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Synthesis of eudistomin D analogues and its effects on adenosine receptors

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Abstract—Six analogues (**1**–**6**) of eudistomin D, a β-carboline alkaloid from a marine tunicate *Eudistoma olivaceum*, were synthesized, and their affinity and selectivity for adenosine receptors A_1, A_{2A}, and A_3 were examined. All the synthetic compounds **1**–**6** did not show affinity to the adenosine A_1 receptor. δ-Carboline **3** exhibited the most potent affinity to the adenosine receptor A_3 among compounds **1**–**6**. δ-Carbolines **3** and **4** showed better affinity than the corresponding β-carbolines **1** and **2**, respectively, while *N*-methylation (**2**, **4**, and **6**, respectively) of the pyrrole ring in **1, 3**, and **5** resulted in the reduced affinity to the adenosin A_3 receptor. On the other hand, an eudistomin D derivative, BED, exhibited modest affinity to all the receptors A_1, A_{2A}, and A_3 but no selectivity.

1. Introduction

During our continuing search for bioactive metabolites from marine organisms, we have found that eudistomin D, a β-carboline alkaloid from a marine tunicate *Eudistoma olivaceum*,[1] and its analogues such as 7-bromoeudistomin D (BED)[2] are potent inducers of Ca^{2+}-release from sarcoplasmic reticulum (SR)[3] as well as inhibitors of phosphodiesterase,[4] and are more potent than caffeine. Caffeine exhibits a variety of physiological activities (or action) including regulation of the blood pressure, respiratory functioning, gastric and colonic activity, urine volume, and exercise performance.[5] The mechanism of actions of caffeine is reported to be competitive antagonism to A_1 and A_{2A} adenosine receptors,[6] induction of Ca^{2+}-release from SR, inhibition of phosphodiesterase, and so on. Previously, our group has found that a hybrid molecule of caffeine and eudistomin D showed a potent affinity for adenosine receptor A_3 subtype.[7] In the present study, the methylated analogues (**1** and **2**, respectively) of eudistomoin D and BED in place of the bromine atom were designed and synthesized. δ-Carboline analogues **3** and **4** were also synthesized. The 2-*N*-methyl derivatives (**5** and **6**, respectively) of **1** and **2** were also prepared to examine effects of the 2-*N*-methyl group on the activity of adenosine receptors.

2. Results and discussion

2.1. Chemistry

The synthesis of β-carboline analogues **1** and **2** is summarized in Scheme 1. Coupling of 2,6-dimethyl-4-bromoanisole (**7**)[8] and 3-aminopyridine (**8**) in DMF with $Pd_2(dba)_3$, Xphos,[9] and NaO^{t}Bu afforded **9** in 88% yield. Photocyclization of **9** in toluene gave **10** and **11** in 31% and 45% yield, respectively, and treatment of **10** with BBr_3 in CH_2Cl_2 furnished **1** in 78% yield. Methylation of **10** with MeI and NaH in DMF provided **12** in 57% yield, which was treated with BBr_3 in CH_2Cl_2 to afford **2** in 78% yield.

In Scheme 2 is shown the synthesis of δ-carboline analogues **3** and **4**. Treatment of **11** with BBr_3 in CH_2Cl_2 furnished **3** in 70% yield. Methylation of **11** with MeI and NaH in DMF provided **13** in 85% yield, which was treated with BBr_3 in CH_2Cl_2 to afford **4** in 83% yield.

Keywords: Eudistomin D analogues; Caffeine; Adenosine receptors; SAR.
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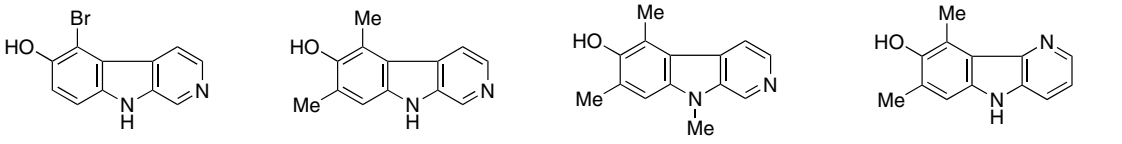
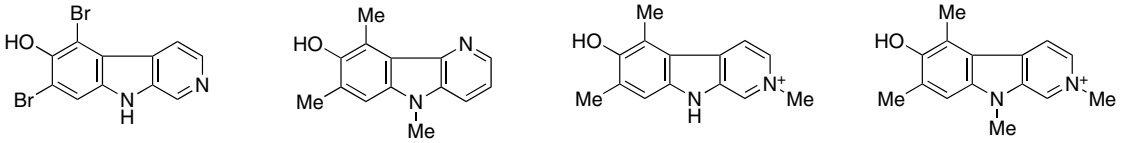


eudistomin D **1** **2** **3**



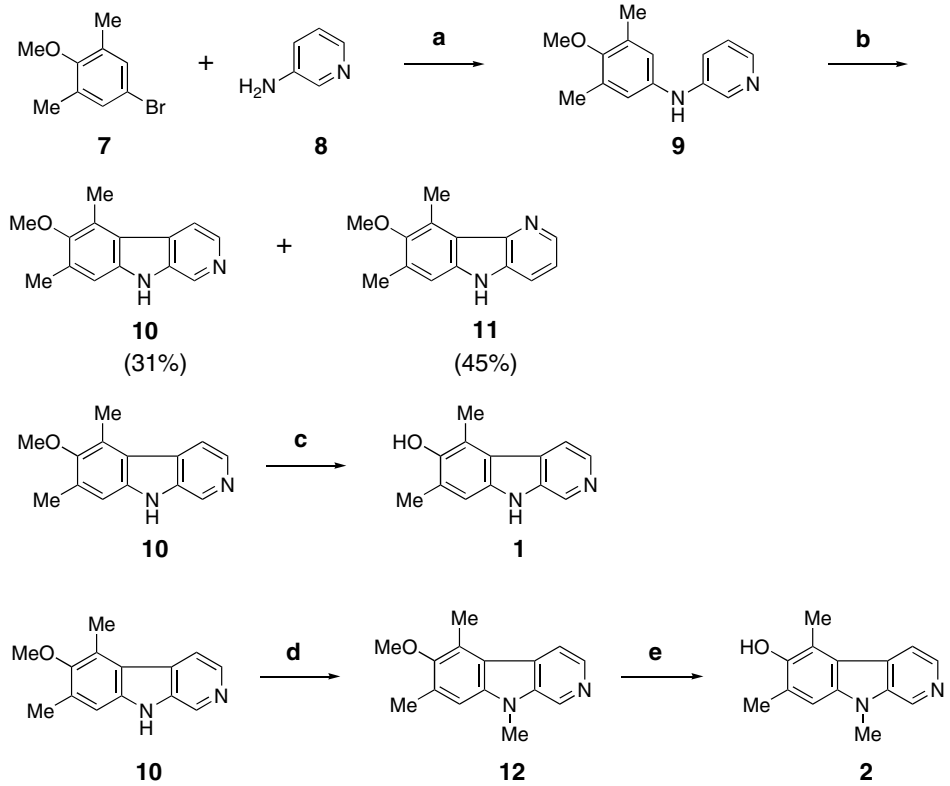
BED **4** **5** **6**

The synthesis of analogues **5** and **6** is summarized in Scheme 3. While methylation of **10** with MeI in acetone followed by treatment with BBr_3 in CH_2Cl_2 furnished **5** in 35% yield for 2 steps, methylation of **10** with MeI and NaH in THF followed by treatment with BBr_3 in CH_2Cl_2 provided **6** in 8% yield for 2 steps.

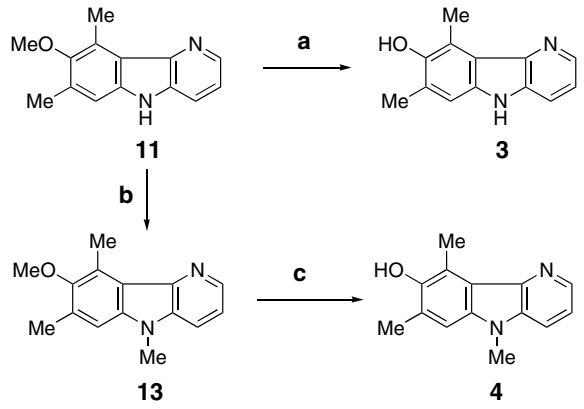
2.2. Biological evaluation

The potency of these synthetic compounds as adenosine receptor ligands was investigated in radioligand binding assays at human recombinant adenosine A_1, A_{2A}, and A_3 receptors expressed in membranes of HEK293T cell as previously reported.[7] The results expressed as K_i values are presented in Table 1. When the affinity of the test compounds was very low, percentage of inhibition at 100 μM was shown. Affinities of reference ligands, that is, the non-selective caffeine, the A_1-selective xanthine amine congener (XAC), the A_{2A}-selective CGS21680, and the A_3-selective 5′-(*N*-ethylcarboxamido)adenosine (NECA), were also shown for comparison. These compounds showed reasonable affinity for each receptor confirming the validity of our assay.

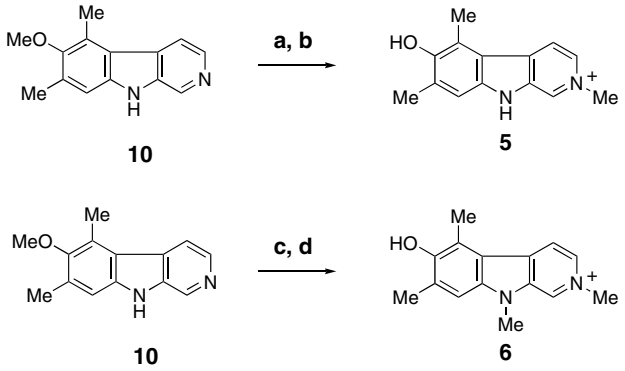
All the synthetic compounds **1**–**6** did not show affinity to the adenosine A_1 receptor (Table 1). δ-Carboline **3** exhibited the most potent affinity to the adenosine receptor A_3 among compounds **1**–**6**. δ-Carbolines **3** and **4** showed better affinity than the corresponding β-carbolines **1** and **2**, respectively, while N-methylation (**2**, **4**, and **6**, respectively) of the pyrrole ring in **1**, **3**, and **5** resulted in the reduced affinity to the adenosine



Scheme 1. Synthesis of compounds **1** and **2**. Reagents and conditions: (a) $Pd_2(dba)_3$, XPhos, NaO^{t}Bu/DMF, 120 °C, 20 h (88%); (b) *hν*/toluene, rt, 15 h; (c) BBr_3/CH_2Cl_2, rt, 3 h (78%); (d) MeI, NaH/THF, rt, 3 h (57%); (e) BBr_3/CH_2Cl_2, rt, 3 h (78%).



Scheme 2. Synthesis of compounds **3** and **4**. Reagents and conditions: (a) BBr_3/CH_2Cl_2, rt, 4 h (70%); (b) MeI, NaH/THF, rt, 3 h (85%); (c) BBr_3/CH_2Cl_2, rt, 4 h (83%).



Scheme 3. Synthesis of compounds **5** and **6**. Reagents and conditions: (a) MeI, acetone, rt, 2 h; (b) BBr_3/CH_2Cl_2, rt, 8 h (35% for 2 steps); (c) MeI, NaH/THF, rt, 1 h; (d) BBr_3/CH_2Cl_2, rt, 8 h (8% for 2 steps).

Table 1. Affinities of caffeine and eudistomin D analogues (**1**–**6**) and BED at human adenosine A_1, A_{2A}, and A_3 receptors

Compound	K_i[a,b] (μM)		
	A_1	A_{2A}	A_3
1	>100 μM	>100 μM	17.7 ± 3.0
2	>100 μM	80.6 ± 10	43.4 ± 1.4
3	>100 μM	24.9 ± 1.2	4.43 ± 2.2
4	>100 μM	44.1 ± 10.6	13.3 ± 3.9
5	>100 μM	>100 μM	29.7 ± 7.4
6	>100 μM	>100 μM	>100 μM
BED (7-bromoeudistomin D)	7.37 ± 2.13	4.92 ± 1.17	2.05 ± 0.69
Caffeine	49.0 ± 19.6	18.1 ± 5.9	>100 μM
XAC	0.009 ± 0.001	nd	nd
CGS21680	nd	0.0462 ± 0.0084	nd
NECA	nd	nd	0.020± 0.009

nd, not determined.

[a] The K_i values are means ± SEM of two or three separate assays, each performed in duplicate.

[b] The binding of each radioactive ligand to membranes prepared from HEK293T cells expressing human adenosine A_1, A_{2A}, or A_3 receptors was best-fitted to a one-site model of binding with estimated K_d (dissociation constant) values of 5, 52, and 6.5 nM, respectively, and B_{max} values of 8600, 7000, and 310 fmol/mg protein, respectively.

A_3 receptor. Likely to this, it has been found for hybrid molecules of caffeine and eudistomin D, in which compounds having a nitrogen at the β-position of the pyridine ring (β-*N* type) showed lower affinity than the corresponding δ-*N* type compounds, while *N*-methylation of a pyrrole ring significantly lowered the potency as adenosine receptor ligands.[7] An eudistomin D derivative, BED, exhibited modest affinity to all the receptors A_1, A_{2A}, and A_3 but no selectivity (Table 1).

3. Experimental

3.1. Chemistry

3.1.1. Instruments and analyses. The IR spectrum was recorded on a JASCO FT/IR-5300 spectrometer. UV spectra were recorded on a Shimadzu UV1600PC spectrophotometer. Proton and carbon NMR spectra were recorded on Bruker 500 and/or 600 MHz and JEOL 400 MHz spectrometers. EI mass spectra were obtained on a DX-303 mass spectrometer.

3.1.2. *N*-(4-Methoxy-3,5-dimethylphenyl)pyridin-3-amine (9). DMF (1.5 mL) was added to an oven-dried Schlenk tube charged with 3-aminopyridine (**8**) (47.7 mg, 0.51 mmol), $Pd_2(dba)_3$ (23 mg, 25 μmol), 2-dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl (21 mg, 43 μmol), and NaO^{t}Bu (67 mg, 0.7 mmol). The mixture was stirred for 10 min at ambient temperature. 2,6-Dimethyl-4-bromoanisole (**7**) (80 μL, 0.51 mmol) was added and then stirred for 20 h at 120 °C. The reaction mixture was quenched with H_2O, and extracted with $CHCl_3$. The extract was washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification with a silica gel column chromatography (hexane/EtOAc = 9:1 → 2:1) provided **9** (101.5 mg, 0.45 mmol) in 88% yield.

Compound **9**: pale yellow amorphous solid; ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.24 (6H, s), 3.69 (3H, d, J = 1.9 Hz), 6.03 (1H, br s), 6.75 (2H, s), 7.11 (1H, dt, J = 1.8 and 5.3 Hz, 1H), 7.30 (1H, dt, J = 8.3 and 1.4 Hz), 8.30 (1H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.17, 59.78, 119.57, 122.15, 123.54, 131.69, 137.06, 138.85, 137.06, 138.85, 138.91, 140.58, 140.80, 152.12; EI-MS *m*/*z* 228 (M^+); HREIMS Calcd for $C_{14}H_{16}N_2O$ (M^+) *m*/*z* 228.1262, found *m*/*z* 228.1263.

3.1.3. 8-Methoxy-7,9-dimethyl-δ-carboline (10) and 6-Methoxy-5,7-dimethyl-β-carboline (11). A solution of **9** (120 mg, 0.53 mmol) in toluene (200 mL) was irradiated with tungsten lamp 15 h at ambient temperature. The mixture was concentrated in vacuo and purified by a flash column chromatography on silica gel (hexane/acetone = 7:1 → 3:1 → $CHCl_3$/MeOH = 20:1 → 10:1 → 5:1) to yield **10** (37 mg, 0.16 mmol, 31%) and **11** (53 mg, 0.24 mmol, 45%).

Compound **10**: pale yellow amorphous solid; UV (MeOH) λ_{max} 309 (ε 27400), 266 (28100), 239 (30100), 217 (55700) nm; IR (KBr) 3360, 2920, 2860, 1630 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.39 (3H, s), 2.88 (3H, s), 3.69 (3H, s), 7.17 (1H, s), 7.30 (1H, dd, J = 8.2

and 5.0 Hz), 7.78 (1H, dd, J = 8.2 and 1.4 Hz), 8.40 (1H, dd, J = 5.0 and 1.4 Hz), 11.18 (1H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ 12.73, 17.33, 60.53, 109.65, 116.90, 118.96, 126.52, 127.97, 131.91, 133.28, 137.00, 141.56, 151.10, 165.70; EI-MS m/z 226 (M^+); HREIMS Calcd for $C_{14}H_{14}N_2O$ (M^+) m/z 226.1106, found m/z 226.1106.

Compound **11**: brown amorphous solid; UV (MeOH) λ_{max} 294 (ε 8000), 240 (15500), 205 (16800) nm; IR (KBr) 3330, 2930, 2860, 1640, 1460 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.43 (3H, s), 2.73 (3H, s), 3.70 (3H, s), 7.35 (1H, s), 8.27 (1H, s), 8.36 (1H, s), 8.99 (1H, s), 11.93 (1H, s); ^{13}C NMR (125 MHz, DMSO-d_6) δ 13.15, 17.33, 60.59, 110.75, 116.04, 118.64, 125.41, 128.23, 132.22, 133.86, 136.59, 137.40, 137.98, 150.08; EI-MS m/z 226 (M^+); HREIMS Calcd for $C_{14}H_{14}N_2O$ (M^+) m/z 226.1106, found m/z 226.1094.

3.1.4. 6-Hydroxy-5,7-dimethyl-β-carboline (1). To a solution of methylether **10** (60 mg, 0.265 mmol) in CH_2Cl_2 (30 mL) was added BBr_3 (1 M in CH_2Cl_2, 5 mL, 5 mmol) and the reaction mixture was stirred for 3 h at ambient temperature. The mixture was concentrated in vacuo and purified by a flash column chromatography on silica gel ($CHCl_3$/MeOH = 5:1) afforded **1** (45 mg, 0.212 mmol) in 78% yield.

Compound **1**: brown amorphous solid; ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.42 (3H, s), 2.74 (3H, s), 7.42, (1H, s), 8.44 (1H, d, J = 6.0 Hz), 8.58 (1H, d, J = 6.0 Hz), 9.17 (1H, s), 12.42 (1H, s); ^{13}C NMR (125 MHz, CD_3OD) δ 13.28, 18.68, 111.17, 117.69, 117.80, 119.67, 133.30, 134.29, 134.62, 139.01, 148.00; EI-MS m/z 212 (M^+); HREIMS Calcd for $C_{13}H_{12}N_2O$ (M^+) m/z 212.0949, found m/z 212.0945.

3.1.5. 6-Methoxy-5,7,9-trimethyl-β-carboline (12). To a solution of **10** (10 mg, 0.044 mmol) in THF (5 mL) was added NaH (25 mg, 1.0 mmol) and the reaction mixture was stirred for 30 min at ambient temperature. To the reaction mixture was added MeI (50 μL, 0.8 mmol) and stirred for 3 h. The reaction mixture was concentrated under reduced pressure. Purification with a silica gel column chromatography ($CHCl_3$/MeOH = 20:1 → 15:1) provided **12** (6.0 mg, 25 μmol) in 57% yield.

Compound **12**: brown amorphous solid; UV (MeOH) λ_{max} 398 (ε 1800), 322 (9200), 266 (14400), 215 (24800) nm; IR (KBr) 3420, 2920, 2850, 1720, 1640 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.52 (3H, s), 2.79 (3H, s), 3.74 (3H, s), 4.02 (3H, s), 7.63 (1H, s), 8.58 (1H, d, J = 6.3 Hz), 8.65 (1H, d, J = 6.3 Hz), 9.56 (1H, s); EI-MS m/z 240 (M^+); HREIMS Calcd for $C_{15}H_{16}N_2O$ (M^+) m/z 240.1263, found m/z 240.1257.

3.1.6. 6-Hydroxy-5,7,9-trimethyl-β-carboline (2). To a solution of methylether **12** (5.5 mg, 0.023 mmol) in CH_2Cl_2 (1 mL) was added BBr_3 (1 M in CH_2Cl_2, 5 mL, 5 mmol) and the reaction mixture was stirred for 4 h at ambient temperature. The mixture was concentrated in vacuo and purified by a flash column chromatography on silica gel ($CHCl_3$/MeOH = 5:1) to provide **2** (4.2 mg, 0.018 mmol) in 78%.

Compound **2**: brown amorphous solid; UV (MeOH) λ_{max} 426 (ε 700), 326 (3000), 273 (4400), 203 (8500) nm; IR (KBr) 3360, 3190, 2930, 1640 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.46 (3H, s), 2.77 (3H, s), 4.00 (3H, s), 7.55, (1H, s), 8.51 (1H, d, J = 6.3 Hz), 8.61 (1H, d, J = 6.3 Hz), 9.52 (1H, s); EI-MS m/z 226 (M^+); HREIMS Calcd for $C_{14}H_{14}N_2O$ (M^+) m/z 226.1106, found m/z 226.1095.

3.1.7. 8-Hydroxy-7,9-dimethyl-δ-carboline (3). To a solution of methylether **11** (10 mg, 0.044 mmol) in CH_2Cl_2 (1 mL) was added BBr_3 (1 M in CH_2Cl_2, 5 mL, 5 mmol) and the reaction mixture was stirred for 4 h at ambient temperature. The mixture was concentrated in vacuo and purified by a flash column chromatography on silica gel ($CHCl_3$/MeOH = 5:1) to yield **3** (6.6 mg, 0.031 mmol) in 70% yield.

Compound **3**: yellow amorphous solid; UV (MeOH) λ_{max} 313 (ε 4800), 272 (5100), 241, (5500), 211 (11400) nm; IR (KBr) 3400, 3180, 1620 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.40 (3H, s), 2.80 (3H, s), 7.35 (1H, s), 7.80 (1H, dd, J = 8.0 and 5.2 Hz), 8.49 (1H, d, J = 8.0 Hz), 8.57 (1H, d, J = 5.2 Hz), 12.25 (1H, s); ^{13}C NMR (150 MHz, DMSO-d_6) δ 14.33, 19.45, 112.94, 114.73, 119.94, 121.08, 128.21, 133.15, 134.27, 137.10, 138.52, 140.11, 150.27; EI-MS m/z 212 (M^+); HREIMS Calcd for $C_{13}H_{12}N_2O$ (M^+) m/z 212.0950, found m/z 212.0934.

3.1.8. 8-Methoxy-5,7,9-trimethyl-δ-carboline (13). To a solution of **11** (20 mg, 0.088 mmol) in THF (5 mL) was added NaH (45 mg, 1.8 mmol) and the reaction mixture was stirred for 30 min at ambient temperature. To the reaction mixture was added MeI (0.5 mL, 8.0 mmol) and stirred for 3 h. The reaction mixture was concentrated under reduced pressure. Purification with a silica gel column chromatography (hexane/acetone = 8:1 → 6:1) provided **13** (18 mg, 0.075 mmol) in 85% yield.

Compound **13**: yellow amorphous solid; UV (MeOH) λ_{max} 312 (ε 2600), 276 (3100), 217 (7500), 204 (8500) nm; IR (KBr) 2930, 2850, 1630, 1600 cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.43 (3H, s), 2.88 (3H, s), 3.67 (3H, s), 3.80 (3H, s), 7.29 (1H, s), 7.36 (1H, dd, J = 8.0 and 4.3 Hz), 7.91 (1H, d, J = 8.0 Hz), 8.42 (1H, d, J = 4.3 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 12.76, 17.54, 29.67, 60.55, 107.42, 114.64, 118.70, 119.16, 126.64, 131.61, 134.66, 138.49, 140.84, 143.35, 150.78; EI-MS m/z 240 (M^+); HREIMS Calcd for $C_{15}H_{16}N_2O$ (M^+) m/z 240.1263, found m/z 240.1263.

3.1.9. 8-Hydroxy-5,7,9-trimethyl-δ-carboline (4). To a solution of methylether **11** (10 mg, 0.042 mmol) in CH_2Cl_2 (1 mL) was added BBr_3 (1 M in CH_2Cl_2, 5 mL, 5 mmol) and the reaction mixture was stirred for 4 h at ambient temperature. The mixture was concentrated in vacuo and purified by a flash column chromatography on silica gel ($CHCl_3$/MeOH = 5:1) to provide **4** (7.8 mg, 0.035 mmol) in 83% yield.

Compound **4**: yellow amorphous solid; UV (MeOH) λ_{max} 351 (ε 560), 313 (2500), 277 (3100), 245 (3000), 217 (5600)

202 (5400) nm; IR (KBr) 3390, 2930, 1650 cm^{-1}; ^1H NMR (400 MHz,CD_3OD) δ 2.58 (3H, s), 2.93 (3H, s), 4.09 (3H, s), 7.54 (1H, s), 7.97 (1H, dd, J = 8.6 and 5.9 Hz), 8.56 (1H, dd, J = 5.9 and 0.9 Hz), 8.73 (1H, dd, J = 8.6 and 0.9 Hz); ^{13}C NMR; δ 12.50, 18.17, 28.72, 107.68, 115.36, 118.66, 119.07, 127.17, 134.11, 136.15, 139,91, 146.82; EI-MS m/z 226 (M^+); HREIMS Calcd for $C_{14}H_{14}N_2O$ (M^+) m/z 226.1106, found m/z 226.1099.

3.1.10. 8-Hydroxy-2,5,7-trimethyl-β-carboline (5). To a solution of **8** (4.8 mg, 21 μmol) in acetone (1 mL) was added MeI (0.1 mL, 1.6 mmol) and stirred for 1 h. The reaction mixture was concentrated and subjected to the next reaction without further purification. To the crude methylether was added BBr_3 (1 M in CH_2Cl_2, 3 mL, 3 mmol) and the reaction mixture was stirred for 8 h at ambient temperature. The mixture was concentrated in vacuo and purified by a flash column chromatography on amino silica gel ($CHCl_3$/MeOH = 2:1) to yield **5** (2.3 mg, 7.5 μmol) in 35% yield.

Compound **5**: brown amorphous solid; UV (MeOH) λ_{max} 325 (ε 8200), 277 (10000) nm; IR (KBr) 3400, 1640 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 2.52 (3H, s), 2.83 (3H, s), 4.50 (3H, s), 7.40 (1H, s), 8.35 (1H, d, J = 6.3 Hz), 8.56 (1H, d, J = 6.3 Hz), 9.04 (1H, s).

3.1.11. 8-Hydroxy-2,5,7,9-tetramethyl-β-carboline (6). To a solution of **8** (10 mg, 47 μmol) in THF (1 mL) was added NaH (6.3 mg, 160 μmol) and the reaction mixture was stirred for 10 min at ambient temperature. To the reaction mixture was added MeI (50 μL, 400 μmol) and stirred for 1 h. The reaction mixture was filtered with SiO_2 pad and subjected to the next reaction without further purification. To the crude methylether was added BBr_3 (1 M in CH_2Cl_2, 2 mL, 2 mmol) and the reaction mixture was stirred for 8 h at ambient temperature. The mixture was concentrated in vacuo and purified by a flash column chromatography on amino silica gel ($CHCl_3$/MeOH = 2:1) to afford **6** (1.2 mg, 3.7 μmol) in 8% yield.

Compound **6**: brown amorphous solid; UV (MeOH) λ_{max} 327 (ε 2500), 280 (3600) nm; IR (KBr) 3400, 1645 cm^{-1}; ^1H NMR (400 MHz,CD_3OD) δ 2.58 (3H, s), 2.85 (3H, s), 4.09 (3H, s), 4.56 (3H, s), 7.58 (1 H, s), 8.43 (1H, d, J = 6.3 Hz), 8.62 (1H, d, J = 6.3 Hz), 9.31 (1H, s).

3.2. Biological assays

3.2.1. Radioligand materials. [^{3}H]-8-Cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX), [^{3}H]-2-[4-(2-carboxyethyl)-phenethylamino]-5′-*N*-ethylcarboxamidoadenosine ([^{3}H]CGS21680), and [^{3}H]-5′-*N*-ethylcarboxamidoadenosine ([^{3}H]NECA) were purchased from Perkin-Elmer (Boston, MA, USA). Unless otherwise stated, all other materials used for ligand binding assay were purchased from Sigma to Aldrich (St. Louis, MO, USA).

3.2.2. Membrane preparations. HEK293T cell lines transiently expressing human adenosine A_1, A_{2A}, and A_3 receptors were used as the receptor source in this study. Plasmids encoding human adenosine A_1, A_{2A}, or A_3 receptor construct obtained from UMR cDNA Resource Center (Rolla, MO, USA) were transiently transfected into HEK293T cells using Effectene (Quiagen). Cells were maintained at 37 °C in humidified air containing 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 μg/mL kanamycin for 48 h. The cells were harvested and homogenized in lysis buffer containing 50 mM Tris–HCl buffer (pH 7.4) with a protease-inhibitor mixture (Roche Diagnostics) and subjected to low-speed centrifugation to remove organelles and nuclei. The resulting supernatant was subjected to centrifugation at 30,000g for 20 min, and precipitated cell membranes were collected, washed twice, resuspended in the lysis buffer, and stored at −80 °C until use.

3.2.3. Adenosine receptor binding assays. Radioligand binding experiments to adenosine A_1, A_{2A}, and A_3 receptors were carried out by using [^{3}H]DPCPX, [^{3}H]CGS21680, and [^{3}H]NECA, respectively. Cell membranes expressing adenosine A_1, A_{2A}, and A_3 receptors were incubated with 4 nM [^{3}H]DPCPX, 15 nM [^{3}H]CGS21680, or 32 nM [^{3}H]NECA, respectively, in the presence of 9 to 10 different concentrations of test compounds in 250 μL of assay buffer containing 50 mM Tris–acetate buffer, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA and 1 U/mL adenosine deaminase for 60 min at 25°C. The incubated mixture was harvested on Whatman GF/B filters pre-soaked in 0.1% polyethyleneimine by a cell harvester, and washed three times with 50 mM Tris–HCl buffer (pH 7.4). The radioactivity on the filter was measured by a scintillation counter. All experiments were carried out two or three times in duplicate. The nonspecific binding for adenosine A_1, A_{2A}, and A_3 receptors was defined as the binding activity in the presence of XAC, CGS21680, and NECA, respectively, at 10 μM each. K_d and B_{max} values in saturation and inhibition studies were determined using one-site binding model by nonlinear regression analysis (GraphPad Prism 4; GraphPad, San Diego, CA, USA).

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