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CuI-mediated synthesis of 1-aryl-5,6,7-trimethoxybenzimidazoles as potent antitubulin agents†

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In situ CuI-mediated cyclization methodology helped yield benzimidazoles with different substitution manner, such as 1,2-diarylbenzimidazoles (**4** and **5**) and 1-arylbenzimidazoles (**6–15**). The result of structure–activity relationship (SAR) study confirmed the significance of the 5,6,7-trimethoxybenzimidazole moiety, and the representative derivatives (**8–10**) exhibited marked antiproliferative activity against A549, HCT-116, and PC-3 cells; in addition, they are able to inhibit the polymerization of tubulin. Among them, compound **10** inhibited the growth of A549, HCT-116, and PC-3 cells with a mean IC_{50} value of 0.07 μM, and its IC_{50} value of tubulin polymerization is 0.26 μM.

Introduction

Cell proliferation is a crucial part of cancer progression, and mitosis is what fuels the spread of cancer cells.[1] Microtubules are polymers of tubulin and play an essential role in cell mitosis. It has been found that disrupting the function of microtubules, either by inhibiting or stabilizing the polymerization of tubulins can slow down the progress of mitosis.[2] Natural products such as vincristine, paclitaxel, and colchicine have been recognized as anti-mitotic agents and are used clinically. Combretastatin A4 (CA-4, **1**) was isolated from the bark of the South African tree *Combretum caffrum* in 1989 and was evaluated as a potent anti-mitotic agent.[3] It binds to the colchicine binding site and inhibits tubulin polymerization.[4] Structurally, CA-4 possesses a *Z*-olefinic bridge connecting the 3,4,5-trimethoxyphenyl (A ring) and the 3′-hydroxy-4′-methoxyphenyl (B-ring). It has been shown that the 3,4,5-trimethoxyphenyl and the *Z*-olefinic bridge as well as the 4′-methoxy group of the B ring are all exceptionally important for binding at the colchicine site and contribute to the potency of the compound. The 3′-hydroxy group of the B ring; however, could be replaced by other substituents without significantly decreasing its potency. The promising anti-mitotic activity of the CA-4 has attracted numerous efforts on the development of potent antitubulin agents (Fig. 1).[5]

There have been several attempts to modify the A ring, particularly replacing the three methoxy groups on the phenyl ring, but the results were mostly underwhelming.[6–9] Nonetheless, the A ring still has the potential to be modified by substituting the phenyl ring into heterocyclic rings. For instance, our previous study found 5,6,7-trimethoxyindole as a surrogate of 3,4,5-trimethoxyphenyl moiety of CA-4 and the resulting compound **2** showed comparable antiproliferative activity to that of CA-4.[10] In 2013, Zhao *et al.* developed C3-substituted *N*-methyl-5,6,7-trimethoxyindoles (**3**) showing

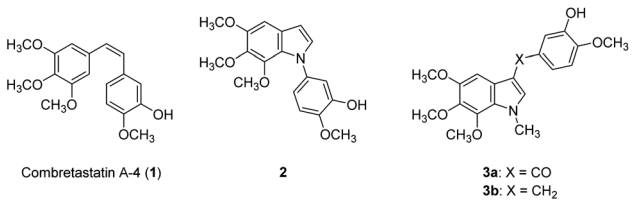

Fig. 1 Combretastatin A4 (**1**, CA-4) and 5,6,7-trimethoxyindoles (**2** and **3**).

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† Electronic supplementary information (ESI) available: HPLC purity data, ^{1}H NMR spectra and ^{13}C NMR spectra of compounds **4–15**. See DOI: https://doi.org/10.1039/d3ra01927f

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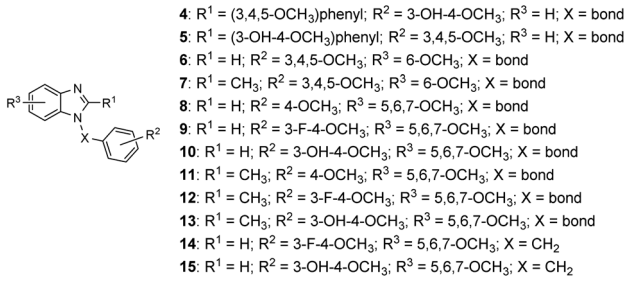
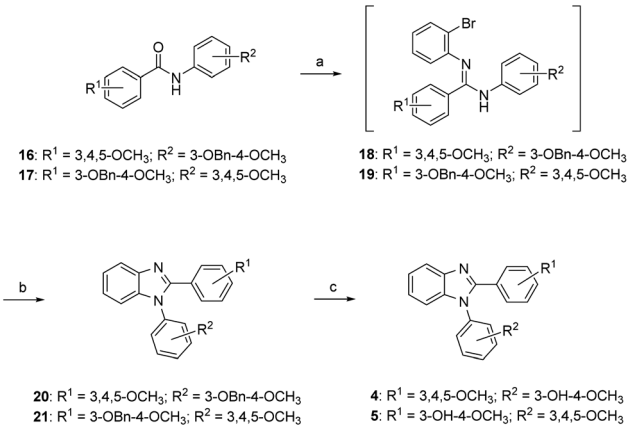
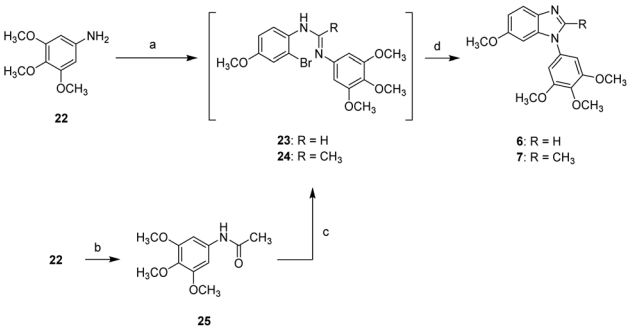


Fig. 2 Structures of 1,2-diarylbenzimidazoles (**4** and **5**) and 1-arylbenzimidazoles (**6–15**).



Scheme 1 Synthetic approaches to compounds **4** and **5**: (a) (i) Tf_2O, 2,6-lutidine, DCM, rt; (ii) *o*-bromoaniline, rt; (b) CuI, 1,10-phenanthroline, ACN, reflux; (c) 10% Pd/C, H_2, MeOH, rt.



Scheme 2 Synthetic approaches to compounds **6** and **7**: (a) (i) formic acid, acetic anhydride, DCM, rt; (ii) 2-bromo-4-methoxyaniline, $POCl_3$, toluene, 0 °C to 90 °C; (b) acetic anhydride, DCM, 0 °C to rt; (c) 2-bromo-4-methoxyaniline, $POCl_3$, toluene, 0 °C to 90 °C; (d) CuI, 1,10-phenanthroline, K_2CO_3, ACN, reflux.

potent antimitotic and vascular disrupting activity.[11] Benzimidazoles are found in a wide range of biologically active compounds, including antimicrobials, antivirals, antiprotozoals, antiproliferative agents, and anti-inflammatory agents;[12,13] in addition, it is considered as bioisostere of indole in the field of medicinal chemistry.[14] Given the above examples, this study attempted to apply a bioisosterism strategy toward compound **2**; as a result, a series of 1,2-diarylbenzimidazoles (**4** and **5**) and 1-arylbenzimidazoles (**6–15**, Fig. 2) were synthesized and investigated for biological activities.

The trial using copper-mediated Ullmann arylation of benzimidazole failed the preparation of anticipated compounds. In addition, the synthesis of 5,6,7-trimethoxybenzimidazole is not feasible; therefore, an alternative methodology is required. Yu *et al.* reported a copper catalyzed approach to obtain 1,2-disubstituted benzimidazoles from imidoyl chlorides and *o*-haloanilines.[15] Liubchak and coworkers published a similar methodology to generate annulated benzimidazoles through CuI-mediated cyclization of amidines which were prepared from lactam and *o*-bromoaniline.[16] Inspired by the cited studies, we applied the reported synthetic methods to the preparation of designed benzimidazole derivatives.

Results and discussion

Chemistry

The synthesis of compounds **4** and **5** is shown in Scheme 1. Compounds **16** and **17** were reacted with triflic anhydride followed by the treatment of *o*-bromoaniline, and the resulting amidine intermediates **18** and **19** underwent copper catalyzed cyclization by reacting with CuI in the presences of 1,10-phenanthroline to afford 1,2-diarylbenzimidazoles **20** and **21**. Removal of benzyl (Bn) protecting group yielded the anticipated compounds **4** and **5**.

Compounds **6** and **7** bearing a 3,4,5-trimethoxyphenyl moiety at *N*1 position were synthesized according to the synthetic route shown in Scheme 2. The reaction of 3,4,5-trimethoxyaniline (**22**) with formic acid and 2-bromo-4-methoxyaniline in the presence of $POCl_3$ gave a unstable amidine intermediate (**23**) which was subjected to CuI-mediated cyclization to afford compound **6**. On the other hand, the acetamide (**25**) obtained from the reaction of 3,4,5-trimethoxyaniline (**22**) with acetic anhydride underwent similar synthetic route toward **6** to generate 2-methylbenzimidazole **7**.

Schemes 3–5 show the synthesis of *N*1-substituted 5,6,7-trimethoxybenzimidazoles (**8–15**). Compounds **26** and **27** were converted into the corresponding formamides followed by reaction with 2-bromo-3,4,5-trimethoxyaniline in the presence of $POCl_3$, yielding the amidine intermediates (**28** and **30**). Following the similar approach, compound **31** generated from **22** through amidation and bromination was converted into the amidine intermediate **29**. All amidine intermediates (**28–30**) subsequently underwent CuI-mediated cyclization to afford compounds **8**, **9**, and **32**. The removal of Bn protecting group of **32** was carried out by hydrogenation to yield compound **10** (Scheme 3).

Scheme 4 shows the synthesis of compounds **11–13**. The reaction of compound **33** with $POCl_3$ or triflic anhydride was followed by addition of **26**, **27**, and **34** to yield the amidine intermediates **35–37**. Without further purification, the resulting intermediates (**35–37**) underwent CuI-mediated cyclization to

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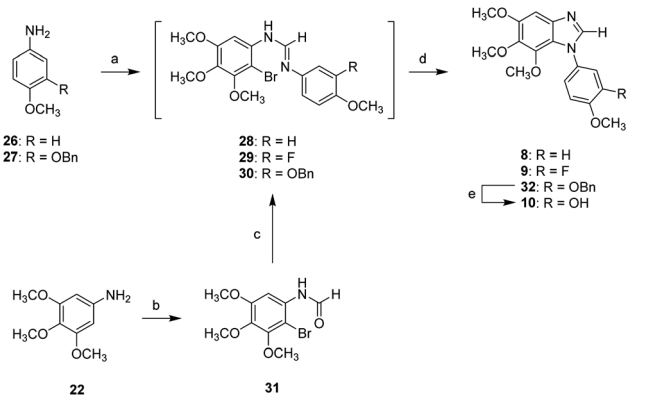
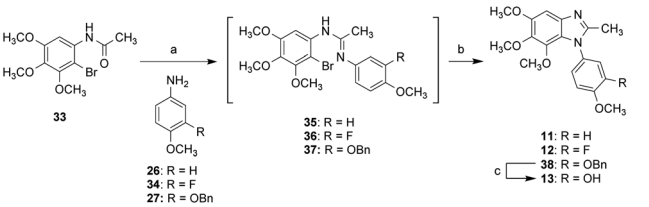
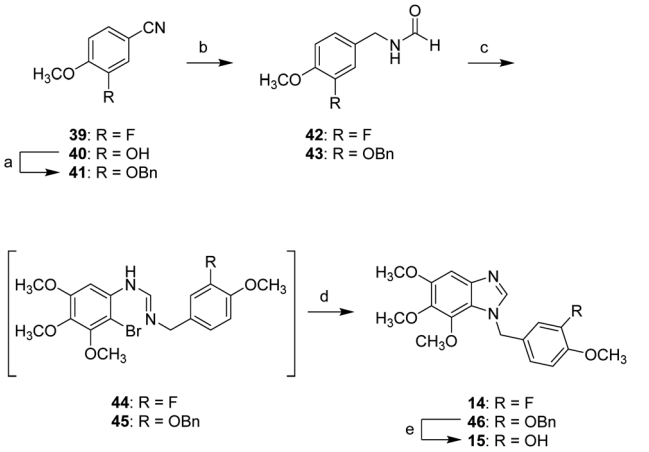


Scheme 3 Synthetic approaches to compounds **8–10**: (a) (i) formic acid, acetic anhydride, DCM, rt; (ii) 2-bromo-3,4,5-trimethoxyaniline, $POCl_3$, toluene, 0 °C to 90 °C; (b) (i) formic acid, acetic anhydride, DCM, rt; (ii) NBS, DCM, 0 °C; (c) 3-fluoro-4-methoxyaniline, $POCl_3$, toluene, 0 °C to 50 °C; (d) CuI, 1,10-phenanthroline, K_2CO_3, ACN, reflux; (e) 5% Pd/C, H_2, MeOH, rt.



Scheme 4 Synthetic approaches to compounds **11–13**: (a) for **35** and **37**: $POCl_3$, **26** or **27**, toluene, 0 °C to 90 °C; for **36**: triflic anhydride, **34**, 2,6-lutidine, DCM, 0 °C to 50 °C; (b) CuI, 1,10-phenanthroline, K_2CO_3, ACN, reflux; (c) 10% Pd/C, H_2, MeOH, rt.



Scheme 5 Synthetic approaches to compounds **14** and **15**: (a) benzyl bromide, K_2CO_3, ACN, reflux; (b) (i): LAH, dry THF, 0 °C to rt; (ii): formic acid, acetic anhydride, DCM, rt; (c) (i) 2-bromo-3,4,5-trimethoxyaniline, $POCl_3$, toluene, 0 °C to 90 °C; (d) CuI, 1,10-phenanthroline, K_2CO_3, ACN, reflux; (e) BCl_3 in hexane, DCM, 0 °C.

afford 2-methyl-5,6,7-benzimidazoles **11**, **12**, and **38**. Compound **38** underwent deprotection of Bn group to afford compound **13**.

Scheme 5 shows the synthesis of compounds **14** and **15** possessing a methylene linkage. The hydroxyl group of **40** was masked by a Bn group. Then, two benzonitriles (**39** and **41**) were reduced by LAH, and the resulting products were subsequently reacted with formic acid in the presence of acetic anhydride to afford the corresponding formamides **42** and **43**. Conversion of the resulting formamides (**42** and **43**) to amidine intermediates (**44** and **45**) followed the similar route to **7** generated compounds **14** and **46**. The removal of benzyl group of **46** was carried out by using BCl_3 to yield compound **15**.

Biological evaluation

All synthesized compounds (**4–15**) were tested for antiproliferative activity against A549, HCT-116, and PC-3 cells (Table 1). The result from **4** and **5** indicated that replacement of the *Z*-geometry C=C bond of CA-4 with 1,2-diarylbenzimidazole moiety led to loss of cellular activity. Compounds **6** and **7** bearing 3,4,5-trimethoxyphenyl moiety at *N*1 position showed no effect on tested cell lines, whereas compounds **8**, **9**, and **10** exhibited marked cellular activity. This comparison revealed that 1-aryl-5,6,7-trimethoxybenzimidazole moiety is favored; however, there are some limitations as follows. The C2-methyl group led to dramatic loss of cellular activity, from the comparison of **8**, **9**, and **10** with **11**, **12**, and **13**. In addition, the insertion of a methylene group between *N*-aryl group and benzimidazole (**13** and **14**) resulted in marked decrease of activity. Compounds **8**, **9**, and **10** showed potent antiproliferative activity. Compound **8** possessing a 4-methoxyphenyl group showed antiproliferative activity against A549, HCT-116, and PC-3 cells with GI_{50} values of 0.4, 0.24, and 0.8 μM, respectively. Addition of an F atom at 3′-position, which results in compound **9**, made a slight increase of cellular activity against A549 and HCT-116 cells, as well as three times of cytotoxicity for PC-3 cell than compound **8**. Replacement of F atom with –OH (**10**) contributed approximately 3-fold enhancement of cytotoxic activity. Compound **10** exhibited potent activity against A549, HCT-116, and PC-3 cells with GI_{50} values of 0.08, 0.05, and 0.09 μM, respectively. In addition, the comparison of **8**, **9**, and **10** showed that the trend of influence of C3′-substitution is –OH > –F > –H.

Compounds **8**, **9**, and **10** were tested for their effects on tubulin polymerization (Fig. 3). Despite their displaying weaker tubulin polymerization inhibition than CA-4, compounds **8**, **9**, and **10** are able to inhibit polymerization of tubulin in a manner of dose-dependent. The influence of substitution on tubulin polymerization is consistent with that on cellular activity shown in Table 1. The addition of 3′-F resulted in 2.6-fold increase of activity, as compared with compound **8**. The placement of 3′-F (**9**) with the 3′-OH (**10**) led to a 3.2-fold enhancement of tubulin polymerization inhibition. Among them, **10** showed marked tubulin polymerization inhibition in a dose-dependent manner, with an IC_{50} value of 0.26 μM.

Fig. 4 demonstrates the overlap of compound **10** and CA-4, which was conducted using the Glide module of Schrödinger Maestro. The trimethoxybenzimidazole overlapped with 3,4,5-trimethoxyphenyl moiety of CA-4. In addition, the *Z*-geometry was retained by benzimidazole ring that had 3′-OH-4′-methoxyphenyl

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Table 1 Antiproliferative activity of compounds 4–15

Compound	Cell type ($GI_{50} \pm SD^a$, μM)		
	A549	HCT116	PC-3
4	>10	>10	>10
5	7.30 ± 0.10	6.30 ± 0.10	7.75 ± 0.05
6	>10	>10	>10
7	>10	>10	>10
8	0.40 ± 0.09	0.24 ± 0.01	0.80 ± 0.03
9	0.27 ± 0.02	0.19 ± 0.01	0.29 ± 0.02
10	0.08 ± 0.01	0.05 ± 0.02	0.09 ± 0.01
11	>10	>10	>10
12	>10	>10	>10
13	>10	>10	>10
14	8.60 ± 0.70	7.80 ± 0.50	8.25 ± 0.15
15	>10	>10	>10
1[b]	0.02	0.079	0.01

[a] SD: standard deviation. GI_{50}: 50% growth inhibition. [b] Data from ref. 17.

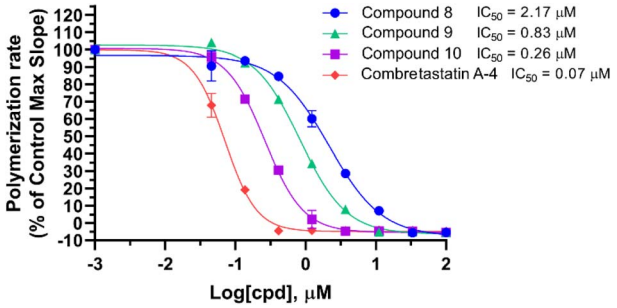

Fig. 3 The effect of compounds 8–10 on *in vitro* tubulin polymerization.

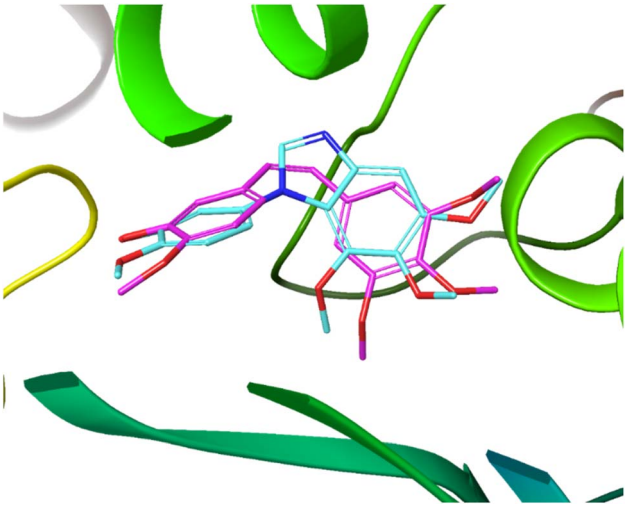

Fig. 4 Overlap of **10** (aqua) and CA-4 (pink) at the binding site of tubulin (PDB ID: 5LYJ).

motif directed toward the same orientation as that of CA-4. This result of docking study explains the contribution of benzimidazole to maintain the similar steric alignment.

Conclusions

In this study, CuI-mediated cyclization *via* amidine intermediate fulfilled the synthesis of 1,2-diarylbenzimidazoles (**4** and **5**) and 1-arylbenzimidazoles (**6–15**), which helped explore the influence of pattern of substitution (1-aryl and 1,2-diaryl) on biological activity. The result showed that 1-arylbenzimidazole scaffold, especially 1-aryl-5,6,7-trimethoxybenzimidazole (**8–10**), contributed potent cellular activity against tested cancer cells and were able to inhibit tubulin polymerization. Among them, compound **10** showed the most potent cytotoxicity against A549, HCT-116, and PC-3 cells with GI_{50} values of 0.08, 0.05, and 0.09 μM, respectively. The IC_{50} value of **10** toward tubulin polymerization is 0.26 μM. Together with our previous study, it is concluded that 5,6,7-trimethoxyindole and 5,6,7-trimethoxybenzimidazole effectively modified A-ring part of CA-4. Recent studies on antitubulin agents have gradually shifted from single pharmacological function to dual activity.[18] Therefore, 5,6,7-trimethoxyindole and 5,6,7-trimethoxybenzimidazole could be templates for advanced development such as multifunctional tubulin agents in the future.

Experimental

Chemistry

Nuclear magnetic resonance spectra were obtained with a Bruker DRX-300 spectrometer operating at 300 MHz and with Agilent DD2 600 MHz NMR Spectrometer operating at 600 MHz, with chemical shifts reported in parts per million (ppm, δ) downfield from TMS, an internal standard. High-resolution mass spectra (HRMS) were measured with a JEOL (JMS-700) electron impact (EI) mass spectrometer. Purity of the final compounds was determined using a Hitachi 2000 series HPLC system using C-18 column (Agilent ZORBAX Eclipse XDB-C18 5 μm. 4.6 mm × 150 mm) and was found to be ≥95% in all cases. Flash column chromatography was carried out using silica gel (Merck Kieselgel 60, no. 9385, 230–400 mesh ASTM). All reactions were done under an atmosphere of dry nitrogen.

2-Methoxy-5-(2-(3,4,5-trimethoxyphenyl)-1*H*-benzo[*d*]imidazol-1-yl)phenol (4). A mixture of compound **20** (0.05 g, 0.1 mmol), 10% Pd/C (0.01 g), and MeOH (5 mL) was stirred under an atmosphere of hydrogen (1 atm) at room temperature for 10 h. The resulting mixture was filtered through a pad of Celite and washed with MeOH. The filtrate was concentrated *in vacuo* and the resulting residue was purified by recrystallization to afford compound **4** as a white solid (0.03 g, 84%). Mp 223.1 °C (dec). ^{1}H NMR (300 MHz, CD_3OD) δ 7.73 (dd, J = 1.3, 6.5 Hz, 1H), 7.20–7.35 (m, 3H), 7.09–7.12 (m, 1H), 6.91 (s, 2H), 6.83 (sextet, J = 2.5 Hz, 2H), 3.93 (s, 3H), 3.77 (s, 3H), 3.68 (s, 6H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 152.4, 151.2, 148.2, 147.5, 142.1, 138.5, 137.5, 129.3, 124.8, 123.0, 122.4, 119.0, 118.4, 114.7, 112.8, 110.3, 106.4, 60.0, 55.9, 55.5. HRMS (ESI) for $C_{23}H_{23}N_2O_5$ $[M + H]^+$ calculated 407.1607, found 407.1607.

2-Methoxy-5-(1-(3,4,5-trimethoxyphenyl)-1*H*-benzo[*d*]imidazol-2-yl)phenol (5). The title compound was obtained from compound **21** in a manner similar to that described for the synthesis of compound **4**; yield 0.03 g (80%). Mp 242.6 °C (dec).

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^{1}H NMR (600 MHz, DMSO-d_6) δ 9.17 (s, 1H), 7.24–7.28 (m, 1H), 7.22–7.24 (m, 2H), 7.17 (d, J = 2.0 Hz, 1H), 6.94 (dd, J = 2.0, 8.4 Hz, 1H), 6.90 (d, J = 8.5 Hz, 1H), 6.75 (s, 2H), 3.77 (s, 3H), 3.75 (s, 3H), 3.70 (s, 6H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 153.4, 148.8, 146.0, 142.3, 137.4, 137.2, 132.2, 122.7, 122.3, 122.3, 120.3, 118.7, 116.1, 111.5, 110.5, 105.4, 60.2, 56.2, 55.4. HRMS (ESI) for $C_{23}H_{23}N_2O_5$ [M + H]$^+$ calculated 407.1607, found 407.1609.

6-Methoxy-1-(3,4,5-trimethoxyphenyl)-1*H*-benzimidazole (6). A mixture of formic acid (1.5 mL) and acetic anhydride (2.0 mL) was stirred at room temperature for 30 min and then was added to a solution of 3,4,5-trimethoxyaniline (**22**, 2.75 g, 15.00 mmol) in DCM (20 mL). After stirring at room temperature for 12 h, the reaction mixture was quenched with H_2O and $NaHCO_{3(aq)}$, extracted with DCM, and purified by flash column chromatography on silica gel (Hex : EA = 1 : 2; R_f = 0.25) to afford crude formamide (2.53 g). To a mixture of resulting formamide (1.14 g, 5.42 mmol) in toluene (10 mL) was added $POCl_3$ (0.60 mL, 2.48 mmol) under ice bath. After stirring at room temperature for 30 minutes, a mixture of 2-bromo-4-methoxyaniline (1.64 g, 8.13 mmol) in toluene (10 mL) was added and stirred at 90 °C for 6 h. The reaction mixture was diluted with H_2O, neutralized with 5 N $NaOH_{(aq)}$, extracted with EA, and purified by flash column chromatography on silica gel (Hex : EA = 2 : 1; R_f = 0.28) to afford crude formamidine **23** (0.55 g). A mixture of the resulting product **23** (0.40 g, 1.00 mmol), CuI (0.01 g, 0.50 mmol), 1,10-phenanthroline (0.09 g, 0.50 mmol), and K_2CO_3 (0.14 g, 1.00 mmol) in ACN (10 mL) was stirred at reflux for 6 h. The reaction mixture was purified by flash column chromatography on silica gel (Hex : EA = 1 : 2; R_f = 0.29) and the residue was washed with ether, DCM, and MeOH to afford compound **6** as a pink solid (0.05 g, 3%). Mp = 166.1–166.8 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.38 (s, 1H), 7.64 (d, J = 9.0 Hz, 1H), 7.12 (d, J = 2.4 Hz, 1H), 6.96 (s, 2H), 6.92 (dd, J = 2.6, 9.0 Hz, 1H), 3.87 (s, 3H), 3.80 (s, 3H), 3.73 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 156.6, 153.6, 142.5, 137.9, 136.6, 133.8, 131.7, 120.3, 111.8, 101.5, 94.0, 60.1, 56.2, 55.5. HRMS (ESI) for $C_{17}H_{19}N_2O_4$ [M + H]$^+$ calculated 315.1345, found 315.1344.

6-Methoxy-2-methyl-1-(3,4,5-trimethoxyphenyl)-1*H*-benzimidazole (7). To a mixture of *N*-(3,4,5-trimethoxyphenyl)acetamide (**25**, 0.45 g, 2.00 mmol) and toluene (10 mL) was added $POCl_3$ (0.20 mL, 2.10 mmol) under ice bath and the resulting mixture was stirred at room temperature for 30 minutes. Then, 2-bromo-4-methoxyaniline (0.51 g, 2.50 mmol) was added to the reaction mixture. After stirring at 90 °C for 6 h, the resulting mixture was diluted with H_2O, neutralized with 5 N $NaOH_{(aq)}$, extracted with EA, and purified by flash column chromatography on silica gel (Hex : EA = 1 : 1; R_f = 0.33) to afford crude amidine **24** (0.65 g). The crude product (0.41 g, 1.0 mmol) was dissolved in ACN (10 mL), and then CuI (0.10 g, 0.5 mmol), 1,10-phenanthroline (0.09 g, 0.57 mmol), and K_2CO_3 (0.14 g, 1.0 mmol) were added. The resulting mixture was heated to reflux for 6 h, and then purified by flash column chromatography on silica gel (Hex : EA = 1 : 1; R_f = 0.25) and the residue was washed with MeOH to afford compound **7** as a white solid (0.06 g, 13%). Mp = 158.7–159.6 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.47–7.50 (d, J = 8.7 Hz, 1H), 6.80–6.84 (m, 3H), 6.67 (d, J = 2.4 Hz, 1H), 3.82 (s, 6H), 3.76 (s, 3H), 3.72 (s, 3H), 2.41 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 155.8, 153.5, 150.4, 137.3, 136.6, 136.5, 131.2, 118.8, 110.8, 104.6, 93.7, 60.1, 56.2, 55.5, 14.1. HRMS (ESI) for $C_{18}H_{21}N_2O_4$ [M + H]$^+$ calculated 329.1501, found 329.1500.

5,6,7-Trimethoxy-1-(4-methoxyphenyl)-1*H*-benzimidazole (8). The title compound was obtained from compound **26** in a manner similar to that described for the synthesis of compound **6**; yield 0.12 g (9%). Mp 163.6–164.5 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.12 (s, 1H), 7.46 (d, J = 8.7 Hz, 2H), 7.12 (s, 1H), 7.07 (d, J = 9.0 Hz, 2H), 3.84 (s, 3H), 3.83 (s, 3H), 3.74 (s, 3H), 3.46 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 161.2, 152.7, 144.9, 141.4, 141.2, 140.9, 130.9, 128.6, 122.9, 115.0, 98.2, 61.9, 61.8, 56.8, 56.0. HRMS (ESI) for $C_{17}H_{19}N_2O_4$ [M + H]$^+$ calculated 315.1345, found 315.1347.

1-(3-Fluoro-4-methoxyphenyl)-5,6,7-trimethoxy-1*H*-benzimidazole (9). The title compound was obtained from compound **31** in a manner similar to that described for the synthesis of compound **7**; yield 0.04 g (24%). Mp 131.5–132.3 °C. ^{1}H NMR (300 MHz, CD_3OD) δ 8.07 (s, 1H), 7.37 (dd, J = 2.4, 11.7 Hz, 1H), 7.31 (ddd, J = 0.9, 2.4, 8.7 Hz, 1H), 7.21–7.27 (m, 1H), 7.07 (s, 1H), 3.97 (s, 3H), 3.92 (s, 3H), 3.85 (s, 3H), 3.57 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 150.5 (d, J = 243.8 Hz), 150.4, 146.8 (d, J = 10.2 Hz), 144.1, 140.1, 139.2 (d, J = 4.4 Hz), 129.3 (d, J = 9.0 Hz), 122.4 (d, J = 3.5 Hz), 121.0, 114.4 (d, J = 20.4 Hz), 113.2, 113.2, 97.9, 60.9, 60.8, 56.2, 56.1. HRMS (ESI) for $C_{17}H_{18}N_2O_4F$ [M + H]$^+$ calculated 333.1251, found 333.1253.

2-Methoxy-5-(5,6,7-trimethoxybenzimidazol-1-yl)phenol (10). The title compound was obtained from compound **32** in a manner similar to that described for the synthesis of compound **4**; yield 0.06 g (70%). Mp = 217.9–218.8 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.44 (br, 1H), 8.08 (s, 1H), 7.10 (s, 1H), 7.03 (dd, J = 1.8, 7.2 Hz, 1H), 6.89–6.92 (m, 2H), 3.84 (s, 3H), 3.84 (s, 3H), 3.74 (s, 3H), 3.47 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 150.3, 147.5, 146.3, 144.1, 140.1, 139.3, 139.2, 129.6, 121.2, 116.6, 113.5, 111.6, 97.9, 61.1, 60.9, 56.1, 55.8. HRMS (ESI) for $C_{17}H_{19}N_2O_5$ [M + H]$^+$ calculated 331.1294, found 331.1295.

5,6,7-Trimethoxy-1-(4-methoxyphenyl)-2-methyl-1*H*-benzimidazole (11). The title compound was obtained from compound **33** in a manner similar to that described for the synthesis of compound **7**; yield 0.17 g (35%). Mp 152.2 °C (dec). ^{1}H NMR (300 MHz, CD_3OD) δ 7.34 (d, J = 9.0 Hz, 2H), 7.09 (d, J = 8.7 Hz, 2H), 6.97 (s, 1H), 3.89 (s, 3H), 3.80 (s, 3H), 3.45 (s, 3H), 2.32 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 159.0, 151.6, 149.8, 138.9, 138.8, 138.5, 129.6, 128.7, 123.2, 113.9, 97.2, 60.9, 60.8, 56.1, 55.3, 13.7. HRMS (ESI) for $C_{18}H_{21}N_2O_4$ [M + H]$^+$ calculated 329.1501, found 329.1501.

1-(3-Fluoro-4-methoxyphenyl)-5,6,7-trimethoxy-2-methyl-1*H*-benzimidazole (12). The title compound was obtained from compound **33** in a manner similar to that described for the synthesis of compound **7**; yield 0.06 g (7%). Mp 154.5–155.7 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.48–7.53 (m, 1H), 7.26–7.33 (m, 2H), 6.99 (s, 1H), 3.93 (s, 3H), 3.82 (s, 3H), 3.71 (s, 3H), 3.41 (s, 3H), 2.25 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 150.5 (d, J = 244.1 Hz), 149.9, 147.2 (d, J = 10.4 Hz), 138.8, 138.7 (d, J = 24.0 Hz), 129.6, 129.4 (d, J = 8.9 Hz), 124.3 (d, J = 3.3 Hz), 123.1, 115.8 (d, J = 19.5 Hz), 113.2, 113.2, 97.3, 60.9, 60.8, 56.1, 56.1,

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13.7. HRMS (ESI) for $C_{18}H_{20}N_2O_4F$ [M + H]$^+$ calculated 347.1407, found 347.1409.

2-Methoxy-5-(5,6,7-trimethoxy-2-methyl-benzimidazol-1-yl) phenol (13). The title compound was obtained from compound **38** in a manner similar to that described for the synthesis of compound **4**; yield 0.06 g (31%). Mp 227.5 °C (dec). ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.40 (s, 1H), 7.03 (d, J = 8.4 Hz, 1H), 6.96 (s, 1H), 6.84 (dd, J = 2.4, 8.4 Hz, 1H), 6.81 (d, J = 2.4 Hz, 1H), 3.85 (s, 3H), 3.81 (s, 3H), 3.70 (s, 3H), 3.39 (s, 3H), 2.23 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 151.5, 149.7, 147.7, 146.3, 138.8, 138.8, 138.5, 129.7, 123.2, 118.2, 114.8, 111.5, 97.2, 61.1, 60.8, 56.1, 55.7, 13.7. HRMS (ESI) for $C_{18}H_{21}N_2O_5$ [M + H]$^+$ calculated 345.1450, found 345.1449.

1-(3-Fluoro-4-methoxybenzyl)-5,6,7-trimethoxy-1*H*-benzoimidazole (14). The title compound was obtained from compound **42** in a manner similar to that described for the synthesis of compound **7**; yield 0.13 g (13%). Mp 101.2–101.9 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.18 (s, 1H), 7.01–7.14 (m, 3H), 6.89 (d, J = 8.1 Hz, 1H), 5.42 (s, 2H), 3.81 (s, 3H), 3.78 (s, 3H), 3.73 (s, 3H), 3.71 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 151.2 (d, J = 243.0 Hz), 150.3, 146.3 (d, J = 10.4 Hz), 144.5, 139.8 (d, J = 15.8 Hz), 138.5, 131.0 (d, J = 5.9 Hz), 122.9 (d, J = 3.3 Hz), 120.1, 114.4 (d, J = 18.6 Hz), 113.9, 113.9, 97.6, 61.2, 60.7, 56.0, 55.9, 48.0. HRMS (ESI) for $C_{18}H_{20}N_2O_4F$ [M + H]$^+$ calculated 345.1407, found 345.1412.

2-Methoxy-5-(5,6,7-trimethoxybenzimidazol-1-ylmethyl) phenol (15). To a mixture of compound **46** (0.06 g, 0.13 mmol) and DCM (6 mL) was added BCl_3 in hexane (1 M, 0.78 mL) under ice bath. After stirring at 0 °C for 2 h, the resulting mixture was concentrated *in vacuo*. The precipitate was filtered and washed by DCM. Then, the filtrate was collected, concentrated *in vacuo*, and purified by recrystallization to afford compound **15** as a transparent crystal (20 mg, 44%). Mp = 176.1–177.5 °C; ^{1}H NMR (300 MHz, CD_3OD) δ 9.17 (s, 1H), 7.07 (s, 1H), 6.95 (dd, J = 2.4, 6.6 Hz, 1H), 6.76–6.78 (m, 2H), 5.64 (s, 2H), 3.96 (s, 3H), 3.94 (s, 3H), 3.87 (s, 3H), 3.85 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 150.1, 146.9, 146.5, 144.5, 140.2, 139.3, 138.5, 130.7, 120.3, 117.3, 113.8, 112.2, 97.5, 61.2, 60.7, 56.0, 55.6, 48.5. HRMS (ESI) for $C_{18}H_{21}N_2O_5$ [M + H]$^+$ calculated 345.1450, found 345.1454.

1-(3-(Benzyloxy)-4-methoxyphenyl)-2-(3,4,5-trimethoxyphenyl)-1*H*-benzo[*d*]imidazole (20). To a mixture of compound **16** (0.21 g, 0.5 mmol) in DCM (15 mL) was added 2,6-lutidine (0.13 mL, 1.13 mmol) and Tf_2O (0.1 mL, 0.6 mmol) at ambient temperature, and the resulting mixture was stirred for 30 min. 2-Bromoaniline (0.15 mL, 1.32 mmol) was added and stirred at rt for 30 min. The reaction was quenched with H_2O (20 mL), and resulting residue was extracted with DCM (20 mL × 2). The organic lay was dried under reduced pressure to afford the amidine residue which was dissolved in ACN (5 mL). The resulting solution was placed in sealed tube and then K_2CO_3 (0.07 g, 0.51 mmol), CuI (0.05 g, 0.26 mmol), and 1,10-phenanthroline (0.05 g, 0.28 mmol) were added. After heating at 85 °C for 1 h, the reaction mixture was cooled and purified by column chromatography to afford an oil **20** (0.05 g, 20%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.84 (d, J = 7.8 Hz, 1H), 7.34–7.28 (m, 6H), 7.19 (dt, J = 0.6, 7.8 Hz, 1H), 7.00–6.96 (m, 2H), 6.88–6.84 (m, 2H), 6.79 (s, 2H), 5.10 (s, 2H), 3.94 (s, 3H), 3.85 (s, 3H), 3.63 (s, 6H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 152.9, 152.0, 149.9, 148.7, 142.6, 139.2, 137.6, 136.1, 129.8, 128.7, 128.2, 127.2, 125.0, 123.3, 123.0, 120.5, 119.6, 113.6, 112.1, 110.4, 106.6, 71.2, 60.9, 56.4, 56.0.

2-(3-(Benzyloxy)-4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)-1*H*-benzo[*d*]imidazole (21). The title compound was obtained from compound **17** in a manner similar to that described for the synthesis of compound **20**; yield 0.04 g (16%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.85 (d, J = 8.4 Hz, 1H), 7.38–7.19 (m, 11H), 6.83 (d, J = 8.4 Hz, 1H), 6.52 (s, 2H), 5.00 (s, 2H), 3.93 (s, 3H), 3.89 (s, 3H), 3.75 (s, 6H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 154.1, 152.1, 150.9, 147.9, 142.9, 138.2, 137.4, 136.7, 132.8, 128.6, 128.0, 127.2, 123.2, 123.0, 122.9, 122.4, 119.7, 114.6, 111.3, 110.4, 105.1, 71.0, 61.2, 56.5, 56.0.

***N*-(3,4,5-Trimethoxyphenyl)acetamide (25).** To a solution of compound **22** (10.00 g, 54.59 mmol) in DCM (40 mL) was added acetic anhydride (5.57 g, 54.59 mmol) under ice bath and stirred at room temperature for 30 minutes. The reaction mixture was quenched with H_2O, filtered, and washed by saturated $NaHCO_3$ and H_2O to afford compound **25** as a gray solid (11.17 g, 91%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.21 (br, 1H), 6.80 (s, 2H), 3.83 (s, 6H), 3.81 (s, 3H), 2.16 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 168.1, 152.7, 135.5, 133.2, 96.8, 60.1, 55.6, 24.0.

***N*-(2-Bromo-3,4,5-trimethoxyphenyl)formamide (31).** Formic acid (1.5 mL) was added to acetic anhydride (2.0 mL) and the resulting mixture was stirred for 30 minutes. The reaction mixture (3.5 mL) was added to the solution of compound **22** (2.75 g, 15.00 mmol) in DCM (20 mL). After stirring at room temperature for 12 h, the reaction mixture was quenched with H_2O and saturated $NaHCO_{3(aq)}$, extracted with DCM, and purified by flash column chromatography on silica gel (Hex : EA = 1 : 2; R_f = 0.25) to afford crude formamide (2.53 g). The resulting product was dissolved in DCM (20 mL), and then NBS (1.1 eq.) was added under ice bath. After stirring at 0 °C for 30 minutes, the reaction mixture was purified by flash column chromatography on silica gel (Hex : EA = 1 : 2; R_f = 0.28) to afford compound **31** as a pale yellow solid (1.44 g, 72%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.47 (d, J = 1.8 Hz, 1H), 7.97 (s, 1H), 3.91 (s, 3H), 3.89 (s, 3H), 3.86 (s, 3H). ^{13}C NMR (75 MHz, CD_3OD) δ 162.1, 154.1, 152.2, 141.5, 132.7, 104.6, 102.1, 61.5, 56.6.

1-(3-Benzyloxy-4-methoxyphenyl)-5,6,7-trimethoxy-1*H*-benzimidazole (32). The title compound was obtained from compound **27** in a manner similar to that described for the synthesis of compound **6**; yield 0.04 g (19%). ^{1}H NMR (300 MHz, CD_3OD) δ 8.07 (br, 1H), 7.45–7.48 (m, 2H), 7.28–7.40 (m, 3H), 7.20 (d, J = 1.8 Hz, 1H), 7.07–7.14 (m, 3H), 5.17 (s, 2H), 3.95 (s, 3H), 3.93 (s, 3H), 3.86 (s, 3H), 3.42 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 150.3, 148.9, 147.4, 139.4, 139.2, 136.8, 129.6, 128.3, 127.8, 127.7, 118.5, 112.2, 111.5, 97.9, 70.2, 61.0, 60.8, 56.1, 55.8.

1-(3-Benzyloxy-4-methoxyphenyl)-5,6,7-trimethoxy-2-methyl-1*H*-benzimidazole (38). The title compound was obtained from compound **33** in a manner similar to that described for the synthesis of compound **7**; yield 0.25 g (23%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.27–7.42 (m, 5H), 6.97–6.99 (m, 2H), 6.91 (dd, J = 2.1, 8.4 Hz, 1H), 6.84 (d, J = 2.4 Hz, 1H), 5.18 (s, 2H), 3.98 (s, 3H), 3.90 (s, 3H), 3.83 (s, 3H), 3.36 (s, 3H), 2.22 (s, 3H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 151.6, 149.8, 149.1, 147.2, 138.8, 138.6, 136.8, 129.5, 128.3, 127.8, 127.7, 123.3, 120.2, 113.5, 111.4, 97.2, 69.9, 60.9, 60.7, 56.1, 55.7, 13.6.

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3-Benzyloxy-4-methoxybenzonitrile (41). A mixture of 3-hydroxy-4-methoxybenzonitrile (**40**, 2.00 g, 13.41 mmol), benzyl bromide (2.75 g, 16.09 mmol), and K_2CO_3 (3.71 g, 26.82 mmol) in ACN (10 mL) was stirred at reflux for 2 h. The reaction was quenched with H_2O and extracted with EA. The organic phase was collected and purified by flash column chromatography on silica gel (Hex : EA = 4 : 1; $R_f = 0.45$) to afford compound **41** as a white solid (2.97 g, 93%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.32–7.49 (m, 7H), 7.14 (d, $J = 8.4$ Hz, 1H), 5.13 (s, 2H), 3.84 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 153.0, 147.8, 136.3, 128.4, 128.0, 127.9, 126.6, 119.2, 115.8, 112.3, 102.5, 70.1, 55.8.

***N*-(3-Fluoro-4-methoxybenzyl)formamide (42).** To a solution of 3-fluoro-4-methoxybenzonitrile (**39**, 2.27 g, 15.00 mmol) in THF (30 mL) was added LAH in THF (1 M, 30 mL) at 0 °C. After stirring at room temperature for 12 h, the reaction mixture was diluted with ether, and quenched sequentially with H_2O (1.2 mL), 5 N $NaOH_{(aq)}$ (1.2 mL) and H_2O (3.5 mL) under ice bath. After stirring at room temperature for 15 minutes, the mixture was filtered through Celite and EA was used as an eluent. The filtrate was concentrated *in vacuo* to afford a crude amine product which was dissolved in DCM (30 mL). Meanwhile, formic acid (1.5 mL) was added to acetic anhydride (2.0 mL) and the resulting mixture was stirred for 30 minutes. Then, the resultant was added to the previously prepared solution of amine in five portions (one portion every 30 minutes). After stirring at room temperature for 12 h, the reaction mixture was quenched with H_2O and $NaHCO_{3(aq)}$, extracted with DCM, and purified by flash column chromatography on silica gel (Hex : EA = 1 : 2; $R_f = 0.14$) to afford compound **42** as a white solid (1.50 g, 55%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.46 (br, 1H), 8.11 (d, $J = 1.8$ Hz, 1H), 7.01–7.14 (m, 3H), 4.23 (d, $J = 6.0$ Hz, 2H), 3.81 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 161.0, 151.3 (d, $J = 242.1$ Hz), 146.0 (d, $J = 10.4$ Hz), 132.0 (d, $J = 5.9$ Hz), 123.4 (d, $J = 3.5$ Hz), 114.9 (d, $J = 17.8$ Hz), 113.7 (d, $J = 1.8$ Hz), 55.9, 39.8 (d, $J = 1.4$ Hz).

***N*-(3-Benzyloxy-4-methoxybenzyl)formamide (43).** The title compound was obtained from compound **41** in a manner similar to that described for the synthesis of compound **42**; yield 1.86 g (69%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.40 (br, 1H), 8.10 (d, $J = 1.8$ Hz, 1H), 7.31–7.47 (m, 5H), 6.99 (d, $J = 2.1$ Hz, 1H), 6.93 (d, $J = 8.1$ Hz, 1H), 6.81 (dd, $J = 2.1$, 8.1 Hz, 1H), 5.03 (s, 2H), 4.21 (d, $J = 6.0$ Hz, 2H), 3.74 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 160.9, 148.2, 147.7, 137.0, 131.3, 128.3, 127.8, 127.8, 119.9, 113.2, 112.0, 70.0, 55.6, 40.4.

1-(3-Benzyloxy-4-methoxybenzyl)-5,6,7-trimethoxy-1*H*-benzimidazole (46). The title compound was obtained from compound **43** in a manner similar to that described for the synthesis of compound **7**; yield 0.1 g (9%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.27–7.36 (m, 6H), 6.98 (s, 1H), 6.91 (d, $J = 7.8$ Hz, 1H), 6.62 (d, $J = 7.5$ Hz, 1H), 5.41 (s, 2H), 5.02 (s, 2H), 3.81 (s, 3H), 3.73 (s, 3H), 3.71 (s, 3H), 3.65 (s, 3H).

Biology

Cell culture. All human cancer cells were purchased from the American Type Cell Culture Collection (Manassas, VA, USA) and cultured by the protocols as previously described.[19]

Anti-proliferation assay. Human cancer cells were seeded onto 96-well plates in a density of 5×10^3 cells per well. Overnight, cells were treated with the tested compounds for 48 h. Then, antiproliferative activity was determined by SRB staining according to the methods described in our previous works.[20]

Tubulin polymerization. All compound dilutions were prepared prior to the following steps. The plate reader was set to 37 °C. Once the internal temperature of the plate reader reached 37 °C a 96-well plate (Corning Costar, Cat. # 3686) was placed in the plate reader and allowed to warm to 37 °C for a minimum of 30 minutes. A total of 12 mg of porcine tubulin was resuspended in 6.5 mL of polymerization buffer 1/tubulin glycerol buffer (75%/25%) to generate a 1.85 mg mL^{-1} tubulin stock in PEM buffer with 15% glycerol and 10 μM DAPI, and this was kept on ice. Compound stocks were spotted 1.0 μL per well in duplicate into the wells of the pre-warmed 96-well plate and allowed to re-warm for 2 minutes at 37 °C. During this incubation, the tubulin solution was placed in a disposable plastic trough and allowed to warm for 1 minute at room temperature. To initiate the reaction, 50 μL per well of tubulin solution was added to each well using a multi-channel pipetor and the plate reader protocol was started immediately.

The absorbance data acquired for this compound screen was analyzed as follows:

The maximal velocity (maximum slope, called V_{max}) of tubulin polymerization was determined using Spectramax Software. The mean of duplicate maximum slope determinations at each concentration were then normalized to reactions that contained only DMSO (1% final concentration, *i.e.* buffer control), and were converted to % activity using the following formula: % polymerization rate = 100 × [V_{max}(cmpd)/V_{max}(DMSO)].

Conflicts of interest

There are no conflicts to declare.

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