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DESIGN, SYNTHESIS, AND EVALUATION OF NOVEL 2-HALOGENATED OR AMINATED CARBOCYCLIC OXETANOCIN A ANALOGS AS POTENTIAL ANGIOGENIC AGENTS

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Abstract – We succeeded in the synthesis of four types of carbocyclic oxetanocin A (2-Chloro-C.OXT-A; COA-Cl) analog: 2-halogenated or aminated carbocyclic oxetanocin A: COA-F, COA-Br, COA-I, and COA-NH₂, which were synthesized and evaluated using human umbilical vein endothelial cells. Among these compounds, 100 μ M COA-Br and COA-I had greater angiogenic activity, with relative tube areas of 2.10 ± 0.32 and 2.01 ± 0.61 (mean \pm SD), respectively, which were comparable to that of COA-Cl (2.30 ± 0.59).

Angiogenesis, the process in which new capillary vessels are built from preexisting ones, plays an important role in a variety of situations, including wound healing, tumor growth, and metastasis. Angiogenesis promoters are of much potential benefit for therapy of various symptoms arising due to a lack of blood flow, such as Buerger's disease or chronic arteriosclerosis obliterans in diabetic patients. However, the clinical applications of these promoters as medical agents remain minimal compared with those of angiogenesis inhibitors, which are utilized as antitumor therapeutics. The lack of angiogenesis promoters used as therapeutic agents may be due to limited knowledge about them, with the exception of growth factors derived from living systems. For example, vascular endothelial growth factor (VEGF) and

fibroblast growth factor (FGF) are macromolecular glycoproteins that are chemically and biologically

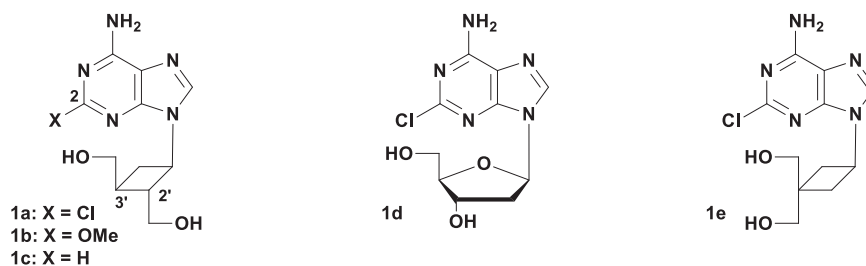


Figure 1. Structures of COA-Cl (**1a**), COA-OMe (**1b**), C.OXT-A (**1c**), cladribine (**1d**), and COA-Cl derivative (**1e**)

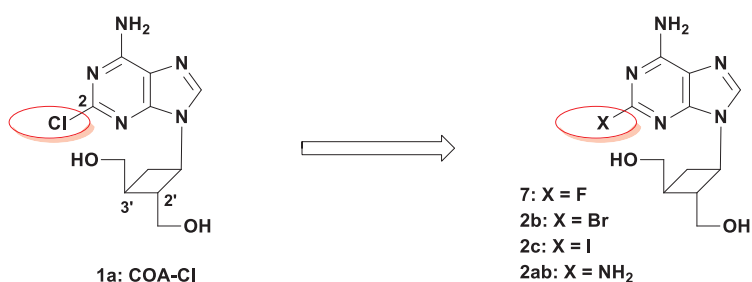
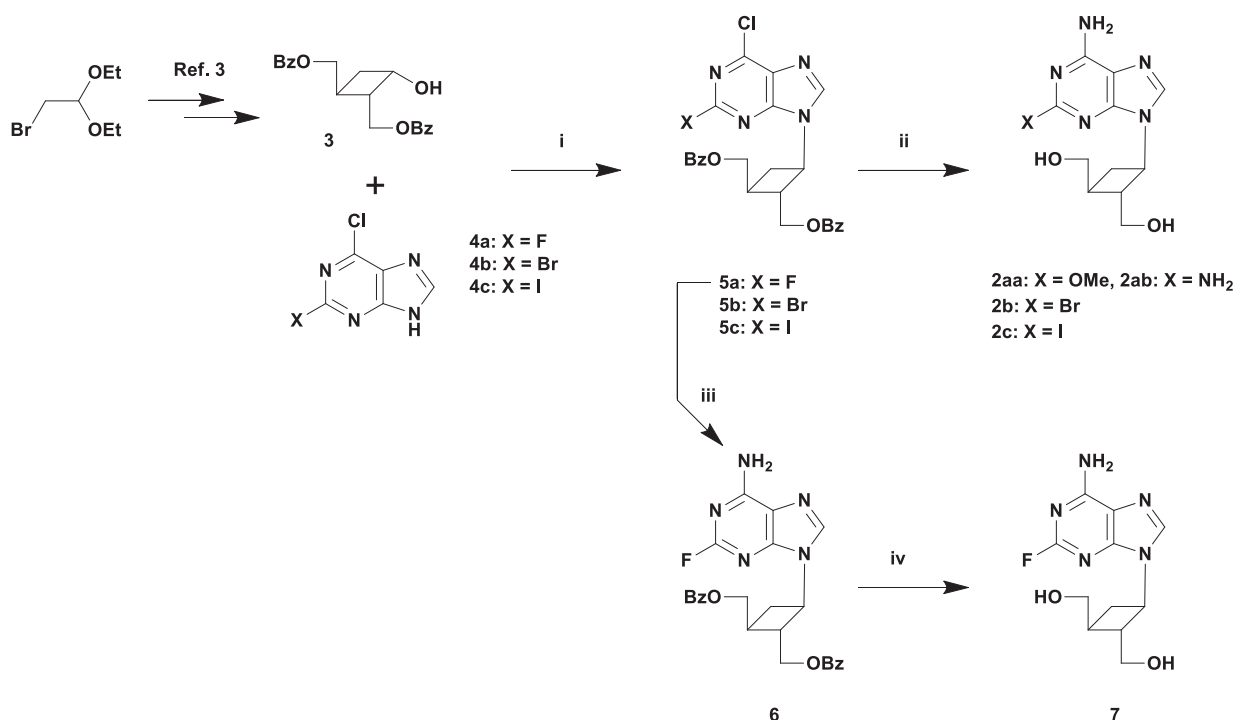


Figure 2. Structures of COA-Cl (**1**) and COA-Cl analogs (**7**, **2b**, **2c** and **2ab**)



Reagents and conditions: i, PPh₃, DIAD, THF, 50 °C; ii, NH₃ aq., MeOH, 70 °C; iii, NH₃ aq., MeOH, 4 °C, 4 h; iv, Na₂CO₃, CD₃OD, rt, 6 h

Scheme 1. Synthesis of compounds **2ab**, **2b**, **2c** and **7**

unstable. Hence, they are used only in treatments administered by injection or gene transfection approaches.¹ Because of this situation, we synthesized the first low-molecular-weight angiogenesis promoter, COA-Cl (**1a**, racemate) (Figure 1),² whose molecular weight (MW = 284) is suitable for pharmaceutical use in percutaneous and transmucosal absorption. Therefore, it is expected to have practical applications as a therapeutic for wound healing, Buerger's disease, or peripheral arterial obliterative disease, and could be developed as an adhesive skin patch, lotion, or oral agent. In addition to its angiogenic effect, we found that COA-Cl protects against oxygen-glucose deprivation in primary cortical neurons in a manner sensitive to suramin.^{2c} Because of its utility, COA-Cl is commercially available (chemical name: 2-Cl-C.OXT-A, Wako Co., Ltd., Japan, order number: 032-21541, quantity: 1 mg, price: 15,000 yen). As for mechanistic studies of it, it was revealed that COA-Cl is a novel and potent angiogenic agent that may behave as a partial agonist of the S1P₁ receptor.^{2a,2d} Understanding of the utility and unique mechanism of COA-Cl **1a** has triggered intensive efforts to discover novel COA-Cl analogs with enhanced angiogenic activity and reduced cytotoxicity.^{2a,2e,2f} However, our previous work resulted in analogs with almost no or less potency than COA-Cl **1a**. For example, as shown in Figure 1, the COA-Cl derivative COA-OMe (**1b**), in which the 2-chloro group at the 2-position in the purine system is substituted with a methoxy group, displayed half the vascularization activity of COA-Cl **1a**.^{2e} Furthermore, substitution of an H in place of the Cl (**1c**: C.OXT-A) or replacement of a cyclobutane moiety with a pentose ring (**1d**: cladribine) showed no angiogenic potency.^{2a} In a single exceptional case, COA-Cl analog **1e**, regioisomer of the two hydroxymethyl groups at the cyclobutane ring of **1a**, exhibited angiogenic activity comparable to that of COA-Cl **1a**.^{2f} Collectively, these studies indicated that the structure of not only the cyclobutane ring moiety but also the 2-substituted purine skeleton in COA-Cl **1a** is essential for its angiogenic activity. In an ongoing effort to discover potential COA-Cl analogs with high angiogenic activity, we were interested in the structure-activity relationship around the functional group at the 2-position of the purine skeleton of COA-Cl. Therefore, here we designed four structurally relevant, novel classes of halogenated or aminated COA-Cl analogs (**7**, **2b**, **2c**, and **2ab**), by replacing the 2-chloro group of the purine skeleton with appropriate groups (X = F, Br, I, NH₂), as shown in Figure 2. The synthesis of COA-Cl analogs **7**, **2b**, **2c**, and **2ab** is described in Scheme 1. The intermediate cyclobutyl alcohol **3** was prepared using commercially available bromoacetaldehyde diethyl acetal as starting material in five-step reactions, according to a modified version of a method in a previous report.³ Secondary alcohol **3** was treated with 2-halogenated 6-chloropurine (**4a**: 6-chloro-2-fluoropurine, **4b**: 2-bromo-6-chloropurine, **4c**: 6-chloro-2-iodopurine) and underwent the Mitsunobu reaction⁴ in the presence of PPh₃ and DIAD to yield 2-halogenated 9-cyclobutyl-6-chloropurine congeners, **5a–c**. The yields for Mitsunobu reaction differ considerably depending on the substrate, at 26–62% yields; in particular, the coupling of 2-bromo-6-chloropurine **4b** and cyclobutanol **3** had a low yield of 26%, which

corresponds to a previous report⁵ that **4b** was coupled with the secondary alcohol under almost the same conditions as a Mitsunobu reaction using PPh₃, DIAD, and THF. Then, **5b** or **5c** was treated with methanolic ammonia at 70 °C to give the desired 2-brominated or 2-iodinated products of 6-aminopurine analog **2b** (COA-Br) and **2c** (COA-I) at 78 and 60% yields, respectively. In contrast, for 6-chloro-2-fluoropurine derivative **5a** under the same reactive conditions, we observed no objective 2-fluorinated substrate, but the simultaneous production of 2-methoxylated and 2-aminated products of 6-aminopurine analog **2aa** (COA-OMe) and **2ab** (COA-NH₂) occurred at 27 and 45% yields because the fluorine group at the 2-position of the purine skeleton of **5a** was substituted under the thermal conditions by a methoxy group or amino group derived from methanol or ammonia in the reaction system, respectively. Subsequently, 2-fluoropurine analog **5a** was treated under the alternative mild ammonolysis reaction with *liq.* NH₃/MeOH at 4 °C to give 2-fluoro-9-[2',3'-bis(benzoyloxymethyl)cyclobutyl]purine derivative **6** at 21% yield. Then, compound **6** was deprotected with Na₂CO₃/CD₃OD condition⁶ to afford the desired 6-chloro-2-fluoropurine analog **7**.

The effects of the four synthesized types of 2-halogenated or aminated COA-Cl analog: COA-F (**7**), COA-Br (**2b**), COA-I (**2c**), and COA-NH₂ (**2ab**), on angiogenesis were examined. We evaluated the analogs using a well-established tube formation assay.⁷ Typical pictures are shown in Figure 3. VEGF was used as a positive control. The area of the formed tube is presented as a value relative to that of tubes formed in wells without additives (saline). Figure 4 shows the dose-dependent tube formation responses, as well as the solvent control (10% saline) and positive control (VEGF, 10 ng/mL).

As shown in Figures 3 and 4, angiogenic potencies were observed for three prepared compounds, namely, COA-F (**7**) at a concentration of 100 μM, COA-Br (**2b**) at 10 and 100 μM, and COA-I (**2c**) at 1, 10, and 100 μM (Figure 4). Among these, at a concentration of 100 μM, **2b** and **2c** exerted greater angiogenic

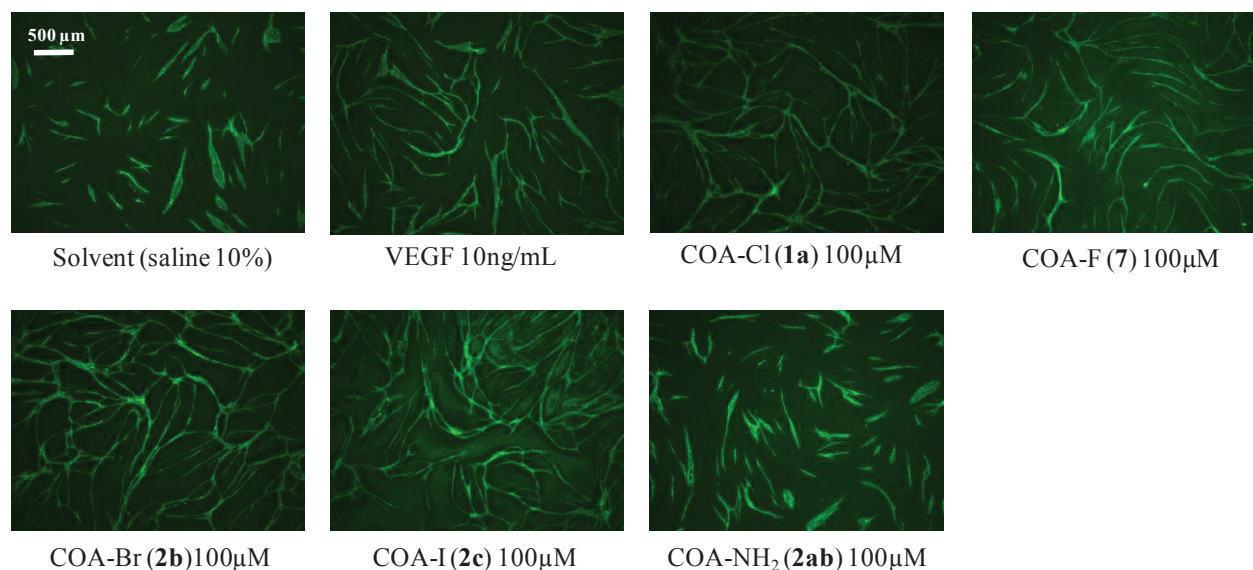


Figure 3. Pictures of stimulated tube formation by **1a**, **7**, **2b**, **2c** and **2ab**. Additives are shown under the pictures.

activities (2.10 ± 0.32 and 2.01 ± 0.61 [mean \pm SD], respectively), which were comparable to that of COA-Cl **1a** (2.30 ± 0.59). In contrast, COA-F (**7**) at 1 and 10 μ M, COA-Br (**2b**) at 1 μ M, and COA-NH₂ (**2ab**) at all concentrations showed no effect on angiogenic potency (Figure 4). Moreover, as shown in Figure 5, we performed a proliferation assay because angiogenesis is intimately associated with complex cellular processes, including proliferation of endothelial cells,^{1a,8} which showed that all examined compound (**7**, **2b**, **2c**, and **2ab**) did not stimulate the proliferation of HUVECs.

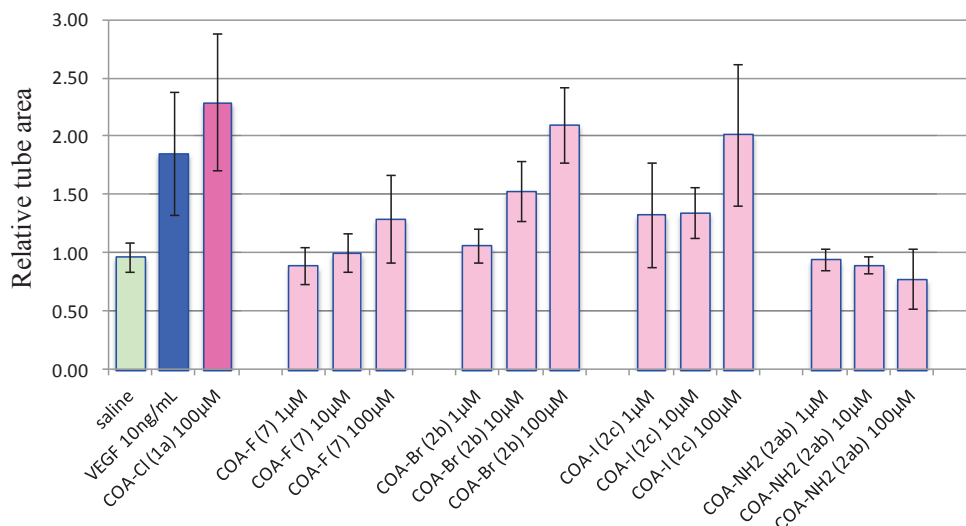


Figure 4. Tube formation assay using compounds **1a**, **7**, **2b**, **2c** and **2ab**. The area of the formed tube is represented as a value relative to that in control wells with no additives. The effects of the solvent (10% saline) and positive control (VEGF; 10 ng/mL) are shown together. Results are expressed as mean \pm SE of 5 – 12 individual experiments.

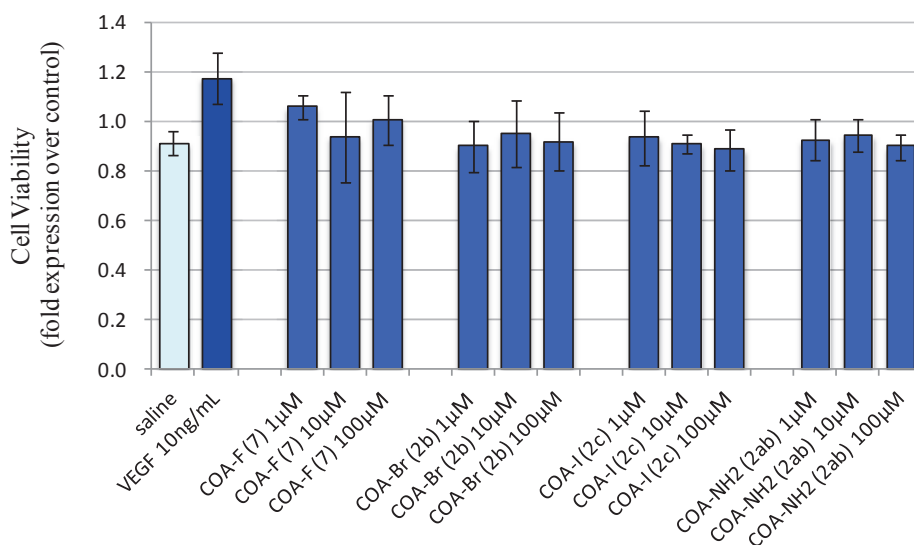


Figure 5. Effects of compounds **7**, **2b**, **2c** and **2ab** on the proliferation. 48 h after the addition of additives, the cell viability was measured with a Cell Counting Kit-8. Cell viability is represented as a value relative to that in control wells with no additive. The effect of the solvent, 10% saline as well as a positive control, VEGF (10 ng/mL) are shown together. Results were expressed as mean \pm SE of 4 – 10 individual experiments.

In conclusion, we designed four structurally relevant COA-Cl analogs: COA-F, COA-Br, COA-I, and COA-NH₂ (**7**, **2b**, **2c** and **2ab**), which of **2b** and **2c** had greater angiogenic activity at 100 μ M, with relative tube areas of 2.10 ± 0.32 , and 2.01 ± 0.61 , respectively. This was comparable with the tube area induced by COA-Cl (**1a**, 2.30 ± 0.59). Overall, our data will be utilized in the future in preliminary structure–activity relationship studies and molecular modeling analyses using COA-Cl or its corresponding analogs as a ligand, performed to explore the major interactions between the S1P₁ receptor and COA-Cl (**1a**), as well as COA-Cl derivatives.

EXPERIMENTAL

Instrumentation

¹H NMR and ¹³C NMR spectra were taken with a Ultrashield™ 400 Plus FT NMR System (BRUKER). Chemical shifts and coupling constants (*J*) were given in δ and Hz, respectively. Melting points were determined on a Yanaco MP-500D. Elementary analyses were determined by a Perkin Elmer Series II CHNS/O Analyzer 2400. High-resolution mass spectrometry was performed on a APEX IV mass spectrometer (BRUKER) with electrospray ionization mass spectroscopy (ESI-MS).

General procedure for the synthesis of **5a–c**

A solution of compound **3** (374.4 mg, 1.10 mmol), triphenylphosphine (288.5 mg, 1.10 mmol), **4a–c** (1.00 mmol) and DIAD (diisopropyl azodicarboxylate, 216.6 μ L, 1.10 mmol) in THF (5.5 mL) was stirred at 50 °C. After 2–17 h stirring, the residual solution was purified by silica gel column chromatography (60% AcOEt in hexane) to give **5a–c**.

6-Chloro-2-fluoro-9-[*trans-trans*-2',3'-bis(benzoyloxymethyl)cyclobutyl]purine (**5a**)

Yield 39%; oil; ¹H NMR (400 MHz, CDCl₃): δ 8.17 (1 H, s, H-8), 7.98–8.12 (3 H, m, 2'-OBz and 3'-OBz), 7.82–7.88 (2 H, m, 2'-OBz and 3'-OBz), 7.53–7.63 (3 H, m, 2'-OBz and 3'-OBz), 7.34–7.50 (2 H, m, 2'-OBz and 3'-OBz), 4.82 (1 H, dd, *J* 17.6 and 8.4, H-1'), 4.45–4.61 (4 H, m, 2'-CH₂ and 3'-CH₂), 3.45–3.53 (1 H, m, H-2'), 2.71–2.81 (2 H, m, H-4'), 2.58–2.66 (1 H, m, H-3'); ¹³C NMR (100 MHz, CDCl₃): δ 166.5, 166.3, 156.9 (d, *J* 219), 156.9 (d, *J* 19), 153.5 (d, *J* 17), 144.9 (d, *J* 3), 133.4, 133.5, 130.8 (d, *J* 5), 129.7, 129.5, 128.7, 128.5, 118.0 (d, *J* 4), 65.9, 64.5, 49.2, 45.4, 31.2, 29.3; HRMS (ESI) Calcd for C₂₅H₂₀ClFN₄NaO₄ [M+Na]⁺: 517.10493. Found: 517.10443.

2-Bromo-6-chloro-9-[*trans-trans*-2',3'-bis(benzoyloxymethyl)cyclobutyl]purine (**5b**)

Yield 26%; oil; ¹H NMR (400 MHz, CDCl₃): δ 8.17 (1 H, s, H-8), 8.04–8.09 (2 H, m, 2'-OBz and 3'-OBz), 7.79–7.84 (2 H, m, 2'-OBz and 3'-OBz), 7.52–7.59 (2 H, m, 2'-OBz and 3'-OBz), 7.44–7.50 (2 H, m, 2'-OBz, 3'-OBz), 7.36–7.41 (2 H, m, 2'-OBz, 3'-OBz), 4.91 (1 H, dd, *J* 17.2 and 9.2, H-1'), 4.49–4.62 (4 H,

m, 2'-CH₂ and 3'-CH₂), 3.35-3.44 (1 H, m, H-2'), 2.74-2.83 (1 H, m, H-4'), 2.59-2.72 (2 H, m, H-3' and H-4'); ¹³C NMR (100 MHz, CDCl₃): δ 166.4, 152.9, 151.6, 144.2, 143.0, 133.4, 129.7, 129.4, 128.6, 66.1, 64.5, 49.5, 31.3, 29.5, 22.0; HRMS (ESI) Calcd for C₂₅H₂₀BrClN₄NaO₄ [M+Na]⁺: 577.02487. Found: 577.02336.

6-Chloro-2-iodo-9-[*trans-trans*-2',3'-bis(benzoyloxymethyl)cyclobutyl]purine (5c)

Yield 62%; oil; ¹H NMR (400 MHz, CDCl₃): δ 8.11 (1 H, s, H-8), 8.02-8.09 (2 H, m, 2'-OBz and 3'-OBz), 7.79-7.84 (2 H, m, 2'-OBz and 3'-OBz), 7.51-7.62 (2 H, m, 2'-OBz and 3'-OBz), 7.43-7.50 (2 H, m, 2'-OBz and 3'-OBz), 7.34-7.41 (2 H, m, 2'-OBz and 3'-OBz), 4.90 (1 H, dd, *J* 17.2 and 8.8, H-1'), 4.50-4.62 (4 H, m, 2'-CH₂ and 3'-CH₂), 3.22-3.41 (1 H, m, H-2'), 2.74-2.82 (1 H, m, H-4'), 2.59-2.70 (2 H, m, H-3', H-4'); ¹³C NMR (100 MHz, CDCl₃): δ 171.1, 166.4, 152.5, 150.5, 143.7, 133.4, 129.6, 128.6, 128.5, 66.2, 64.6, 49.4, 46.1, 31.2, 29.5; HRMS (ESI) Calcd for C₂₅H₂₀IClN₄NaO₄ [M+Na]⁺: 625.0112. Found: 625.01033.

General procedure for the synthesis of 2aa, 2ab, 2b and 2c

Compound **5a–c** (0.16 mmol) was dissolved in NH₃ (14.0 mL)/MeOH (3.0 mL), and then sealed and stirred for several hours at 70 °C. The mixture was evaporated, and the residue was purified by silica gel column chromatography (20–30% MeOH in CH₂Cl₂) to afford **2aa**, **2ab**, **2b** and **2c**.

6-Amino-2-methoxy-9-[*trans-trans*-2',3'-bis(hydroxymethyl)cyclobutyl]purine (2aa)

Yield 27%; pale yellow oil; ¹H NMR (400 MHz, CD₃OD); δ 7.91 (1 H, s, H-8), 4.52 (1 H, dd, *J* 17.2 and 8.4, H-1'), 3.85 (3 H, s, 2-OCH₃), 3.53-3.63 (4 H, m, 2'-CH₂ and 3'-CH₂), 2.77-2.84 (1 H, m, H-2'), 2.41-2.50 (1 H, m, H-4'), 2.29-2.37 (1 H, m, H-4'), 2.07-2.17 (1 H, m, H-3'); ¹³C NMR (100 MHz, CDCl₃): δ 162.1, 156.7, 151.2, 138.6, 115.2, 64.0, 62.2, 53.7, 33.4, 28.1, 13.1; HRMS (ESI) Calcd for C₁₂H₁₇N₅NaO₃ [M+Na]⁺: 302.12236. Found: 302.12229.

2,6-Diamino-9-[*trans-trans*-2',3'-bis(hydroxymethyl)cyclobutyl]purine (2ab)

Yield 45%; pale yellow crystals; ¹H NMR (400 MHz, CD₃OD); δ 7.91 (1 H, s, H-8), 4.51 (1 H, dd, *J* 17.2 and 8.8, H-1'), 3.63-3.77 (4 H, m, 2'-CH₂, 3'-CH₂), 2.72-2.81 (1 H, m, H-3'), 2.53-2.61 (1 H, m, H-4'), 2.28-2.38 (1 H, m, H-4'), 2.13-2.24 (1 H, m, H-2'); ¹³C NMR (100 MHz, CDCl₃): δ 168.2, 160.0, 156.2, 136.7, 113.1, 63.7, 62.5, 33.4, 28.0; HRMS (ESI) Calcd for C₁₁H₁₆N₆NaO₂ [M+Na]⁺: 287.12269. Found: 287.12246; mp 179.3 °C.

6-Amino-2-bromo-9-[*trans-trans*-2',3'-bis(hydroxymethyl)cyclobutyl]purine (2b)

Yield 78%; pale yellow crystals; ¹H NMR (400 MHz, CD₃OD); δ 8.08 (1 H, s, H-8), 4.56 (1 H, dd, *J* 17.2 and 8.4, H-1'), 3.55-3.63 (4 H, m, 2'-CH₂ and 3'-CH₂), 2.73-2.81 (1 H, m, H-2'), 2.45-2.53 (1 H, m, H-4), 2.07-2.18 (1 H, m, H-4'); ¹³C NMR (100 MHz, MeOD): δ 156.4, 150.4, 144.2, 140.0, 118.2, 63.8, 62.3,

33.3, 28.4, 27.5; HRMS (ESI) Calcd for $C_{11}H_{14}BrN_5O_2$ $[M+H]^+$: 328.04036. Found: 328.04041; mp 146.7–148.0 °C.

6-Amino-2-iodo-9-[*trans-trans*-2',3'-bis(hydroxymethyl)cyclobutyl]purine (2c)

Yield 60%; pale yellow crystals; 1H NMR (400 MHz, CD_3OD): δ 8.01 (1 H, s, H-8), 4.55 (1 H, dd, J 17.2 and 8.4, H-1'), 3.56–3.63 (4 H, m, 2'-CH₂ and 3'-CH₂), 2.72–2.82 (1 H, m, H-2'), 2.43–2.52 (1 H, m, H-4'), 2.20–2.30 (1 H, m, H-4'), 2.06–2.17 (1 H, m, H-3'); ^{13}C NMR (100 MHz, MeOD): δ 155.6, 149.8, 139.6, 118.9, 63.9, 62.4, 48.5, 33.4, 28.4, 20.7; HRMS (ESI) Calcd for $C_{11}H_{14}IN_5NaO_2$ $[M+Na]^+$: 398.00844. Found: 398.00860; mp 188.9–190.6 °C.

6-Amino-2-fluoro-9-[*trans-trans*-2',3'-bis(benzoyloxymethyl)cyclobutyl]purine (6)

Compound **5a** (33.0 mg, 0.067 mmol) was dissolved in NH_3 (14.0 mL)/MeOH (2.0 mL), and then sealed and stirred for 10 h at 4 °C. The mixture was evaporated, and the residue was purified by silica gel column chromatography (AcOEt) to give **6** as pale yellow oil (6.8 mg, 0.041 mmol, 21%). 1H NMR (400 MHz, $CDCl_3$): δ 7.98–8.14 (2 H, m, 2'-OBz and 3'-OBz), 7.86–7.90 (2 H, m, 2'-OBz and 3'-OBz), 7.82 (1 H, s, H-8), 7.36–7.62 (6 H, m, 2'-OBz and 3'-OBz), 5.99 (2 H, brs, NH_2), 4.75 (1 H, dd, J 18.0 and 9.2, H-1'), 4.48–4.60 (4 H, m, 2'-CH₂ and 3'-CH₂), 3.42–3.50 (1 H, m, H-2'), 2.67–2.77 (1 H, m, H-4'), 2.52–2.62 (1 H, m, H-3'); ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.6, 166.4, 158.7 (d, J 207), 157.0 (d, J 20), 151.7 (d, J 20), 139.7 (d, J 3), 133.3, 129.7, 129.5, 128.6, 128.4, 118.0 (d, J 4), 65.9, 64.5, 49.2, 45.4, 31.2, 29.3; HRMS (ESI) Calcd for $C_{25}H_{22}FN_5NaO_4$ $[M+Na]^+$: 498.15480. Found: 498.15010.

6-Amino-2-fluoro-9-[*trans-trans*-2',3'-bis(hydroxymethyl)cyclobutyl]purine (7)

A solution of compound **6** (15.6 mg, 0.033 mmol) and Na_2CO_3 (35.0 mg, 0.33 mmol), in CD_3OD (0.45 mL) was stirred at ambient temperature. After 6 h stirring, the mixture was evaporated in vacuo and the residue was filtered through sintered glass, concentrated, and subjected to silica gel column chromatography (20% MeOH in CH_2Cl_2) to give a pale yellow solid **7** (4.8 mg, 0.018 mmol, 55%). 1H NMR (400 MHz, CD_3OD): δ 8.08 (1 H, s, H-8), 4.54 (1 H, dd, J 17.6 and 8.8, H-1'), 3.55–3.63 (4 H, m, 2'-CH₂ and 3'-CH₂), 2.73–2.82 (1 H, m, H-2'), 2.42–2.51 (2 H, m, H-4'a), 2.20–2.29 (2 H, m, H-4'b), 2.08–2.16 (1 H, m, H-3'); ^{13}C NMR (100 MHz, CD_3OD): δ 160.4 (d, J 207), 159.0 (d, J 20), 152.2 (d, J 19), 141.5 (d, J 3), 118.6 (d, J 4), 65.3, 63.6, 55.1, 37.9, 34.8, 29.8; HRMS (ESI) Calcd for $C_{11}H_{14}FN_5NaO_2$ $[M+Na]^+$: 290.10237. Found: 290.10194; mp 180.0–181.5 °C.

Assay⁷

Human umbilical vein endothelial cells (HUVEC), normal human dermal fibroblasts (NHDF), endothelial cell growth medium (EGM-2), endothelial cell basal medium (EBM-2) and fibroblast growth medium (FGM-2) were purchased from Lonza Japan (Tokyo, Japan). Vascular endothelial growth factor-A

(VEGF) was from Kurabo Co. (Osaka, Japan). Cell Counting Kit 8 was supplied by Dojindo Molecular Technologies (Kumamoto, Japan). Anti-CD31 monoclonal antibody Human, Mouse from Dako Japan (Tokyo, Japan) and anti-Mouse IgG antibody conjugated with Alexa Fluor 488, Gout, from Life Technologies (Carlsbad, CA, USA) were used for the immuno staining. HUVEC and NHDF were cultured in EGM-2 and FGM-2, respectively.

Tube formation assay⁷

HUVEC and NHDF were trypsinized and suspended separately, and were then mixed at 20,000 of HUVEC and 140,000 of NHDF in 500 μ L of culture medium and seeded on gelatin-coated 24-well plates. After seeding, the plates were incubated for 24 h to permit anchorage, and then, the medium was changed with the assay medium, consisting of 450 μ L of EBM-2 with 2% heat-inactivated FBS and 50 μ L of saline containing additives **7**, **2b**, **2c** and **2ab**. The contents of EBM-2 are almost identical to those of EGM-2, except for the fact that the former does not include FBS, growth factors, or antibiotics. After 96 h, HUVEC was stained with first antibody against CD31 and second antibody conjugated with Alexa fluor 488. The area of the formed tube was measured by the ImageJ program. Two pictures from each well were provided for the estimation. VEGF (10 ng/mL) was used as a positive control.

Proliferation assay^{2a,8}

HUVECs were seeded on gelatin-coated 96-well plates, typically at 3000 cells/well in 100 μ L of EGM-2. After seeding, the plates were incubated for 24 h to permit anchorage, and then, the culture medium was changed with the assay medium, consisting of 90 μ L of EBM-2 with 2% heat-inactivated FBS and 10 μ L of saline containing additives. The proliferation assay was performed using a Cell Counting Kit-8 48 h after the addition of compounds **7**, **2b**, **2c** and **2ab**.

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