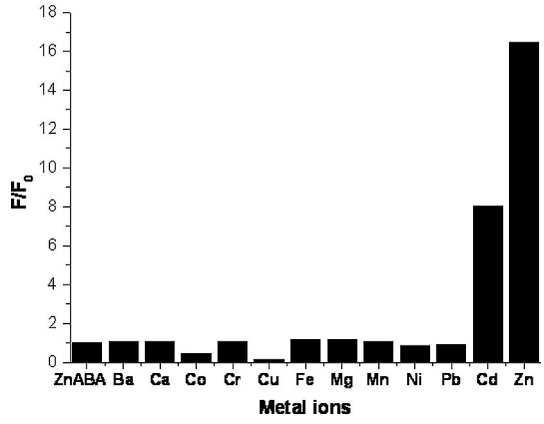


Figure 6. Selectivity of ZnABA towards Zn^{2+} and other metal ions. Experimental conditions: ZnABA (10 μM; in 25 mM HEPES buffer, 0.1 M $NaClO_4$, pH = 7.4, I = 0.1), 10 μM Ba^{2+}, Ca^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Cd^{2+}, and Zn^{2+}. λ_{ex} = 260 nm, λ_{em} = 446 nm.

For compounds **3** and **4**, the BPEA and indazolone moieties are interchanged on the aromatic ring with respect to ZnABA. Although the electron-donating ability of the 2-amino group decreased as a result of Zn^{2+} binding, the electron-withdrawing ability of the 1-carbamoyl group was not affected, because the 1-carbamoyl group cannot participate in the binding of Zn^{2+} along with BPEA. Therefore, the fluorescence emission intensities of **3** and **4** upon addition of Zn^{2+} did not increase (Figures S4 and S5). For compound **5**, there is no indazolone substituent in its scaffold. It employs photoinduced electron transfer (PET) and exhibits an on–off response with Co^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} (Figure S6).

As a Zn^{2+}-specific fluorescent probe, the water-soluble ZnABA has the potential to be used for the quantitative determination of Zn^{2+} in an aqueous environment, because there is a good linear relationship between the fluorescence intensity of ZnABA and the concentration of Zn^{2+} between 0 and 10 μM (Figure S7).

Conclusions

Four novel Zn^{2+} fluorescent sensors, ZnABA, **3**, **4**, and **5**, have been designed with BPEA as the chelator and the 2-aminobenzamide scaffold as the fluorophore. Both the use of the weaker base K_2CO_3 in the aromatic nucleophilic substitution and a low substrate concentration in the Buchwald–Hartwig coupling reaction benefited the efficient preparation of these probes. Of the four designed sensors, ZnABA was found to be the most desirable Zn^{2+}-specific fluorescent probe and exhibited good solubility in biological buffer, a large Stokes shift (186 nm), a high off–on fluorescence response (16-fold enhancement), and a distinct selectivity towards Zn^{2+} over other metal ions. It has also been demonstrated that ZnABA employs an ICT mechanism, which is quite different from **5** for which PET operates, which suggests that indazolone substitution at the 4-postion of the benzamide is necessary for ICT fluorescence. Moreover, it is important that BPEA should be located at the 2-position of the aromatic ring, which can efficiently form a stable Zn^{2+} complex together with the 1-carbamoyl group. In addition, the excellent linear relationship between fluorescence emission intensity and Zn^{2+} concentration indicates that ZnABA may be used in the quantitative determination of Zn^{2+} in aqueous systems. Owing to the special properties of fluorescence and the pharmacological activity of its core structure, it is hoped that ZnABA can be used in biological applications.

Experimental Section

General: All chemicals were purchased as reagent grade and used without further purification. Buchwald–Hartwig cross-coupling reactions were performed in flame-dried glassware under argon. Toluene was distilled from calcium hydride. The reactions were monitored by analytical thin-layer chromatography (TLC) on silica gel F_{254} glass plates and visualized under UV light (254 and 365 nm) and/or by staining with ninhydrin. Flash column chromatography was performed on silica gel (200–300 mesh). ^{1}H NMR spectra were recorded with a Bruker Avance III 400 MHz NMR spectrometer at 20 °C. Chemical shifts (in ppm) were determined relative to tetramethylsilane (δ = 0 ppm) in deuteriated solvents. Coupling constants in Hz were measured from the one-dimensional spectra. ^{13}C NMR or ^{13}C attached-proton-test (^{13}C-Apt) spectra were recorded with the 400 MHz NMR spectrometer (100 MHz) and calibrated with $CDCl_3$ (δ = 77.23 ppm). High-resolution mass spectra were recorded with a Waters LCT Premier XE mass spectrometer. UV absorption and emission spectra were recorded with a GBC Cintra 10e UV/Vis spectrophotometer and a Varian Cary Eclipse spectrofluorimeter, respectively, in a quartz cell with a 1 cm path length.

2-Bromo-4-(3,6,6-trimethyl-4-oxo-4,5,6,7-tetrahydroindazol-1-yl)-benzonitrile (8): Compound **7**[25] (107 mg, 0.60 mmol, 1.20 equiv.) was dissolved in DMF, and 2-bromo-4-fluorobenzonitrile (**6**; 100 mg, 0.5 mmol, 1.00 equiv.) and K_2CO_3 (118 mg, 0.86 mmol, 1.72 equiv.) were added. The reaction mixture was stirred at room temperature for 30 min and then heated in a 40 °C oil bath until the starting material had been completely consumed as detected by TLC. The solution was then allowed to cool to room temperature, and the DMF was evaporated in vacuo to leave a yellowish oil. The crude oil was then diluted with dichloromethane (150 mL), washed with saturated sodium chloride solution (3 × 30 mL), and dried with magnesium sulfate. After removal of the solvent, the mixture was purified by column chromatography (hexanes/EtOAc, 4:1) and then recrystallized (CH_2Cl_2/hexanes) to give **8** (145 mg, 0.41 mmol, 82%) as a colorless solid. Its ^{1}H NMR spectrum was consistent with that reported in ref.[19a]

2-({2-[Bis(pyridin-2-ylmethyl)amino]ethyl}amino)-4-(3,6,6-trimethyl-4-oxo-4,5,6,7-tetrahydroindazol-1-yl)benzonitrile (10): A Schlenk flask was charged with compound **8** (100 mg, 0.28 mmol, 1.00 equiv.), BPEA[26] (100 mg, 0.41 mmol, 1.50 equiv.), sodium *tert*-butoxide (70 mg, 0.73 mmol, 2.60 equiv.), palladium chloride (20 mg, 0.11 mmol, 0.40 equiv.), 1,1′-bis(diphenylphosphanyl)ferrocene (DPPF; 40 mg, 0.072 mmol, 0.26 equiv.), and toluene (11 mL) under argon. The flask was immersed in an oil bath at 100 °C with stirring until the starting material had completely disappeared as judged by TLC analysis. The solution was then allowed to cool to room temperature, was diluted with dichloromethane (100 mL), filtered through Celite, and concentrated. The crude product was then purified further by column chromatography (CH_2Cl_2/MeOH, 45:1) on silica gel to give **10** (74 mg, 0.14 mmol, 50%) as a dark-red viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.09 (s, 6 H), 2.39 (s, 2 H), 2.52 (s, 3 H), 2.78 (s, 2 H), 2.94 (t, J = 5.47 Hz, 2 H), 3.30 (d, J = 4.81 Hz, 2 H), 3.91 (s, 4 H), 6.09 (br. s, 1 H), 6.69 (dd, J = 8.29, 1.74 Hz, 2 H), 6.74 (d, J = 1.56 Hz, 1 H), 7.16 (dd, J = 6.93, 5.64 Hz, 1 H), 7.48 (d, J = 8.28 Hz, 1 H), 7.58 (d, J = 7.82 Hz, 2 H), 7.69 (dt, J = 7.64, 1.66 Hz, 2 H), 8.56 (d, J = 4.31 Hz) ppm. ^{13}C-Apt (100 MHz, $CDCl_3$): δ = 13.37, 28.37, 29.65, 35.83, 37.64, 40.33, 51.53, 52.30, 60.04, 94.81, 105.60, 110.42, 117.45, 117.51, 122.25 (2 C), 123.26 (2 C), 133.84, 136.85 (2 C), 143.41, 149.04, 149.09 (2 C), 150.28, 151.26, 158.69, 191.23 ppm. HRMS (ESI): calcd. for $C_{31}H_{34}N_7O$ 520.2825; found 520.2826.

2-({2-[Bis(pyridin-2-ylmethyl)amino]ethyl}amino)-4-(3,6,6-trimethyl-4-oxo-4,5,6,7-tetrahydroindazol-1-yl)benzamide (ZnABA, 2): KOH (22 mg, 0.39 mmol, 5.65 equiv.) and compound **10** (36 mg, 0.069 mmol, 1 equiv.) were added to a solution of EtOH and DMSO (4:1, 1.12 mL). The reaction mixture was stirred in a 50 °C oil bath, and H_2O_2 (30%, 0.11 mL) was slowly added dropwise through a syringe over 0.5 h. Then the resulting solution was stirred for another 1.5 h until the disappearance of benzonitrile, as shown by TLC. After removal of the solvent by rotary evaporation, the mixture was diluted with dichloromethane (150 mL), washed with saturated sodium chloride solution (3 × 30 mL), dried with magnesium sulfate, filtered, and concentrated. The mixture was purified by column chromatography (CH_2Cl_2/MeOH, 20:1) to give ZnABA (32 mg, 0.060 mmol, 87%) as a colorless solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.09 (s, 6 H), 2.39 (s, 2 H), 2.54 (s, 3 H), 2.77 (s, 2 H), 2.91 (t, J = 6.04 Hz, 2 H), 3.32 (t, J = 6.08 Hz, 2 H), 3.90 (s, 4 H), 6.63 (dd, J = 8.40, 1.84 Hz, 1 H), 6.71 (d, J = 1.72 Hz, 1 H), 7.12 (t, J = 6.06 Hz, 1 H), 7.49 (d, J = 8.40 Hz, 1 H), 7.65 (t, J = 7.62 Hz, 2 H), 7.73 (d, J = 7.80, 2 H), 8.48 (d, J = 4.12 Hz, 2 H) ppm. ^{13}C-Apt (100 MHz, $CDCl_3$): δ = 13.39, 28.38 (2 C), 35.77, 37.50, 40.42, 52.36, 52.53, 53.38, 60.35, 106.38, 109.07, 112.95, 117.14, 122.04 (2 C), 123.24 (2 C), 129.48, 136.59 (2 C), 142.57, 148.81 (2 C), 149.03, 149.88, 150.64, 159.14, 171.17, 193.39 ppm. HRMS (ESI): calcd. for $C_{31}H_{36}N_7O_2$ 538.2930; found 538.2924.

4-Bromo-2-(3,6,6-trimethyl-4-oxo-4,5,6,7-tetrahydroindazol-1-yl)-benzonitrile (12): Compound **7** (214 mg, 1.2 mmol, 1.2 equiv.) was dissolved in DMF, and 4-bromo-2-fluorobenzonitrile (**11**; 200 mg, 1.0 mmol, 1.0 equiv.) and K_2CO_3 (236 mg, 1.7 mmol, 1.7 equiv.) were added. The reaction mixture was stirred at room temperature until the starting material had been completely consumed as judged

by TLC. After removal of DMF by rotary evaporation in vacuo, the mixture was then diluted with dichloromethane (150 mL), washed with saturated sodium chloride solution (3 × 30 mL), and dried with magnesium sulfate. After removal of the solvent, the mixture was purified by column chromatography (hexanes/EtOAc, 4:1) and then recrystallized (CH_2Cl_2/hexanes) to give **12** (330 mg, 0.92 mmol, 92%) as a colorless solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.13 (s, 6 H), 2.42 (s, 2 H), 2.55 (s, 3 H), 2.70 (s, 2 H), 7.67–7.75 (m, 3 H) ppm. ^{13}C-Apt (100 MHz, $CDCl_3$): δ = 13.32, 28.31, 36.09, 52.4, 109.07, 115.30, 117.30, 128.54, 131.32, 132.79, 134.58, 141.36, 151.26, 193.12 ppm. HRMS (ESI): calcd. for $C_{17}H_{17}BrN_3O$ 358.0555; found 358.0558.

4-({2-[Bis(pyridin-2-ylmethyl)amino]ethyl}amino)-2-(3,6,6-trimethyl-4-oxo-4,5,6,7-tetrahydroindazol-1-yl)benzamide (3): A Schlenk flask was charged with compound **12** (70 mg, 0.20 mmol, 1.00 equiv.), BPEA (70 mg, 0.29 mmol, 1.48 equiv.), sodium *tert*-butoxide (36 mg, 0.37 mmol, 1.85 equiv.), palladium chloride (19 mg, 0.11 mmol, 0.56 equiv.), DPPF (42 mg, 0.076 mmol, 0.38 equiv.), and toluene (5 mL) under argon. The flask was immersed in an oil bath at 100 °C with stirring until the starting material had been completely consumed as determined by TLC. The solution was then allowed to cool to room temperature, taken up in dichloromethane (100 mL), filtered, and concentrated. The crude product was then purified further by column chromatography (CH_2Cl_2/MeOH, 40:1) on silica gel to give **13** (79 mg, 0.15 mmol, 78%) as a viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.11 (s, 6 H), 2.40 (s, 2 H), 2.55 (s, 3 H), 2.70 (s, 2 H), 2.97 (s, 2 H), 3.23 (s, 2 H), 3.96 (s, 4 H), 6.58 (s, 1 H), 6.71 (d, J = 8.60 Hz, 1 H), 7.21 (br. s, 2 H), 7.38 (br. s, 2 H), 7.48 (d, J = 8.65 Hz, 2 H), 7.66 (br. s, 2 H), 8.60 (d, J = 4.43 Hz, 2 H) ppm. Then, by using the same procedure as that described for the preparation of ZnABA, compound **13** was hydrated with KOH catalyzed by H_2O_2 to give **3** in 90% yield as a colorless solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.06 (s, 6 H), 2.36 (s, 2 H), 2.43 (s, 2 H), 2.55 (s, 3 H), 2.94 (s, 2 H), 3.22 (s, 2 H), 3.95 (s, 4 H), 6.38 (s, 1 H), 6.75 (dd, J = 8.73, 2.28 Hz, 1 H), 7.18–7.21 (m, 2 H), 7.39 (br. s, 2 H), 7.63–7.67 (m, 2 H), 7.81 (d, J = 8.66 Hz, 1 H), 8.58 (d, J = 4.60 Hz, 2 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 13.46, 28.21, 35.38, 35.68, 40.93, 51.91, 52.54, 59.85, 110.33, 113.63, 116.36, 118.90, 122.29, 123.24, 132.37, 136.61, 137.00, 149.15, 150.19, 151.86, 152.32, 158.74, 167.79, 193.40 ppm. HRMS (ESI): calcd. for $C_{31}H_{36}N_7O_2$ 538.2930; found 538.2933.

4-Bromo-2,6-bis(3,6,6-trimethyl-4-oxo-4,5,6,7-tetrahydroindazol-1-yl)benzonitrile (15): Compound **7** (180 mg, 1.01 mmol, 2.2 equiv.) was dissolved in DMF, and 4-bromo-2,6-difluorobenzonitrile (**14**; 100 mg, 0.46 mmol, 1.0 equiv.) and K_2CO_3 (180 mg, 1.30 mmol, 2.8 equiv.) were added. The reaction mixture was stirred at room temperature until the starting material had been completely consumed as judged by TLC. After removal of DMF by rotary evaporation in vacuo, the mixture was then diluted with dichloromethane (150 mL), washed with saturated sodium chloride solution (3 × 30 mL), dried with magnesium sulfate, and filtered. After removal of the solvent, the mixture was purified by column chromatography (hexanes/EtOAc, 3.5:1) and then recrystallized (CH_2Cl_2/hexanes) to give **15** (176 mg, 0.33 mmol, 72%) as a colorless solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.14 (s, 12 H), 2.43 (s, 4 H), 2.57 (s, 6 H), 2.74 (s, 4 H), 7.82 (s, 2 H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 13.39, 14.13, 22.65, 28.32, 31.58, 35.07, 36.18, 52.34, 60.39, 107.25, 112.55, 117.61, 128.75, 131.34, 142.43, 151.52, 151.78, 193.01 ppm. HRMS (ESI): calcd. for $C_{27}H_{29}BrN_5O_2$ 534.1505; found 534.1503.

4-({2-[Bis(pyridin-2-ylmethyl)amino]ethyl}amino)-2,6-bis(3,6,6-trimethyl-4-oxo-4,5,6,7-tetrahydro-1*H*-indazol-1-yl)benzamide (4): A Schlenk flask was charged with compound **15** (70 mg, 0.13 mmol, 1.0 equiv.), BPEA (50 mg, 0.21 mmol, 1.6 equiv.), sodium *tert*-butoxide (35.7 mg, 0.37 mmol, 2.8 equiv.), palladium chloride (9 mg, 0.05 mmol, 0.38 equiv.), DPPF (20 mg, 0.036 mmol, 0.27 equiv.), and toluene (5 mL) under argon. The flask was immersed in an oil bath at 100 °C with stirring until the starting material had been completely consumed as judged by TLC. The solution was then allowed to cool to room temperature, taken up in dichloromethane (100 mL), filtered, and concentrated. The crude product was then purified further by column chromatography [CH_2Cl_2/MeOH, 40:1, containing 0.1% Et_3N (v/v)] on silica gel to give **16** (40 mg, 0.057 mmol, 44%) as a viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.13 (s, 12 H), 2.41 (s, 4 H), 2.57 (s, 6 H), 2.74 (s, 4 H), 2.98 (s, 2 H), 3.29 (s, 2 H), 3.97 (s, 4 H), 6.75 (s, 2 H), 7.22 (br. s, 2 H), 7.34 (d, J = 6.16 Hz, 2 H), 7.67 (t, J = 6.84 Hz, 2 H), 8.58 (d, J = 3.90 Hz, 2 H) ppm. Then, by using a procedure similar to that described for the preparation of ZnABA, compound **15** was hydrated by KOH catalyzed by H_2O_2 to give **4** in 83% yield as a colorless solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.08 (s, 12 H), 2.36 (s, 4 H), 2.53 (s, 6 H), 2.57 (s, 4 H), 2.95 (s, 2 H), 3.24 (s, 2 H), 3.94 (s, 4 H), 6.65 (s, 2 H), 7.20 (s, 2 H), 7.37 (s, 2 H), 7.66 (s, 2 H), 8.56 (d, J = 3.90 Hz, 2 H) ppm. ^{13}C-Apt (100 MHz, $CDCl_3$): δ = 13.45, 28.24, 29.69, 35.52, 35.80, 40.89, 51.70, 52.52, 59.34, 112.18, 116.18, 122.52, 123.54, 136.92, 138.16, 148.94, 149.82, 150.60, 151.79, 165.00, 193.42 ppm. HRMS (ESI): calcd. for $C_{41}H_{48}N_9O_3$ 714.3880; found 714.3889.

2-({2-[Bis(pyridin-2-ylmethyl)amino]ethyl}amino)benzamide (5): A Schlenk flask was charged with compound **17** (60 mg, 0.33 mmol, 1.14 equiv.), BPEA (71 mg, 0.29 mmol, 1.00 equiv.), sodium *tert*-butoxide (70 mg, 0.73 mmol, 2.52 equiv.), palladium chloride (19 mg, 0.11 mmol, 0.37 equiv.), DPPF (42 mg, 0.076 mmol, 0.26 equiv.), and toluene (13 mL) under argon. The flask was immersed in an oil bath at 100 °C with stirring until the starting material had been completely consumed as judged by TLC. The solution was then allowed to cool to room temperature, taken up in dichloromethane (100 mL), filtered, and concentrated. The crude product was purified further by column chromatography (CH_2Cl_2/MeOH, 45:1) on silica gel to give **18** (54 mg, 0.17 mmol, 59%) as a viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.95 (s, 2 H), 3.26 (s, 2 H), 3.95 (s, 4 H), 6.54 (d, J = 8.47 Hz, 1 H), 6.63 (t, J = 7.54 Hz, 1 H), 7.18 (t, J = 5.94 Hz, 2 H), 7.30–7.39 (m, 2 H), 7.65–7.75 (m, 4 H), 8.55 (d, J = 4.86 Hz, 2 H) ppm. Then, by using a procedure similar to that described for the preparation of ZnABA, compound **18** was hydrated by KOH catalyzed by H_2O_2 to give **5** in 83% yield as a colorless solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.92 (s, 2 H), 3.32 (s, 2 H), 3.95 (s, 4 H), 6.55–6.61 (m, 2 H), 7.16 (br. s, 2 H), 7.39 (d, J = 7.37 Hz, 2 H), 7.69 (t, J = 7.55 Hz, 2 H), 7.79 (d, J = 7.81 Hz, 2 H), 8.51 (d, J = 4.22 Hz, 2 H) ppm. ^{13}C-Apt (100 MHz, $CDCl_3$/CD_3OD): δ = 40.08, 52.74, 60.16, 111.69, 113.68, 114.61, 122.28, 123.34, 128.47, 133.31, 137.14, 148.31, 149.54, 159.10, 172.71 ppm. HRMS (ESI): calcd. for $C_{23}H_{26}N_3O$ 362.1981; found 362.1987.

Spectroscopic Materials and Methods: Stock solutions (0.001 M) of zinc perchlorate were prepared in HEPES buffer (25 mM HEPES, 0.1 M $NaClO_4$, pH = 7.4, I = 0.1). Stock solutions (0.001 M) of **1a**, ZnABA, **3**, **4**, and **5** were prepared in EtOH. All the fluorescence spectra were also measured in HEPES buffer [25 mM HEPES, 0.1 M $NaClO_4$, pH = 7.4, I = 0.1, 1% (v/v) EtOH], and the excitation wavelength was 260 nm with excitation and emission slit widths of 5 nm at room temperature.

Supporting Information (see also the footnote on the first page of this article): Figures S1–S7 and the ^{1}H and ^{13}C NMR spectra of new compounds.

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