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NOVEL SYNTHESIS OF CARBOCYCLIC OXETANOCIN ANALOGS (2-ALKOXY-C.OXT-A) AND THEIR TUBE FORMATION ACTIVITIES OF HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVEC)

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Abstract – We succeeded in the effective synthesis of five types of 2-alkoxy-C.OXT-A analogs (**9**, **10a–d**) by applying the de novo synthesis of 2-alkoxyadenosine derivatives (**2**), which was developed in our laboratory. For these synthesized compounds, the angiogenic activity was evaluated using human umbilical vein endothelial cells. Four products (**10a–d**) enhanced the activity of the cell; in particular, 2-methoxy-C.OXT-A (**10a**) and 2-isopropoxy-C.OXT-A (**10c**) at a concentration of 100 μ M exhibited the same or stronger potency than the vascular endothelial cell growth factor.

INTRODUCTION

Angiogenesis is the process that builds new capillary vessels from preexisting ones, and it plays an important role in a variety of situations such as wound treatment, tumor growth, and metastasis. Angiogenesis promoters are very essential for detecting various symptoms arising because of lack of blood flow, for instance, chronic arteriosclerosis obliterans in diabetic patients or Buerger's disease. However, the clinical applications of these promoters as medical agents remain minimal compared to the angiogenesis inhibitors that are utilized as antitumor medicines, etc. Such fewer adaptations of the neoangiogenesis promoter as therapeutic agents appear to be because of limited knowledge about the promoter except for the growth factors derived from living systems, such as the vascular endothelial cell growth factor (VEGF) and fibroblast growth factor (FGF).

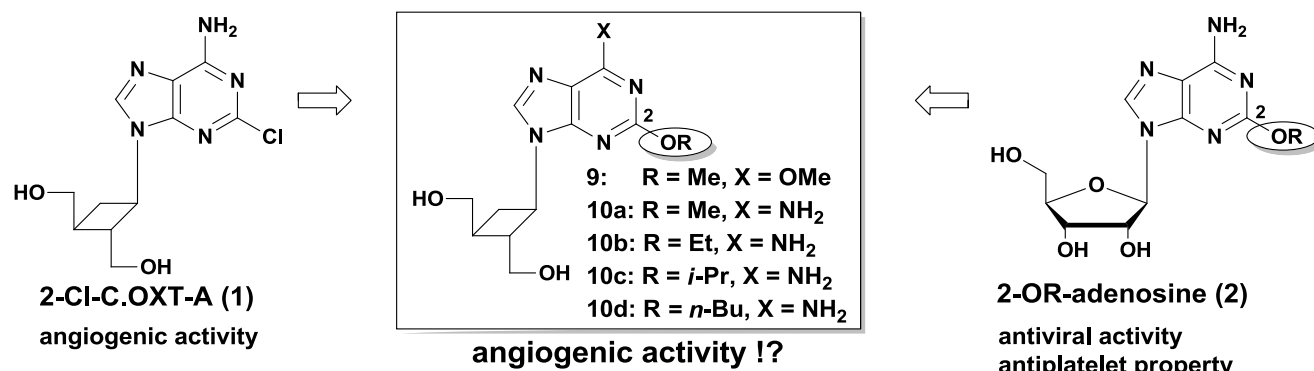
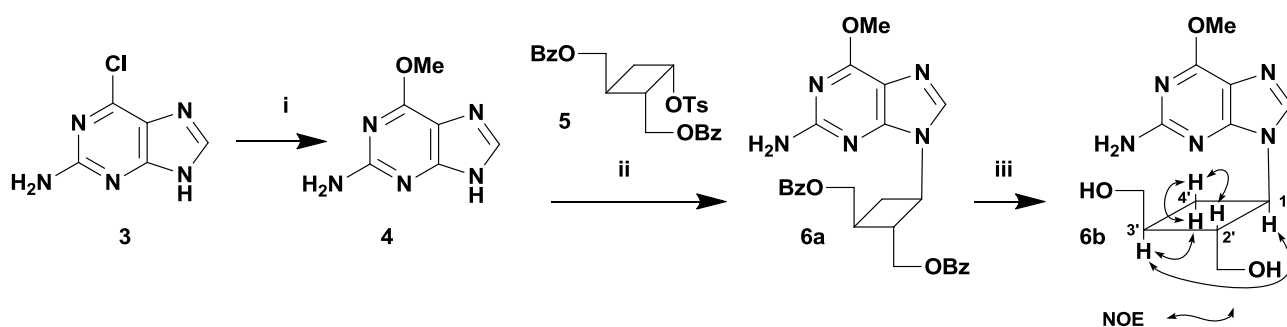


Figure 1. Structure of 2-Cl-C.OXT-A (**1**), 2-alkoxyadenosine (**2**) and 2-alkoxy-C.OXT-A analogs (**9**, **10a-d**)

In recent years, the newly synthesized angiogenesis promoter, 2-Cl-C.OXT-A (**1**), which is the first active substance on low-molecular compounds, was discovered by us.¹ The macromolecular glycoproteins, VEGF and FGF, which also possess the lumen formation stimulatory effect in the endocapillary cells, are chemically and biologically unstable. Hence, they are used only in treatment by the injection or gene transfection approach.² The molecular size of compound **1**, with a molecular weight of 284, is suitable for pharmaceutical use in percutaneous and transmucosal absorption. Therefore, it is expected to have practical applications as a wound healing agent, a topical cream for hair growth tonics and therapeutic drugs for dementia, an adhesive skin patch, and a lotion. In our previous work,¹ however, the various analogs of **1** were simultaneously synthesized and assayed for angiogenesis activity, resulting in no potency that is similar to the lumen formation promoter, except for **1**. As a result, it seemed that the structure of the 2-chloropurine skeleton and the cyclobutane ring moiety in **1** was essential to exert angiogenesis activity.¹ To overcome this challenge, we changed of our point of view and presumed that the 2-alkoxy-C.OXT-A derivatives (**9**, **10a-d**), in which the 2-chloro group at the 2-position in the purine system is substituted by an alkoxy group in **1**, are expected to possess higher vascularization activity in synthetic targets. Some 2-alkoxyadenosine derivatives (**2**) are known worldwide to exhibit a variety of biological activities such as antiviral activities and antiplatelet properties (Figure 1).³ Therefore, the synthetic procedures attempted by past researchers introduced the alkoxy group at the 2-position in the purine moiety of adenosine analogs. Nevertheless, products with low yields were formed or required multiple steps for synthesis.⁴ Recently, however, we developed an efficient synthetic process to convert the amino group at the 2-position of the purine skeleton into the alkoxy group.⁵ Herein, by applying our method,⁵ we succeeded in the effective synthesis of five types of 2-alkoxy-C.OXT-A analogs (**9**, **10a-d**). For the above compounds, the angiogenic activity was evaluated using human umbilical vein endothelial cells (HUVECs). This resulted in an increase to their activities, especially in 2-methoxy-C.OXT-A (**10a**) and 2-isopropoxy-C.OXT-A (**10c**) at a concentration of 100 μ M, and a same or stronger potency than VEGF.

RESULTS AND DISCUSSION

The synthesis of compound **6a** began with methoxylation at the 6-position in the purine skeleton of the readily available 2-amino-6-chloropurine (**3**) (Scheme 1).⁶ That is, **3** was refluxed with methanol in the presence of sodium methoxide to give 2-amino-6-methoxypurine (**4**) in 97% yield. The resulting product **4** was alkylated with cyclobutane derivative **5**, which has been synthesized in the past in our laboratory according to a previous report,^{7a} in the presence of potassium carbonate and DMF solvent at 120 °C, providing the corresponding alkylation product **6a** in 49% yield. To determine the structure of **6a**, **6a** was debenzoylated to give the corresponding compound **6b**. Then, **6b** was confirmed as N9 alkylation product by measurement of UV spectrum: UV absorption spectrum of **6b** (282.9 nm, EtOH) was very similar to that of N9-alkylated 2-amino-6-methoxypurine derivative (282.5 nm, EtOH) as Geen reported,^{7b} while N7-alkylated isomer shifts to 295.0 nm towards long-wavelength side. As to the configurations of cyclobutane skeleton of **6b**, NOESY correlations of between the 1' proton (δ 4.37, 1 H, m) and the 3' proton (δ 2.18, 1 H, m) were detected, while those of between the 1' proton and the 2' proton (δ 2.75, 1 H, m) were not significantly correlated; this means that the 1' proton and the 2' proton display a trans configuration (Scheme 1). Moreover, ¹H NMR spectrum of **6b** was analogous to that of *trans-trans*-2-amino-9-[2,3-bis(hydroxymethyl)cyclobutyl]-6-benzyloxypurine as Slusarchyk reported.^{7c} Subsequently, we adopted our previous method concerning the introduction of the alkoxy group at the 2-position in the purine moiety,⁵ and the conversion of the amino group at the 2-position of the purine skeleton into the alkoxy group was attempted by using compound **6a** as the starting material in the presence of appropriate alcohols for treating isoamyl nitrite: compound **6a** was treated with 5 equivalent amounts of isoamyl nitrite in methanol at 50 °C for 36 h to provide the corresponding 2-methoxy-C.OXT-A (**7a**) in 90% yield, and simultaneously giving the xanthosine analog **8** as a by-product in 8% yield (Table 1, entry 1).



Reagents and conditions: i, NaOMe, MeOH, 80 °C, 2 days; ii, compound **5**, K₂CO₃, DMF, 120 °C, 4 h; iii, NaOMe, MeOH, 40 °C, 20 min

Scheme 1. Synthesis and structural determination of compound **6a**

Table 1. 2-Alkoxylation of **6a** with alcohols

entry	solvent	time (h)	yield of 7a-d	yield(s) of other product(s)
1	R = Me	36	7a: 90 %	8: 8 %
2	R = Et	12	7b: 87 %	8: 10 %
3	R = <i>i</i> -Pr	12	7c: 56 %	8: 14 % and 6a (SM rec.):10 %
4	R = <i>n</i> -Bu	11	7d: 69%	8: 23 % and 6a (SM rec.):7 %

Similarly, in ethanol, the 2-ethoxylated product **7b** was obtained with 87% yield, and the xanthosine derivative **8** in 10% yield (entry 2). In contrast, in isopropanol and *n*-butanol, we observed a decrease in the yield of the objective 2-alkoxylated substrates (**7c** and **7d**) and an increase in the yield of by-product **8**. Additionally, starting material **6a** was recovered in a yield of a few percentages (entry 3 and 4).

Next, based on Katagiri's procedure,⁸ the 2-alkoxy analogs (**7a–d**) were treated with methanolic ammonia solution in a sealed tube at 110–120 °C to produce the target materials (**10a–d**) in 50–75% yields with the two functional group transformations: the amino substitution at the 6-position and deprotection at the two benzoyl groups of the cyclobutane ring (Table 2). With regard to the 2,6-dimethoxy product **9**, using the conventional approach of sodium methoxide in methanol, **9** was synthesized from substrate **7a** by carrying out the debenzoylation at the two hydroxyl groups.

Table 2. Debzoylation of compounds **7a–d**

entry	substrate	time (days)	temp (°C)	yield of 10a-d
1	7a: R = Me	3	120	10a: 75 %
2	7b: R = Et	2	110	10b: 62 %
3	7c: R = <i>i</i> -Pr	4	120	10c: 50%
4	7d: R = <i>n</i> -Bu	2	110	10d: 73 %

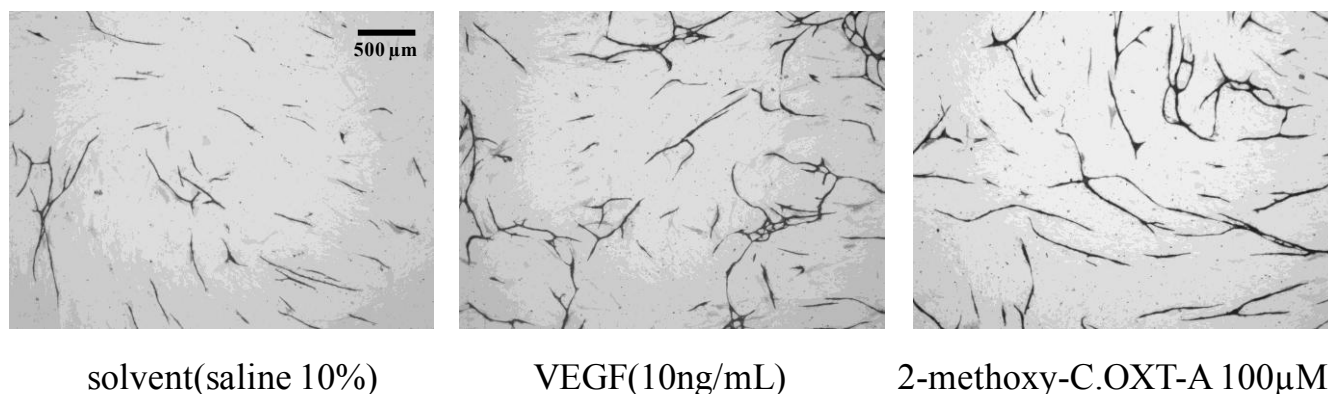


Figure 2. Typical pictures of stimulated tube formation by 2-methoxy-C.OXT-A 100 μ M (**10a**).

Additives are shown under the pictures. Tube area was estimated from two pictures from each well.

The effects of the synthesized nucleoside analogs mentioned above (**9**, **10a–d**) on angiogenesis were examined. We explored the analogs to a well established tube formation assay.^{1,9} Ten days following incubation periods with co-cultured fibroblast and additives, HUVEC were stained using Tubule Staining Kit for CD31. Typical pictures were shown in Figure 2. VEGF (vascular endothelial growth factor-A) was used as a positive control. The area of the formed tube was represented as a relative value to that formed in the well with no additive. Figure 3 shows the dose dependence of tube formation responses together with the solvent control (10% saline) and positive control.

As shown in Figures 2 and 3, the angiogenic potencies were observed in compounds **10a–d**. Among those, 2-methoxy and 2-isopropyl derivatives, **10a** and **10c** were more angiogenic. The relative area of the formed tube in the presence of **10a** and **10c** were 1.87 ± 0.19 (100 μ M, $n = 4$) and 1.90 ± 0.45 (100 μ M, $n = 5$) (mean \pm SD), respectively, which were comparable with that of 10 ng/mL of VEGF (1.87 ± 0.50 ($n = 12$)). The potency of **10a–d** became maximum around 100 μ M, and then, rather decreased at 1mM. However, the 2,6-dimethoxy analog, **9** caused no effect on tube formation. Comparing the molecular structures of the non-potent **9** and potent **10a** compounds, only the amino group at the 6-position in the purine moiety of **10a** was substituted by the methoxy group. It appears that the 6-amino group in the purine skeleton is essential for the activity.

We have previously examined the structure-activity relationship of the most angiogenic compound **1** and its analogs.¹ The compound **1** stimulated tube formation much stronger than VEGF. The relative area reached 3.78 ± 1.09 (100 μ M, $n = 9$). However, a substitution of its adenine moiety with guanine, or a conversion of cyclobutane ring into the pentose ring diminished its potency. Even a single exchange of chlorine with hydrogen in the purine moiety had the same effect. Only the substitution of chlorine with the thiomethoxy group at the position 2 showed weak potency. In this work, we confirmed 2-alkoxy analogs were also capable.

Stimulating agents of angiogenesis are in clinical use, for example to the patients who suffer from deep skin ulcers associated with diabetes or burn wounds. The small molecular size of angiogenic 2-alkoxy analogs is desirable to consider various routes of administration, potentially including topical way to skin. Moreover, it is stable even when stored at room temperature. Thus, these will have advantages to innovate novel angiogenic drugs.

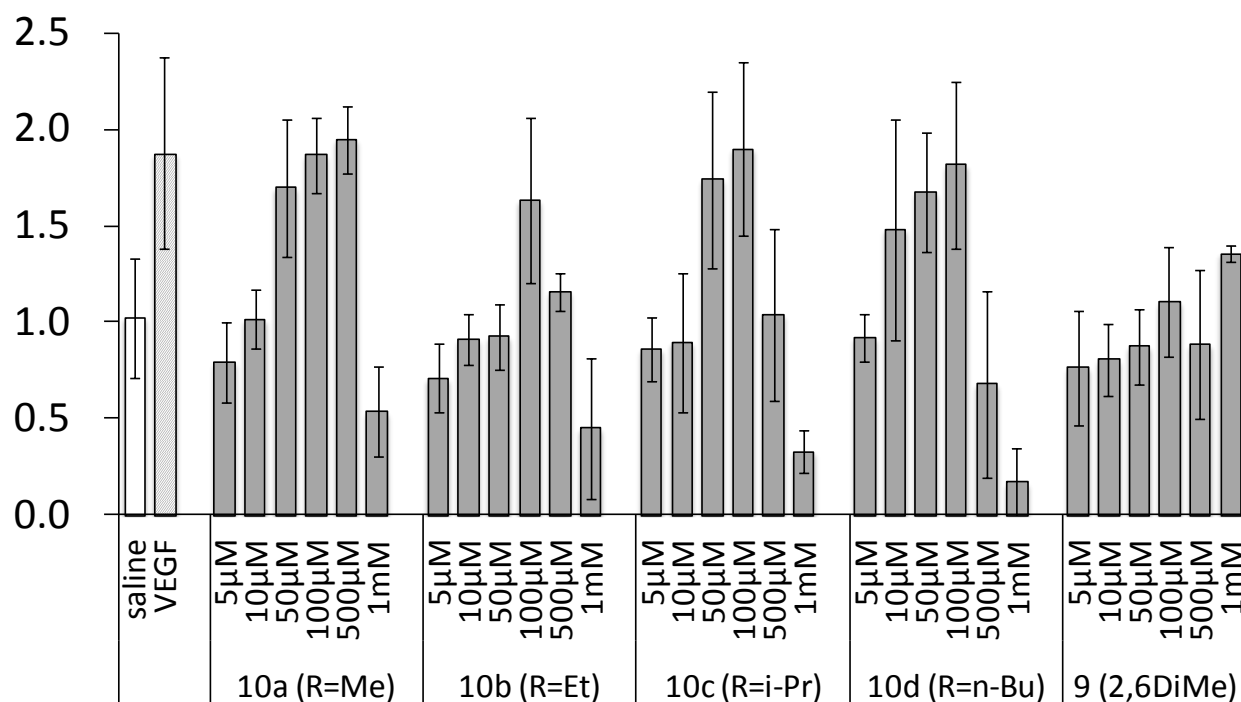


Figure 3. Tube formation assay of compounds **9** and **10a–d**. The tube formation assay was performed using an Angiogenesis Kit and the estimation was done 10 days after the incubation with various additives (5–1000 μM). The area of the formed tube was represented as a relative value to that formed in the control well with no additive. The effect of the solvent, 10% saline as well as a positive control, VEGF (10 ng/mL) were shown together. Results were expressed as mean ± SE of more than three separated experiments.

EXPERIMENTAL

Instrumentation

^1H NMR and ^{13}C NMR spectra were taken with a UltrashieldTM 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). UV spectrum was obtained on a Perkin Elmer Lambda 35 UV/VIS Spectrometer in ethanol solution. Angiogenesis Kit, Tubule Staining Kit for CD31, human umbilical vein endothelial cells (HUVEC), fetal bovine serum

(FBS), Vascular endothelial growth factor-A (VEGF), HuMedia EG2, and HuMedia EB2 were purchased from Kurabo Co. (Osaka, Japan). Cell Counting Kit 8 was supplied by Dojindo Molecular Technologies (Kumamoto, Japan).

2-Amino-6-methoxypurine (4)

Compound **3** (1.70 g, 10.0 mmol) was dissolved in dry MeOH (100 mL), and sodium methoxide (5.7 g, 100.0 mmol) was added to the solution at room temperature, and then stirred for 2 days at 80 °C. The mixture was evaporated, and the residue was purified by silica gel column chromatography (20% MeOH in CH₂Cl₂) to white powder **4** (1.60 g, 9.7 mmol, 97%). ¹H NMR (400MHz, DMSO-*d*₆): δ 12.44 (1H, s, 9-NH), 7.82 (1H, s, H-8), 6.22 (2H, s, 2-NH₂), 3.95 (3H, s, 6-OMe).

***trans-trans*-3-[2-Amino-6-methoxy-9*H*-purin-9-yl]-1,2-cyclobutanedimethanol, 1,2-dibenzoate ester (6a)**

A solution of compound **4** (817.5 mg, 5.0 mmol) and K₂CO₃ (348.3 mg, 2.5 mmol), in dry DMF (54.7 mL) was stirred at 120 °C. After 1 h stirring, compound **5** (1484.0 mg, 3.0 mmol) was added to the solution and stirred for 4h at 120 °C, and the mixture was evaporated in vacuo and the residue was extracted with AcOEt. The organic extracts were washed with water and saturated aqueous sodium chloride solution, and dried with sodium sulfate, and then evaporated. The residue was purified by silica gel column chromatography (8% MeOH in AcOEt) to white powder **6a** (607.0 mg, 1.23 mmol, 42%). ¹H NMR (400MHz, CDCl₃): δ 8.06 (2H, d, *J* = 7.2, Bz), 7.88 (2H, d, *J* = 7.2, Bz), 7.68 (1H, s, H-8), 7.36-7.59 (6H, m, Bz), 4.80 (2H, s, 2-NH₂), 4.67 (1H, dd, *J* = 17.6 and 8.8, H-1'), 4.55 (4H, m, 2'-CH₂OBz and 3'-CH₂OBz), 4.04 (3H, s, 6-OMe), 3.38 (1H, m, H-2'), 2.66 (2H, m, H-4'), 2.59 (1H, m, H-3'); ¹³C NMR (100 MHz, CDCl₃): δ 166.5, 166.4, 161.6, 159.2, 153.9, 137.9, 133.2, 129.9, 129.7, 129.6, 129.5, 128.6, 128.4, 116.1, 66.3, 64.9, 53.8, 48.4, 45.4, 31.1, 29.0; HRMS (ESI) Calcd for C₂₆H₂₅N₅O₅ [M+H]⁺: 488.1928. Found 488.1925.

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

Compound **6a** (48.8 mg, 0.1 mmol) was dissolved in dry MeOH (1.6 mL), and sodium methoxide (14.0 mg, 0.25 mmol) was added to the solution at room temperature, and then stirred for 40 minutes at 40 °C. The mixture was evaporated, and the residue was purified by silica gel column chromatography (20% MeOH in CH₂Cl₂) to white powder **6b** (21.0 mg, 0.08 mmol, 75%). ¹H NMR (400MHz, CDCl₃): δ 7.64 (1H, s, H-8), 4.90 (2H, s, 2-NH₂), 4.37 (1H, m, H-1'), 4.90 (3H, s, 6-OMe), 3.63-3.87 (4H, m, 2'-CH₂, 3'-CH₂), 2.75 (1H, m, H-2'), 2.62 (1H, m, H-4'a), 2.44 (1H, m, H-4'b), 2.18 (1H, m, H-3'); ¹³C NMR (100 MHz, CD₃OD): δ 162.7, 161.6, 154.8, 139.5, 115.6, 65.2, 63.8, 54.2, 49.9, 48.4, 34.8, 29.4; HRMS (ESI) Calcd for C₁₂H₁₇N₅NaO₃ [M+H]⁺: 302.1224. Found 302.1224. UV: max 282.9 nm (EtOH).

***trans-trans*-3-[2,6-(Dimethoxy)-9*H*-purin-9-yl]-1,2-cyclobutanedlmethanol, 1,2-dibenzoate ester (7a)**
and ***trans-trans*-3-[1,9-dihydro-6-methoxy-9*H*-purin-2-one-9-yl]-1,2-cyclobutanedlmethanol, 1,2-dibenzoate ester (8)**

Compound **6a** (73.0 mg, 0.15 mmol) was dissolved in dry MeOH (1.2 mL) and isoamyl nitrite (105.0 μ L, 0.75 mmol) was added to the solution at room temperature, and then stirred for 36 h at 50 °C. The mixture was evaporated, and the residue was purified by silica gel column chromatography (AcOEt then 10% MeOH in AcOEt) to give **7a** as pale yellow oil (67.8 mg, 0.14 mmol, 90%). Evaporation of second fraction gave **8** as pale yellow oil (5.8 mg, 0.012 mmol, 8%). **7a**: ^1H NMR (400 MHz, CDCl_3): δ 8.06 (2H, d, $J = 8.4$, Bz), 7.88 (2H, d, $J = 8.4$, Bz), 7.82 (1H, s, H-8), 7.52 (2H, m, Bz), 7.45 (2H, m, Bz), 7.38 (2H, m, Bz), 4.78 (1H, dd, $J = 16.8$ and 8.8 , H-1'), 4.55 (4H, m, 2'- CH_2 , 3'- CH_2), 4.14 (3H, s, 6-OMe), 3.98 (3H, s, 2-OMe), 3.36 (1H, m, H-2'), 2.62 (3H, m, H-3' and 4'ab); ^{13}C NMR (100 MHz, CDCl_3): 166.4, 166.3, 162.1, 161.6, 153.4, 139.2, 133.2, 129.8, 129.6, 129.4, 129.5, 128.5, 128.4, 117.9, 66.7, 64.8, 55.0, 54.2, 48.6, 46.0, 31.0, 29.0; HRMS (ESI) Calcd for $\text{C}_{27}\text{H}_{26}\text{N}_4\text{O}_6$ $[\text{M}+\text{H}]^+$: 503.1925. Found 503.1923. **8**: ^1H NMR (400 MHz, CDCl_3): δ 8.05 (2H, d, $J = 8.4$, Bz), 7.75 (1H, s, H-8), 7.70 (2H, d, $J = 8.4$, Bz), 7.58 (1H, m, Bz), 7.43-7.50 (3H, m, Bz), 7.25-7.30 (2H, m, Bz), 4.78 (1H, dd, $J = 17.2$ and 8.8 , H-1'), 4.48-4.60 (4H, m, 2'- CH_2 and 3'- CH_2), 4.19 (3 H, s, 6-OMe) 3.15 (1H, m, H-2'), 2.85 (1H, m, H-4'a), 2.67 (1H, m, H-3'), 2.44 (1H, m, H-4'b); ^{13}C NMR (100 MHz, CDCl_3): δ 166.5, 166.0, 163.3, 147.7, 136.6, 133.4, 133.1, 129.7, 129.5, 129.3, 128.6, 128.3, 65.9, 65.2, 60.4, 55.5, 48.9, 46.4, 31.0, 29.6; HRMS (ESI) Calcd for $\text{C}_{26}\text{H}_{24}\text{N}_4\text{NaO}_6$ $[\text{M}+\text{H}]^+$: 511.1588. Found 511.1588.

***trans-trans*-3-[2-Ethoxy-6-methoxy-9*H*-purin-9-yl]-1,2-cyclobutanedlmethanol, 1,2-dibenzoate ester (7b)**

Compound **6a** (73.0 mg, 0.15 mmol) was dissolved in dry EtOH (1.2 mL) and isoamyl nitrite (105.0 μ L, 0.75 mmol) was added to the solution at room temperature, and then stirred for 11 h at 50 °C. The mixture was evaporated, and the residue was purified by silica gel column chromatography (AcOEt then 10% MeOH in AcOEt) to give **7b** as pale yellow oil (67.0 mg, 0.13 mmol, 87%). Evaporation of second fraction gave **8** as pale yellow oil (7.2 mg, 0.015 mmol, 10%). **7b**: ^1H NMR (400 MHz, CDCl_3): δ 8.03 (2H, dd, $J = 8.4$ and 1.2 , Bz), 7.86 (2H, dd, $J = 8.4$ and 1.2 , Bz), 7.81 (1 H, s, H-8), 7.57 (2H, m, Bz), 7.45 (2H, m, Bz), 7.39 (2H, m, Bz), 4.81 (1H, dd, $J = 17.2$ and 8.8 , H-1'), 4.55 (4H, m, 2'- CH_2 and 3'- CH_2), 4.38 (2H, q, $J = 7.2$, 2-OEt), 4.14 (3H, s, 6-OMe), 3.33 (1H, m, H-2'), 2.66 (3H, m, H-3' and 4'ab), 1.41(3H, t, $J = 7.2$, 2-OEt); ^{13}C NMR (100 MHz, CDCl_3): δ 166.4, 166.3, 162.1, 161.2, 153.3, 139.0, 133.3, 133.2, 129.8, 129.5, 129.4, 128.6, 128.4, 117.8, 66.7, 64.8, 63.7, 54.3, 48.5, 46.1; HRMS (ESI) Calcd for $\text{C}_{28}\text{H}_{28}\text{N}_4\text{O}_6$ $[\text{M}+\text{H}]^+$: 517.2082. Found 517.2067.

***trans-trans*-3-[2-*i*-Propoxy-6-(methoxy)-9*H*-purin-9-yl]-1,2-cyclobutanedlmethanol, 1,2-dibenzoate**

ester (7c)

Compound **6a** (73.0 mg, 0.15 mmol) was dissolved in dry *i*PrOH (1.2 mL) and isoamyl nitrite (105.0 μ L, 0.75 mmol) was added to the solution at room temperature, and then stirred for 12 h at 50 °C. The mixture was evaporated, and the residue was purified by silica gel column chromatography (AcOEt then 10% MeOH in AcOEt) to give **7c** as pale yellow oil (44.6 mg, 0.084 mmol, 56%). Evaporation of second fraction gave starting material **6a** (7.0 mg, 0.015 mmol, 10%) and third fraction gave **8** as oil (10.1 mg, 0.021 mmol, 14%). **7c**: ^1H NMR (400 MHz, CDCl_3): δ 8.03 (2H, dd, $J = 8.4$ and 1.2 , BzO), 7.86 (2H, dd, $J = 8.4$ and 1.2 , BzO), 7.81 (1H, s, H-8), 7.56 (2H, m, BzO), 7.45 (2H, m, BzO), 7.38 (2H, m, BzO), 5.25 (1H, heptet, $J = 6.4$, 2-O-*i*Pr), 4.82 (1H, dd, $J = 17.2$ and 8.8 , H-1'), 4.53 (4H, m, 2'-CH₂, 3'-CH₂), 4.14 (3H, s, 6-OMe), 4.14 (3H, s, 6-OMe), 3.33 (1H, m, H-2'), 2.75 (1H, m, H-3'), 2.64 (2H, m, H-4'ab), 1.37 (6H, d, $J = 6.4$, 2-O-*i*Pr); ^{13}C NMR (100 MHz, CDCl_3): δ 166.4, 166.3, 162.2, 160.8, 153.3, 139.0, 133.3, 133.2, 130.0, 129.6, 129.5, 128.6, 128.4, 117.6, 70.6, 66.8, 64.8, 54.3, 48.4, 46.1 31.0, 29.3, 21.8; HRMS (ESI) Calcd for $\text{C}_{29}\text{H}_{30}\text{N}_4\text{O}_6$ $[\text{M}+\text{H}]^+$: 531.2238. Found 531.2223.

***trans-trans*-3-[2-*n*-butoxy-6-(methoxy)-9*H*-purin-9-yl]-1,2-cyclobutanedlmethanol, 1,2-dibenzoate ester (7d)**

Compound **6a** (73.0 mg, 0.15 mmol) was dissolved in dry *n*-BuOH (1.2 mL) and isoamyl nitrite (105.0 μ L, 0.75 mmol) was added to the solution at room temperature, and then stirred for 11 h at 50 °C. The mixture was evaporated, and the residue was purified by silica gel column chromatography (AcOEt then 10% MeOH in AcOEt) to give **7d** as pale yellow oil (56.3 mg, 0.10 mmol, 69%). Evaporation of second fraction gave starting material **6a** (5.1 mg, 0.010 mmol, 7%) and third fraction gave **8** as oil (17.1 mg, 0.035 mmol, 23%). **7d**: ^1H NMR (400 MHz, CDCl_3): δ 8.03 (2H, dd, $J = 8.0$ and 0.8 , Bz), 7.86 (2H, dd, $J = 8.0$ and 0.8 , Bz), 7.82 (1H, s, H-8), 7.52 (2H, m, Bz), 7.43 (2H, m, Bz), 7.36 (2H, m, Bz), 4.80 (1H, dd, $J = 16.8$ and 8.8 , H-1'), 4.54 (4H, m, 2'-CH₂ and 3'-CH₂), 4.32 (2H, t, $J = 6.4$, 2-*n*-Bu), 4.14 (3H, s, 6-OMe), 3.32 (1 H, m, H-2'), 2.75 (1 H, m, H-3'), 2.64 (2 H, m, H-4'ab), 1.77 (2 H, m, 2-*n*-Bu), 1.44 (2 H, m, 2-*n*-Bu), 0.94 (3H, t, $J = 7.2$, 2-*n*-Bu); ^{13}C NMR (100 MHz, CDCl_3): δ 166.4, 166.3, 162.1, 161.4, 153.3, 138.9, 133.3, 133.2, 129.8, 129.6, 129.4, 128.6, 128.4, 117.8, 67.8, 66.7, 64.8, 54.3, 48.4, 46.1, 31.0, 30.9, 29.2, 19.2, 13.9; HRMS (ESI) Calcd for $\text{C}_{30}\text{H}_{32}\text{N}_4\text{O}_6$ $[\text{M}+\text{H}]^+$: 545.2395. Found 545.2376.

***trans-trans*-9-[2,3-bis(Hydroxymethyl)cyclobuthyl]-2,6-dimethoxypurine (9)**

Compound **7a** (54.0 mg, 0.11 mmol) was dissolved in dry MeOH (1.8 mL), and sodium methoxide (15.6 mg, 0.28 mmol) was added to the solution at room temperature, and then stirred for 30 min at 40 °C. The mixture was evaporated, and the residue was purified by silica gel column chromatography (20% MeOH in CH_2Cl_2) to white powder **9** (32.0 mg, 0.11 mmol, 100%). ^1H NMR (400 MHz, CDCl_3): δ 7.80 (1H, s, H-8), 4.51 (1H, dd, $J = 17.6$ and 8.4 , H-1'), 4.16 (3H, s, 6-OMe), 4.04 (3H, s, 2-OMe), 3.82 (2H, m,

2'a-CH₂ and 3'a-CH₂), 3.71 (2H, m, 2'b-CH₂ and 3'b-CH₂), 3.55 (1H, m, OH), 2.82 (1H, m, H-2'), 2.62 (1H, m, H-4'a), 2.48 (1H, dd, $J = 20.4$ and 9.6 , H-4'b), 2.23 (1H, m, H-3'), 2.16 (1H, m, OH); ¹³C NMR (100 MHz, CDCl₃): δ 162.0, 161.5, 153.1, 139.3, 117.6, 64.5, 63.6, 55.1, 54.3, 49.6, 48.8, 34.9, 27.6; HRMS (ESI) Calcd for C₁₃H₁₈N₄O₄ [M+Na]⁺: 317.1220. Found 317.1201; mp 130.9-133.4 °C.

***trans-trans*-6-Amino-9-[2,3-bis(hydroxymethyl)cyclobutyl]-2-methoxypurine (10a)**

Compound **7a** (61.7 mg, 0.12 mmol) was dissolved in NH₃ (14.0 mL)/MeOH (2.0 mL), and then sealed and stirred for 3 d at 120 °C. The mixture was evaporated, and the residue was purified by silica gel column chromatography (20% MeOH in CH₂Cl₂) to give **10a** as pale yellow oil (25.7 mg, 0.092 mmol, 75%). ¹H NMR (400 MHz, CD₃OD): δ 8.01 (1H, s, H-8), 4.60 (1H, dd, $J = 17.6$ and 8.8 , H-1'), 3.94 (3H, s, 2-OMe), 3.69 (4H, m, 2'-CH₂, 3'-CH₂), 2.90 (1H, m, H-2'), 2.54 (1H, m, H-4'a), 2.45 (1H, dd, $J = 20.4$ and 9.6 , H-4'b), 2.22 (1H, m, H-3'); ¹³C NMR (100 MHz, MeOD): δ 162.1, 156.7, 151.2, 138.6, 115.2, 64.0, 62.2, 53.7, 48.3, 47.9, 33.4, 28.1; HRMS (ESI) Calcd for C₁₂H₁₇N₅O₃ [M+Na]⁺: 302.1224. Found 302.1209.

***trans-trans*-6-Amino-9-[2,3-bis(hydroxymethyl)cyclobutyl]-2-ethoxypurine (10b)**

Compound **7b** (48.0 mg, 0.093 mmol) was dissolved in NH₃ (14.0 mL)/MeOH (3.0 mL), and then sealed and stirred for 2 d at 110 °C. The mixture was evaporated, and the residue was purified by silica gel column chromatography (20% MeOH in CH₂Cl₂) to give **10b** as white crystals (17.0 mg, 0.058 mmol, 62%). ¹H NMR (400 MHz, DMSO-*d*₆): δ 7.99 (1H, s, H-8), 7.14 (2H, s, 6-NH₂), 4.70 (1H, t, $J = 5.2$, OH), 4.60 (1H, t, $J = 5.2$, OH), 4.50 (1H, dd, $J = 17.2$ and 8.4 , H-1'), 4.26 (2H, q, $J = 7.2$, 2-OEt), 3.52 (4H, m, 2'-CH₂ and 3'-CH₂), 2.79 (1H, m, H-2'), 2.38 (1H, m, H-4'a), 2.28 (1H, dd, $J = 20.0$ and 9.6 , H-4'b), 2.08 (1H, m, H-3'), 1.30 (3H, t, $J = 7.2$, 2-OEt); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 161.4, 157.3, 151.6, 138.8, 116.1, 64.3, 62.2, 62.1, 48.0, 47.9, 33.7, 29.2, 15.1; HRMS (ESI) Calcd for C₁₃H₁₉N₅O₃ [M+Na]⁺: 316.1380. Found 316.1361; mp 195.7-197.1 °C.

***trans-trans*-6-Amino-9-[2,3-bis(hydroxymethyl)cyclobutyl]-2-*i*-propoxypurine (10c)**

Compound **7c** (52.0 mg, 0.098 mmol) was dissolved in NH₃ (14.0 mL)/MeOH (4.0 mL), and then sealed and stirred for 4 d at 120 °C. The mixture was evaporated, and the residue was purified by silica gel column chromatography (20% MeOH in CH₂Cl₂) to give **10c** as pale yellow oil (15.0 mg, 0.049 mmol, 50%). ¹H NMR (400 MHz, MeOD): δ 8.01 (1H, s, H-8), 5.30 (1H, heptet, $J = 6.0$, 2-O-*i*Pr), 4.61 (1H, dd, $J = 17.6$ and 8.8 , H-1'), 3.70 (4H, m, 2'-CH₂ and 3'-CH₂), 2.94 (1H, m, H-2'), 2.56 (1H, m, H-4'a), 2.48 (1H, m, H-4'b), 2.24 (1H, m, H-3'), 1.38 (6H, d, $J = 6.0$, 2-O-*i*Pr); ¹³C NMR (100 MHz, MeOD): δ 161.1, 156.7, 151.2, 138.6, 115.1, 69.4, 64.2, 62.2, 48.3, 48.0, 33.4, 28.2, 21.0, 20.9; HRMS (ESI) Calcd for C₁₄H₂₁N₅O₃ [M+H]⁺: 308.1717. Found 308.1706.

***trans-trans*-6-Amino-9-[2,3-bis(hydroxymethyl)cyclobutyl]-2-*n*-butoxypurine (10d)**

Compound **7d** (28.0 mg, 0.051 mmol) was dissolved in NH₃ (14.0 mL)/MeOH (2.0 mL), and then sealed and stirred for 2 d at 110 °C. The mixture was evaporated, and the residue was purified by silica gel column chromatography (20% MeOH in CH₂Cl₂) to give **10d** as white crystal (12.0 mg, 0.037 mmol, 73%). ¹H NMR (400 MHz, MeOD): δ 8.02 (1H, s, H-8), 4.62 (1H, dd, *J* = 17.6 and 8.4, H-1'), 4.36 (2H, T, *J* = 6.4, 2-*n*-Bu), 3.71 (4H, m, 2'-CH₂, 3'-CH₂), 2.92 (1H, m, H-2'), 2.56 (1H, m, H-4'a), 2.46 (1H, dd, *J* = 20.4 and 9.6, H-4'b), 2.24 (1H, m, H-3'), 1.78 (2H, m, 2-*n*-Bu), 1.52 (2H, m, 2-*n*-Bu), 1.01 (3H, T, *J* = 7.6, 2-*n*-Bu); ¹³C NMR (100 MHz, MeOD): δ 161.7, 156.7, 151.2, 138.6, 115.2, 66.7, 64.1, 62.2, 48.3, 48.0, 33.4, 30.9, 28.1, 18.9, 12.8; HRMS (ESI) Calcd for C₁₅H₂₃N₅O₃ [M+H]⁺: 322.1874. Found 322.1863; mp 151.6-153.0 °C.

Cell culture

A co-culture system of HUVEC and human fibroblasts (Angiogenesis Kit) was supplied in 24-well plates by Kurabo. Cells were incubated for 10 days prior to analysis with 450 μL of the culture medium and a 50 μL of saline that includes various additives. Culture medium was changed every 3 days, each time including freshly prepared additives.

Tube formation assay

Ten days following incubation periods with co-cultured fibroblasts and substrates (**9**, **10a-d**), HUVEC were stained using Tubule Staining Kit for CD31. 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.

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