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Optimization of fluorescence property of the 8-oxodGclamp derivative for better selectivity for 8-oxo-2′-deoxyguanosine

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ABSTRACT

2′-Deoxyguanosine (dG) suffers from oxidation by reactive oxygen species (ROS) to form 8-oxo-2′-deoxyguanosine (8-oxo-dG), which is regarded as a marker of oxidative stress in the cells. In our continuous study for the recognition molecule of 8-oxo-dG, 8-oxoGclamp and its derivatives have been identified as the selective fluorescent probe. However, it is an obstacle for further application that dG also forms a complex with 8-oxoGclamp, resulting in fluorescence quenching in less polar solvents. Quenching of the fluorescence of 8-oxoGclamp is thought to involve photo-induced electron transfer in the complex. It was hypothesized that the energy level of the excited state of 8-oxoGclamp and the HOMO energy of dG are the preliminary determinant of the quenching efficiency. Thus, fluorescence properties of the substituted derivatives at the 7-position of the 1,3-diazaphenoxazine part of 8-oxoGclamp were investigated. Among the new derivatives, fluorescence of the 7-phenyl substituted 8-oxoGclamp was not quenched by dG even in the stable complex, exhibiting the highest selectivity for 8-oxo-dG.

1. Introduction

Reactive oxygen species (ROS), such as hydroxyl radical, hydrogen peroxide, single oxygen, etc., induce oxidative damage to lipids, proteins, and nucleic acids in living organisms.[1] 2′-Deoxyguanosine (dG) is oxidized to form 8-oxo-2′-deoxyguanosine (8-oxo-dG) as a representative oxidative damage. 8-Oxo-dG is a potent mutagen, which induces G:C to T:A transversion mutations.[2] The 8-oxo-dG level inside and outside cells is regarded as a marker of the oxidative stress of the cells,[3] and its contents in DNA are correlated with some diseases[4] or aging.[5] 8-Oxo-dG is routinely analyzed by HPLC–EC,[6] HPLC/GC–MS,[7] antibodies in an enzyme-linked immunosorbant assay (ELISA), and immunohistochemical staining of tissues or cells, and so on.[7e,8,9] In an attempt to develop a simpler method, we have developed a new series of fluorescent recognition molecules for 8-oxo-dG based on the nucleoside derivatives having the 1,3-diazaphenoxazine skeleton (Fig. 1A).[10] Early studies have shown that selective fluorescence quenching takes place through the complex formation by multiple hydrogen bonds between 8-oxoGclamp (**2**) and 8-oxo-dG. In the subsequent structure–activity investigation, more selective molecules including 8-oxoGclamp (**2-et-naph**) have been determined, together with dG-selective Gclamp (**1-urea**) (Fig. 1B and C).[10b] However, it is an obstacle for further application that dG also forms a complex with 8-oxoGclamp resulting in fluorescence quenching in a less polar solvent. In this study, we attempted to optimize the selectivity for 8-oxo-dG by minimizing fluorescence quenching by dG.

2. Results and discussion

Efficiency of the fluorescence quenching of 8-oxoGclamp by 8-oxo-dG and dG is highly dependent on the stability of their complex, that is, the distance between them within the ground-state complexes. The quenching mechanism most likely involves a photo-induced electron transfer (PET) interaction.[11] The difference in the hydrogen bonds with 8-oxo-dG or dG arises from the *N*7-position, where 8-oxoGclamp (**2**) forms a hydrogen bond with 8-oxo-dG but not with dG, reflecting 10–30 times higher binding affinity for 8-oxo-dG than for dG. Nevertheless, dG causes fluorescence quenching to some extent in less polar organic solvents. In a previous approach to improve selectivity for 8-oxo-dG in fluorescent quenching, we determined new 8-oxoGclamp derivatives such as those shown in Fig. 1A with more selective binding affinity 8-oxo-dG. In this study, we adopted a different strategy for higher quenching selectivity for 8-oxo-dG by minimizing the quenching efficiency by dG so that dG would not cause efficient quenching even in the stable complex formation. According to the PET mechanism, the excited state of 8-oxoGclamp (**2**) serves as the electron acceptor from dG as the ground-state electron donor (Fig. 2A). 8-Oxo-dG has higher HOMO energy than dG and therefore is a better quencher. We hypothesized that quenching efficiency by

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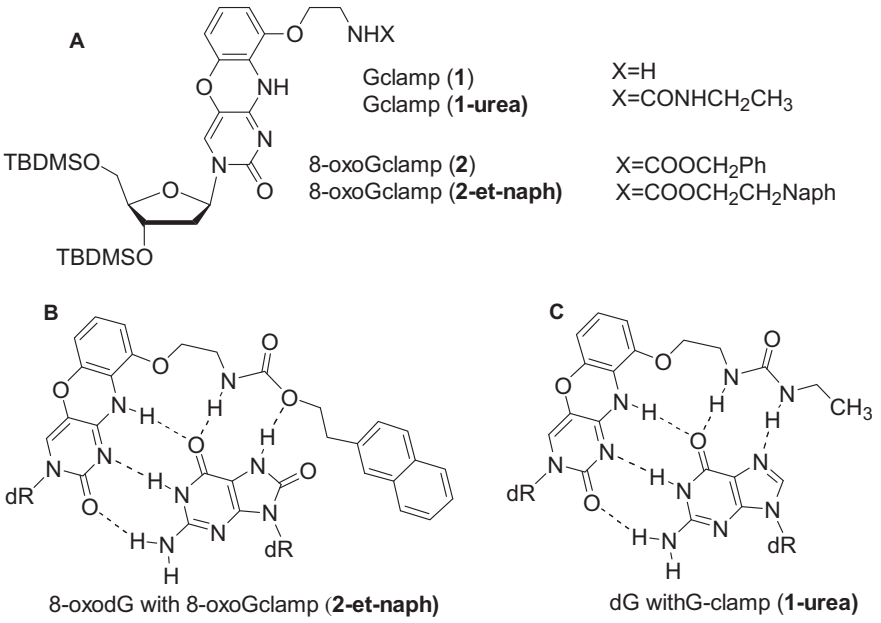


Fig. 1. Structure of Gclamp derivatives (A), a complex with 8-oxo-dG (B) and dG(C).

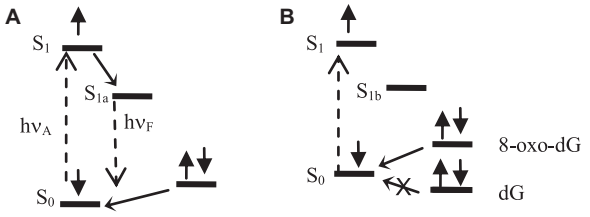


Fig. 2. Relative orbital energies for explaining fluorescence quenching by photo-induced electron transfer mechanism.

dG may be controlled by adjusting the S_0 level of the excited state of 8-oxoGclamp derivatives as schematically shown in Fig. 2B. As it is difficult to theoretically predict the S_0 level and quenching efficiency, we investigated the substituent effects of 8-oxoGclamp on the fluorescent property. To avoid through-space and/or though-bond interactions with the oxygen atoms at the 5-position or the substitution at the 9-position, we have chosen the position 7 of the 1,3-diazaphenoxazine skeleton for introduction of a variety of the compounds (Fig. 3, **3**–**11**).

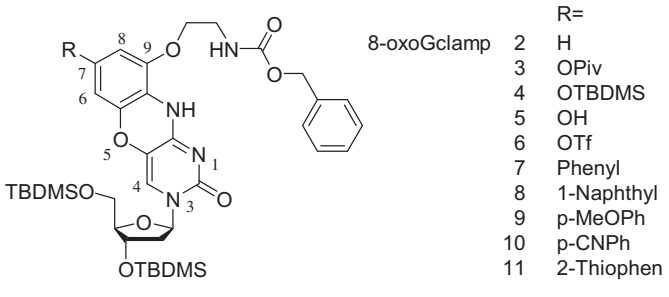


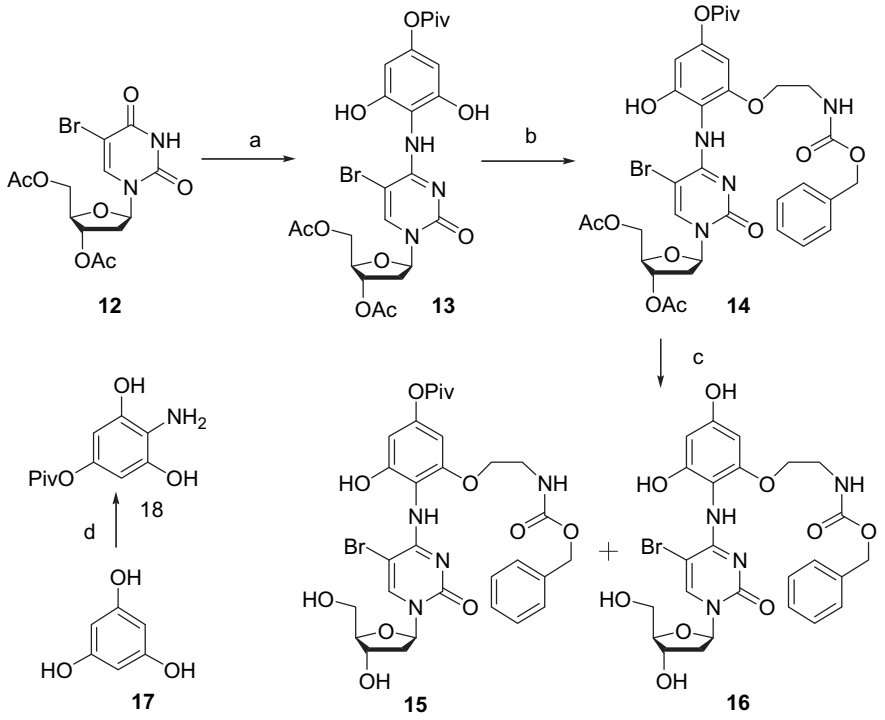
Fig. 3. The structure of the 8-oxoGclamp derivatives investigated in this study.

The synthesis started from the diacetate of 2′-deoxy-5-bromouridine (**12**) (Scheme 1). 1,3,5-Trihydroxybenzene (**17**) was first nitrated at the 4-position, and the 1-hydroxyl group of the resulting nitrated compound was protected with the pivaloyl group; then the nitro group was reduced to 4-amino-1,3,5-trihydroxybenzene derivative **18**. The 4-carbonyl group of **12** was activated using PPh_3 and CCl_4 and subjected to the reaction with **18** to give **13**. $PhCH_2OCO{-}NHCH_2CH_2OH$ was introduced into **14** under the Mitsunobu conditions to produce **14**, which was treated with K_2CO_3 in MeOH to afford dihydroxyl compound (**15**) and trihydroxy compound (**16**).

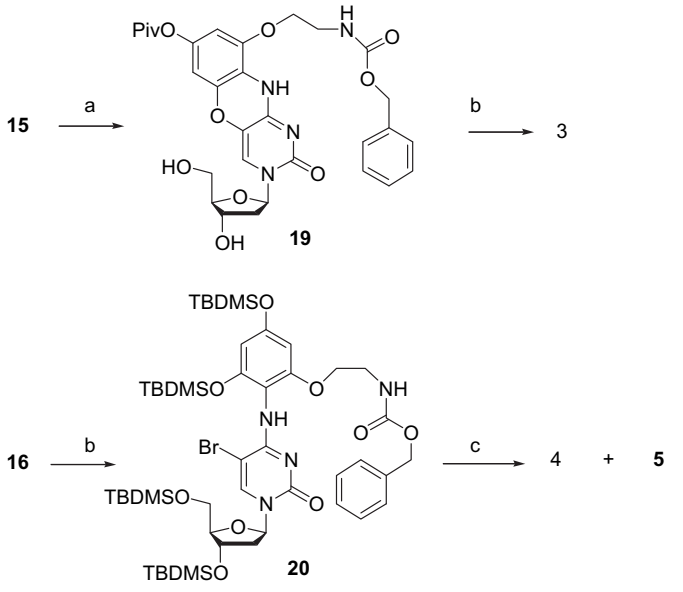
The synthesis of **3**, **4**, and **5** is summarized in Scheme 2. The cyclized compound **19** was obtained by the treatment of the pivaloyl protected **15** with TEA in refluxing ethanol, and the hydroxyl groups were protected with the TBDMS group to produce **3**. The trihydroxy compound **16** was first protected with the TBDMS group, then cyclized in refluxing methanol in the presence of TEA to form **4** and **5**.

The hydroxyl group of **5** was treated with Tf_2NPh in the presence of TEA to produce the triflate **6**, which was used as the common intermediate for the synthesis of the aromatic group-substituted 8-oxoGclamp (**7**–**11**) (Scheme 3). The triflate group of **6** was substituted with an aromatic group under Suzuki–Miyaura coupling conditions using $Pd(OAc)_2$, phosphine ligand, and the corresponding aromatic boric acid.

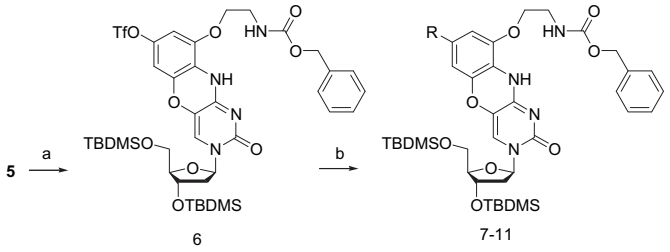
The UV and fluorescent spectra of the 8-oxoGclamp derivatives were measured in the presence and absence of the nucleoside substrates. The quantum yield was obtained using quinine as the standard compound. The maximum absorbance (Abs_{max}), emission spectra (Em_{max}), and quantum yield (φ) are summarized in Table 1. The fluorescence quenching titration was performed at 25 °C using the 3′-*O*-, 5′-*O*-di-TBDMS protected nucleoside and the 8-oxoGclamp derivative in a $CHCl_3$ solution containing 0.3 mM AcOH–TEA. The fluorescence spectra were recorded with the excitation wavelength at 365 nm, and fluorescence quenching was titrated by the addition of the nucleoside derivative. Some examples are shown in Fig. 4. The fluorescence spectra of **2** and **7** were almost equally quenched by 8-oxo-dG (Fig. 4, A and C). In contrast, dG induced fluorescence quenching for **2**, but not for **7** (Fig. 4, B vs D). A bathochromic shift of 10 nm suggests the ground-state complex formation between **7** and dG (Fig. 4D). The ratio of the fluorescent intensity at the maximum wavelength in the absence (F_0) and presence of 10 equiv of the nucleoside (F_q), F_q/F_0, represents an index of the quenching efficiency. For example, the F_q/F_0 values of each combination are as follows, **2** with 8-oxo-dG, 0.08; **2** with dG, 0.76; **7** with



Scheme 1. The synthesis of the intermediates **15** and **16**. (a) PPh_3, CCl_4, CH_2Cl_2, then **18**, DBU, rt, 12 h, 83%, (b) $PhCH_2OCO{-}NHCH_2CH_2OH$, DIAD, PPh_3, CH_2Cl_2, rt, 25 h, 62%, (c) K_2CO_3, MeOH, rt 30 min, **15** (46%), **16** (23%), (d) (1) HNO_3, H_2SO_4, rt, 3 h, 55%, (2) pivaloyl chloride, dry pyridine, (3) H_2, Pd/C, MeOH, 0 °C, 2 h, 41% in two steps.



Scheme 2. The synthesis of **3**, **4**, and **5**. (a) TEA, EtOH, reflux, 54 h, 48%, (b) TBDMSCl, imidazole, DMAP, dry DMF, rt, 13 h, 72% for **3**, 82% for **20**, (c) TEA, MeOH, reflux, 46 h, 22% (**4**) and 38% (**5**).



Scheme 3. The synthesis of the aromatic ring substituted compounds (**7**–**11**). (a) Tf_2NPh, TEA, CH_2Cl_2, rt, 22 h, 78%, (b) $Ar{-}B(OH)_2$, $Pd(OAc)_2$, phosphine ligand, $K_3PO_4{-}nH_2O$, dry solvent: 88% for **7**, phenyl boric acid, SPhos, toluene, 100 °C, 2 h; 51% for **8**, 1-naphthyl boric acid, SPhos, toluene, 2 h; 59% for **9**, 4-methoxyphenyl boric acid, JohnPhos, dioxane, 6 h; 29% for 10, 4-cyanophenyl boric acid, XPhos, dioxane, 6 h; 12% for **11**, 2-thiophene boric acid, XPhos, dioxane, 100 °C, 2 h.

8-oxo-dG, 0.1; **7** with dG, 0.98. These results have clearly indicated that **7** is a more selective fluorescence probe than the original **2** for 8-oxo-dG. The equilibrium binding constants (K_a) between the 8-oxoGclamp derivative and 8-oxo-dG or dG were obtained from the fluorescence titration experiments and are summarized in Table 1.

The quantum yield was not significantly affected by the substituted group except for the case of **4**, **5**, and **11**. These molecules have a lone pair of an oxygen or a sulfur atom, which might serve as an electron donor for the intramolecular quenching. The aromatic substitution caused an emission shift to a longer wavelength (438 nm to longer than 446 nm), indicating that the aromatic substituted group affects the energy level of the excited states. The F_q/F_0 values for 8-oxo-dG of less than 0.1 represent efficient fluorescence quenching of 8-oxodGclamp derivatives by 8-oxo-dG. In contrast, F_q/F_0 values for dG ranged from 0.64 of **3** to 0.98 of **7**. There is no clear relationship between the association constants of the complex (K_a) and the F_q/F_0 values. For example, compound **9** showed a high K_a value of 2.57×10^6 M^{-1}; however, only inefficient fluorescence quenching (F_q/F_0=0.84) was observed with dG. It should be mentioned that phenyl and naphthyl groups enhanced the binding affinity for 8-oxo-dG without changing the affinity for dG.

It was initially anticipated according to the PET quenching mechanism that adjusting the S_0 level of the excited state of 8-oxoGclamp derivatives might affect the quenching efficiency by dG. The HOMO of the excited state of the 8-oxoGclamp derivative serves as an electron acceptor and the HOMO of the ground-state dG serves as the electron donor. The higher HOMO energy of the excited state of the 8-oxoGclamp derivative is expected to be a poorer acceptor, that is, lower quenching efficiency is expected for dG. The maximum wavelength of UV absorbance reflects the energy difference between the HOMO and the LUMO of the 8-oxoGclamp derivative. The energies of the maximum wavelength of the

Table 1
UV, fluorescent, and binding properties of the 8-oxodGclamp[a]

entry	oxoGclamp R=	Abs_{max} (nm)	Em_{max} (nm)	φ[a]	8-oxo-dG		dG	
					F_q/F_0[c]	K_a (10^6 M^{-1})	F_q/F_0[c]	K_a (10^6 M^{-1})
1	**2** H	360	438	0.36	0.08	4.9	0.76	0.48
2	**3** OPiv	362	442	0.29	0.04	12.9	0.64	0.60
3	**4** OTBDMS	360	459	0.09	0.15	41.0	0.70	0.74
4	**5** OH	365	463	0.05	0.24[b]	10.0[b]	0.70[b]	0.51[b]
5	**6** OTf	360	430	0.32	0.04	30.7	0.39	1.95
6	**7** Phenyl	371	447	0.34	0.10	17.4	0.98	0.80
7	**8** Naphthyl	365	447	0.38	0.08	19.8	0.81	0.52
8	**9** MeOPh	368	456	0.28	0.08	6.7	0.84	2.57
9	**10** CNPh	372	447	0.33	0.08	8.1	0.95	2.80
10	**11** Thiophene	364	458	0.11	0.16	67.8	0.85	1.38

[a] UV and fluorescent spectra were measured in $CHCl_3$ solution at 25 °C using 3′-*O*- and 5′-*O*-TBDMS protected nucleosides. The fluorescence quenching titration was performed in the $CHCl_3$ solution containing 0.3 mM AcOH–TEA.
[b] Measured in $CHCl_3$ solution containing 10 mM TEA and 2.7 mM AcOH.
[c] The fluorescence quenching ratio, where Fq and F_0 represent the fluorescence intensity of the 1:10 mixture of 8-oxoGclamp and 8-oxo-dG or dG and the fluorescence intensity of 8-oxoGclamp, respectively.

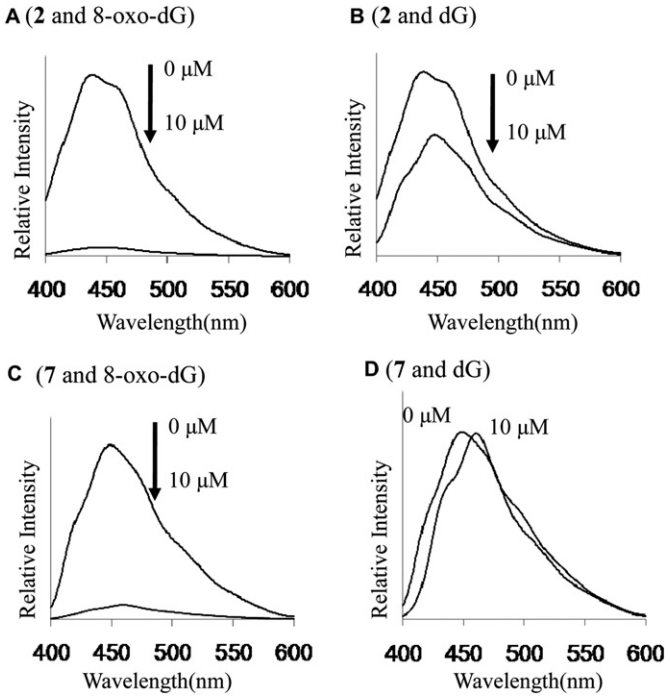


Fig. 4. Comparison of the quenching behavior of **2** and **7**. Quenching experiment was performed using 1 μM 8-oxoGclamp (3′*O*,5′*O*-di-TBDMS), 0 and 10 μM nucleoside (3′*O*,5′*O*-di-TBDMS) at 25 °C with excitation at 365 nm in $CHCl_3$ containing 0.3 mM TEA–AcOH.

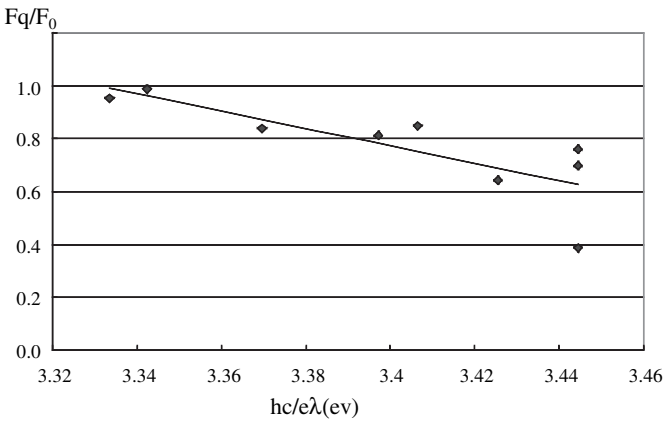


Fig. 5. Comparison of the F_q/F_0 value and the energy of maximum wavelength. Data were taken from Table 1. The energy of the maximum wavelength was calculated by the following equation: $E(eV)=hc/e\lambda$, where h, c, e, and λ represent the Planck constant, the speed of light, the elementary charge, and the wavelength, respectively.

8-oxoGclamp derivatives are compared with the F_q/F_0 values (Fig. 5). There is a tendency that the compound with the higher energy, i.e., shorter wavelength, produces a higher quenching efficiency by dG. Although the maximum wavelength does not simply correlate with the S_0 level of the excited state, it is likely that optimizing the quenching selectivity for 8-oxo-dG has been achieved by adjusting the electronic property of the 1,3-diazaphenoxazine skeleton.

In conclusion, we have improved the selectivity of 8-oxodGclamp for detection of 8-oxo-dG in fluorescence quenching. The molecular design was based on the assumption that the substitution at the 7-position of its 1,3-diazaphenoxazine part might affect the energy level of the excited state of 8-oxodGclamp and inhibit quenching pathway by dG. Among a variety of substituted 8-oxodGclamp derivatives, the phenyl-substituted 8-oxodGclamp (**7**) was found to have the best profile in the sense that its fluorescence was not quenched by dG even when a stable complex was formed. The new phenyl-substituted 8-oxodGclamp (**7**) may be a good candidate for further application to fluorescent detection of 8-oxo-dG.

3. Experimental

3.1. General

^1H NMR (400 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a Varian UNITY 400 or 500 spectrometer. Infrared (IR) spectra were obtained using a Perkin–Elmer FTIR-SpectrumOne. The high-resolution mass spectra were recorded by an Applied

Biosystems Mariner System 5299 spectrometer using nicotinic acid, bradykinin, neurotensin, and angiotensin as the internal standard.

*3.1.1. 4-O-Pivaloyl-2-aminophloroglucinol (**18**).* Under argon atmosphere, pivaloyl chloride (2.0 mL, 13 mmol) was added slowly to a solution of 2-nitrophloroglucinol (2.0 g, 12 mmol) in pyridine (20 mL) at 0 °C; then the reaction mixture was allowed to warm to room temperature and stirred for 2 h. The reaction mixture was quenched with water and extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated. The residue was purified by column chromatography (silica gel, Hexane/AcOEt=10:1 $CHCl_3$/MeOH=12:1) to give pale yellow solids. Under H_2 atmosphere, the solution of this yellow solid in MeOH (105 mL) in the presence of 5% Pd/C (170 mg) was stirred for 2.5 h at room temperature. The reaction mixture was filtered through a Celite pad, and the filtrate was evaporated. The residue was purified by column chromatography (silica gel, $CHCl_3$/MeOH=12:1) to give **18** as pale yellow solids (1.1 g, 41%, in two steps). ^{1}H NMR (400 MHz, CD_3OD) δ ppm: 1.29 (9H, s), 6.02 (2H, s); ^{13}C NMR (125 MHz, CD_3OD) δ ppm: 179.2, 147.4, 144.7, 121.1, 101.2, 39.9, 27.4; IR (cm^{-1}): 1729, 1157; HRMS (ESI) *m*/*z*: calcd for $C_{11}H_{15}NO_4$ $[M+H]^+$: 226.1074; found 226.1088

*3.1.2. 3′,5′-Di-O-acetyl-N-[4-(2,6-dihydroxy-4-O-pivaloylphenyl)]-2′-deoxy-5-bromouridine (**13**).* Under argon atmosphere, a solution of 3′,5′-di-*O*-acetyl-2′-deoxy-5-bromouridine (**12**) (910 mg, 2.3 mmol), triphenylphosphine (1.2 g, 4.7 mmol) in CH_2Cl_2 (5 mL), and CCl_4 (5 mL) was stirred for 3 h at 70 °C. The reaction mixture was allowed to cool to room temperature, and then DBU (0.77 ml, 5.1 mmol) and **18** (830 mg, 3.7 mmol) were added. The reaction mixture was stirred for 12 h at room temperature, and then diluted with CH_2Cl_2 (9 mL), hexane (9 mL), and 5% aqueous citric acid (20 mL). The mixture was extracted with CH_2Cl_2, and the organic layer was dried over Na_2SO_4 and evaporated. The residue was purified by column chromatography (silica gel, $CHCl_3$/MeOH=30:1) to give pale yellow oil, which was purified by column chromatography (silica gel, Hexane/AcOEt=2:3) to give **13** as a pale yellow form (1.2 g, 83%). ^{1}H NMR (400 MHz, DMSO) δ ppm: 1.27 (9H, s), 2.05 (3H, s), 2.08 (3H, s), 2.34 (1H, ddd, *J*=2.7, 3.4, 6.7 Hz), 2.41 (1H, q, *J*=7.3 Hz), 4.20 (1H, dd, *J*=2.7, 4.3 Hz), 4.27 (2H, d, *J*=4.3 Hz), 5.18 (1H, t, *J*=3.4 Hz), 6.08 (2H, s), 6.12 (1H, dd, *J*=6.7, 7.3 Hz), 8.00 (1H, s), 8.19 (1H, br), 9.86 (2H, br); ^{13}C NMR(125 MHz, DMSO) δ ppm: 176.1, 170.1, 170.0, 158.9, 153.8, 152.9, 150.0, 141.3, 110.8, 100.5, 88.1, 85.8, 81.6, 74.1, 63.6, 38.9, 38.5, 26.7, 20.7, 20.6; IR (cm^{-1}): 2977, 1626, 1746; HRMS (ESI) *m*/*z*: calcd for $C_{13}H_{15}N_2O_7Br$ $[M+H]^+$: 598.1017, 600.0973; found 598.1031, 600.1015.

*3.1.3. 3′,5′-Di-O-acetyl-N-[4-(2-N-benzylcarbamyl-ethoxy-6-hydroxy-4-O-pivaloylphenyl)]-2′-deoxy-5-bromouridine (**14**).* Under argon atmosphere, a solution of **13** (1.15 g, 1.9 mmol), $PhCH_2O$-CO−$NHCH_2CH_2OH$ (480 mg, 2.5 mmol), and triphenylphosphine (810 mg, 3.1 mmol) in CH_2Cl_2 (16 mL) was stirred at 0 °C for 15 min and warmed to room temperature. DIAD (1.6 mL, 3.1 mmol) was added to the mixture and the whole was stirred for 26 h at room temperature. The reaction mixture was washed with water and brine. The organic layer was dried over Na_2SO_4 and evaporated. The residue was purified by column chromatography (silica gel, $CHCl_3$/AcOEt=2:1) to give pale yellow solids, which were purified by column chromatography (silica gel, $CHCl_3$/MeOH=50:1) to give **14** as a pale yellow form (910 mg, 62%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 1.31 (9H, s), 2.09 (3H, s), 2.11 (3H, s), 2.68 (1H, dd, *J*=2.1, 2.7 Hz), 3.64 (2H, d, *J*=4.2 Hz), 4.10 (2H, d, *J*=4.2 Hz), 4.30 (1H, dd, *J*=3.1, 4.0 Hz), 4.36 (2H, t, *J*=3.1 Hz), 5.09 (2H, s), 5.19 (1H, dd, *J*=2.7, 4.0 Hz), 6.20−6.26 (2H, m), 6.40 (1H, d, *J*=2.1 Hz), 7.32−7.31 (5H, m), 7.91 (1H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm: 176.1, 175.9, 170.1, 170.0, 159.3, 159.0, 156.2, 155.1, 154.6, 153.8, 153.0, 150.4, 150.0, 147.7, 112.0, 117.0, 85.9, 85.8, 81.6, 79.1, 74.1, 65.4, 63.6, 26.7, 26.4; IR (cm^{-1}): 2354, 1748; HRMS (ESI) *m*/*z*: calcd for $C_{34}H_{39}N_4O_{12}Br$ $[M+H]^+$: 775.1821, 777.1808; found 775.1841, 777.1777.

*3.1.4. N-[4-(2-N-Benzylcarbamylethoxy-6-hydroxy-4-O-pivaloylphenyl)]-2′-deoxy-5-bromouridine (**15**) and N-[4-(2-N-benzylcarbamylethoxy-4,6-dihydroxyphenyl)]-2′-deoxy-5-bromouridine (**16**).* A suspension of **14** (3.8 g, 4.91 mmol) and K_2CO_3 (950 mg 6.9 mmol) in MeOH (190 mL) was stirred for 30 min at room temperature. The reaction mixture was diluted with water (200 mL) then MeOH was removed by fractional distillation. Brine was added to the residue, mixture was extracted with CH_2Cl_2, and the organic layer was dried over Na_2SO_4, then evaporated. The residue was purified by column chromatography (silica gel, $CHCl_3$/MeOH=12:1 → $CHCl_3$/MeOH=8:1) to give **15** as a pale yellow oil (1.57 g, 46%) and **16** as a pale yellow oil (670 mg, 23%). Compound **15**: ^{1}H NMR (400 MHz, CD_3OD) δ ppm: 1.33 (9H, s), 2.19−2.13 (1H, m), 2.40−2.34 (1H, m), 3.47 (2H, t, *J*=5.2 Hz), 3.73 (1H, ddd, *J*=3.4, 3.6, 11.9 Hz), 3.84 (1H, dd, *J*=3.1, 11.9 Hz), 3.93 (1H, dd, *J*=3.4, 3.6 Hz), 4.09 (2H, t, *J*=5.2 Hz), 4.38−4.34 (1H, m), 5.05 (2H, s), 6.15 (1H, t, *J*=6.1 Hz), 6.27−6.33 (2H, m), 7.32−7.25 (5H, m), 8.51 (1H, s); IR (cm^{-1}): 3328, 1624, 1577; MS (ESI) *m*/*z*: $[M+H]^+$: found 691.05, 693.05. Compound **16**: ^{1}H NMR (400 MHz, CD_3OD) δ ppm: 2.18−2.12 (1H, m), 2.39−2.33 (1H, m), 3.47 (2H, dd, *J*=5.2, 5.5 Hz), 3.73 (1H, dd, *J*=3.4, 11.9 Hz), 3.83 (1H, dd, *J*=2.7, 11.9 Hz), 3.92 (1H, q, *J*=3.4 Hz), 4.05 (2H, dd, *J*=5.2, 5.5 Hz), 4.36 (1H, dt *J*=4.3, 6.1 Hz), 5.05 (2H, s), 6.02−6.06 (2H, m), 6.15 (1H, dd, *J*=6.1, 6.4 Hz), 7.32−7.23 (5H, m), 8.45 (1H, s); IR (cm^{-1}): 3330, 2928, 1624, 1573; MS (ESI) *m*/*z*: $[M+H]^+$: found 691.05, 693.05.

*3.1.5. 3-(2′-Deoxy-D-ribofuranosyl)-9-(2-N-benzylcarbamylethoxy)-1,3-diaza-7-O-pivaloyl-2-oxophenoxadine (**19**).* Under argon atmosphere, a solution of **15** (1.57 g, 2.27 mmol) and Et_3N (80 mL) in EtOH (80 mL) was stirred at 80 °C for 54 h. The reaction mixture was evaporated to give the residue, which was purified by column chromatography (silica gel, $CHCl_3$/MeOH=12:1 → $CHCl_3$/MeOH=8:1) to give **19** as a pale yellow oil (660 mg, 48%). ^{1}H NMR (400 MHz, CD_3OD) δ ppm: 1.31 (9H, s), 2.18−2.11 (1H, m), 2.36−2.30 (1H, m), 3.52 (2H, dd, *J*=4.9, 5.2 Hz), 3.70−3.75 (1H, m), 3.78−3.83 (1H, m), 3.92 (1H, dd, *J*=3.0, 3.4 Hz), 4.03 (2H, dd, *J*=4.9, 5.2 Hz), 4.34−4.38 (1H, m), 5.10 (2H, s), 6.21−6.18 (2H, m), 6.35 (1H, s), 7.33−7.24 (5H, m), 7.69 (1H, s); ^{13}C NMR (125 MHz, CD_3OD) δ ppm: 178.4, 159.1, 152.2, 148.0, 144.3, 143.5, 129.4, 128.9, 128.7, 114.9, 112.2, 105.2, 103.4, 102.6, 99.2, 89.1, 89.0, 88.2, 87.6, 72.0, 71.4, 69.9, 68.7, 67.6, 62.7, 62.2, 41.9, 41.2, 40.1, 27.4; IR (cm^{-1}): 3323, 2956, 1501; HRMS (ESI) *m*/*z*: calcd for $C_{30}H_{34}N_4O_{10}$ $[M+H]^+$: 611.2348; found 611.2351.

*3.1.6. 3-(3′,5′-Di-O-tert-butyl-dimethyl-silane-D-ribofuranosyl)-9-(2-N-benzylcarbamyl- ethoxy)-1,3-diaza-7-O-pivaloyl -2-oxophenoxadine (**3**).* Under argon atmosphere, a solution of imidazole (350 mg, 5.16 mmol), DMAP (63 mg, 0.516 mmol), TBDMSCl (625 mg, 4.13 mmol), and **19** (630 mg, 1.03 mmol) in DMF (7.7 mL) was stirred at room temperature for 20 h. Water (15 mL) was added to the reaction mixture, and the mixture was extracted with AcOEt. The organic layer was dried over Na_2SO_4 and evaporated. The residue was purified by column chromatography (silica gel, Hexane/AcOEt=1:1) to give **3** as a pale yellow form (620 mg, 72%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 0.04 (6H, s), 0.11 (3H, s), 0.13 (3H, s), 0.86 (9H, s), 0.93 (9H, s), 1.31 (9H, s), 2.08−2.02 (1H, m), 2.36−2.33 (1H, m), 3.56 (2H, q, *J*=4.9 Hz), 3.74 (1H, d, *J*=9.5 Hz), 3.89−3.92 (2H, m), 4.01 (2H, t, *J*=4.9 Hz), 4.37−4.35 (1H, m), 5.10 (2H, s), 5.95 (1H, br), 6.09 (1H, s), 6.19 (1H, s), 6.24 (1H, t, *J*=6.1 Hz), 7.34−7.25 (5H, m), 7.57 (1H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm: 176.7, 166.4, 156.6, 152.1, 146.8, 141.7, 128.5, 128.1, 128.0, 127.1, 102.5, 87.7, 86.1, 71.1, 68.8, 66.8, 62.4, 41.9, 40.4, 39.1, 27.1, 26.0, 25.7, 24.9, 18.5, 18.0, −4.6, −4.9, −5.4, −5.5; IR

(cm^{-1}): 2930, 1676, 1558, 1499; HRMS (ESI) *m/z*: calcd for $C_{42}H_{62}N_4O_{10}Si_2$ $[M+H]^+$: 839.4077; found 839.4090.

*3.1.7. 3′,5′-Di-O-tert-butyldimethylsilyl-N-[4-(2-N-benzylcarbamylethoxy-4,6-O-tert-butyldimethylsilylphenyl)]-2′-deoxy-5-bromouridine (**20**).* The title compound **20** was obtained as described for the synthesis of **3** using **16** (112 mg, 0.185 mmol). Compound **20** as a pale yellow oil (162 mg, 82%). ^1H NMR (400 MHz, $CDCl_3$) δ ppm: 0.03–0.18 (24H, m), 0.85–0.96 (36H, m), 2.02 (2H, s), 2.37 (1H, br), 3.44 (2H, br), 3.73 (1H, d, *J*=11.0 Hz), 3.86–3.89 (2H, m), 4.07–4.13 (3H, m), 4.32 (1H, br), 5.05 (2H, s), 5.97 (1H, s), 6.07 (1H, s), 6.19 (1H, t, *J*=5.8 Hz), 6.64 (1H, s), 7.29–7.30 (5H, m), 8.01 (1H, s); MS (ESI) *m/z*: $[M+H]^+$: found 1063.08, 1065.06.

*3.1.8. 3-(3′,5′-Di-O-tert-butyldimethylsilyl-D-ribofuranosyl)-9-(2-N-benzylcarbamylethoxy)-1,3-diaza-7-O-tert-butyldimethylsilyl-2-oxophenoxadine (**4**) and 3-(3′,5′-di-O-tert-butyldimethylsilyl-D-ribofuranosyl)-9-(2-N-benzylcarbamylethoxy)-1,3-diaza-7-hydroxy-2-oxophenoxadine (**5**).* Under argon atmosphere, a solution of **20** (85 mg, 0.08 mmol) and Et_3N (4 mL) in MeOH (4 mL) was stirred at 80 °C for 47 h. The reaction mixture was evaporated, and the residue was purified by column chromatography (silica gel, $CHCl_3$/MeOH=25:1) to give pale yellow oil, which was purified by column chromatography (silica gel, Hexane/AcOEt=4:1→Hexane/AcOEt=2:1) to give **4** as a pale yellow form (15 mg, 22%) and **5** as a pale yellow form (23 mg, 38%). Compound **4**: ^1H NMR (400 MHz, $CDCl_3$) δ ppm: 0.04 (6H, s), 0.12 (3H, s), 0.14 (3H, s), 0.16 (6H, s), 0.86 (9H, s), 0.94 (18H, d, *J*=2.1 Hz), 2.02–2.09 (1H, m) 2.32–2.35 (1H, m), 3.57 (2H, t, *J*=4.5 Hz), 3.75 (1H, d, *J*=9.5 Hz), 3.88 (1H, d, *J*=9.5 Hz), 3.93 (1H, s), 3.97 (2H, t, *J*=4.5 Hz), 4.34–4.38 (1H, m), 5.10 (2H, s), 5.87 (1H, d, *J*=2.2 Hz), 5.97 (1H, s), 6.21 (1H, t, *J*=5.8 Hz), 6.27 (1H, br), 7.25–7.35 (5H, m), 7.61 (1H, s); ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm: 163.8, 156.7, 153.5, 151.3, 147.6, 143.4, 136.7, 128.4, 128.0, 127.9, 127.0, 100.4, 100.1, 87.7, 86.0, 70.7, 68.6, 66.6, 62.3, 41.9, 40.4, 29.7, 26.0, 25.7, 25.6, 18.5, 18.2, 17.9, −1.3, −4.5, −4.6, −4.9, −5.5, −5.6; IR (cm^{-1}): 2928, 2856, 1716, 1555, 1503; HRMS (ESI) *m/z*: calcd for $C_{43}H_{68}N_4O_9Si_3$ $[M+H]^+$: 869.4367; found 869.4406. Compound **5**: ^1H NMR (400 MHz, $CDCl_3$) δ ppm: 0.02 (3H, s), 0.04 (3H, s), 0.13 (6H, s), 0.84 (9H, s), 0.94 (9H, s), 2.18 (1H, br), 2.31 (1H, br), 3.45 (2H, br), 3.71 (1H, d, *J*=9.1 Hz), 3.85 (4H, s), 4.37 (1H, s), 4.97 (2H, s), 5.85 (1H, d, *J*=7.1 Hz), 6.32 (2H, br), 6.27 (1H, br), 7.14 (5H, br); ^{13}C NMR (125 MHz, $CDCl_3$) δ ppm: 156.8, 143.3, 136.8, 136.6, 128.3, 128.0, 127.8, 121.8, 96.4, 87.8, 86.0, 71.8, 69.6, 68.1, 66.9, 66.5, 62.8, 41.4, 40.3, 26.1, 25.7, 18.5, 17.9, −4.6, −4.9, −5.5, −5.5; IR (cm^{-1}): 3219, 2930, 2858, 1671, 1556, 1505, 1472; HRMS (ESI) *m/z*: calcd for $C_{37}H_{54}N_4O_9Si_2$ $[M+H]^+$: 755.3502; found 755.3545.

*3.1.9. 3-(3′,5′-Di-O-tert-butyldimethylsilyl-D-ribofuranosyl)-9-(2-N-benzylcarbamyl-ethoxy)-1,3-diaza-7-O-triflate-2-oxophenoxadine (**6**).* Under argon atmosphere, Et_3N (0.3 mL, 2.27 mmol) was added to a solution of **5** (570 mg, 0.757 mmol) and *N*-phenylbis(trifluoromethanesulfonimide) (406 mg, 1.14 mmol) in CH_2Cl_2 (9.6 mL) and the mixture was stirred at room temperature for 22 h. The reaction mixture was washed successively with saturated aqueous $NaHCO_3$, brine, and water. The organic layer was dried over Na_2SO_4 and evaporated. The residue was purified by column chromatography (silica gel, Hexane/AcOEt=1:1→Hexane/AcOEt=1:2) to give **6** as a pale yellow form (520 mg, 78%). ^1H NMR (400 MHz, $CDCl_3$) δ ppm: 0.04 (6H, s), 0.12 (3H, s), 0.13 (3H, s) 0.86 (9H, s), 0.93 (9H, s), 2.08–2.11 (1H, m), 2.33–2.38 (1H, m), 3.59 (2H, d, *J*=4.3 Hz), 3.75 (2H, d, *J*=10.1 Hz), 3.89–3.94 (2H, m), 4.01 (2H, d, *J*=10.1 Hz), 4.36 (1H, d, *J*=4.9 Hz), 5.08 (2H, s), 6.24 (1H, t, *J*=5.5 Hz), 6.26 (1H, s), 6.37 (1H, s), 7.26–7.30 (5H, m), 7.71 (1H, s); ^{13}C NMR(125 MHz, $CDCl_3$) δ ppm: 156.7, 152.4, 151.3, 147.3, 145.3, 143.3, 136.6, 128.4, 128.0, 128.0, 126.6, 123.8, 122.5, 119.9, 117.4, 114.8, 102.3, 101.3, 87.7, 86.2, 70.6, 69.2, 66.7, 62.2, 41.8, 40.7, 40.1, 29.7, 27.1, 26.0, 25.7, −4.6, −4.9, −5.5; IR (cm^{-1}): 2951, 1679, 1561, 1497, 1450, 1425; HRMS (ESI) *m/z*: calcd for $C_{38}H_{53}F_3N_4O_{11}Si_2$ $[M+H]^+$: 887.2995; found 887.2963.

*3.1.10. 3-(3′,5′-Di-O-tert-butyldimethylsilyl-D-ribofuranosyl)-9-(2-N-benzylcarbamyl-ethoxy)-1,3-diaza-7-phenyl-2-oxophenoxadine (**7**).* A solution of **6** (50 mg, 0.056 mmol), $Pd(OAc)_2$ (3 mg, 0.012 mmol), SPhos (12 mg, 0.028 mmol), K_3PO_4–nH_2O (45 mg), and phenyl boronic acid (14 mg, 0.11 mmol) in degassed toluene (5 mL) was heated at 100 °C for 2 h. The reaction mixture was diluted with AcOEt, filtered through a Celite pad, and then the filtrate was evaporated. The residue was purified by column chromatography (silica gel, Hexane/AcOEt=1:1→Hexane/AcOEt=1:2) to give **7** as a pale yellow form (40 mg, 88%). ^1H NMR (400 MHz, CD_3OD) δ ppm: 0.11 (6H, s), 0.16 (3H, s), 0.18 (3H, s), 0.91 (9H, s), 0.97 (9H, s), 2.06–2.13 (1H, m), 2.28–2.35 (1H, m), 3.55 (2H, t, *J*=4.9 Hz), 3.67–3.71 (1H, m), 3.80–3.83 (1H, m), 3.92 (2H, dd, *J*=2.7, 7.0 Hz), 4.12 (2H, t, *J*=4.9 Hz), 4.45–4.46 (1H, m), 5.10 (2H, s), 6.16 (1H, t, *J*=6.1 Hz), 6.57 (1H, s), 6.81 (1H, s), 7.22–7.32 (6H, m), 7.39 (2H, t, *J*=7.6 Hz), 7.49 (2H, d, *J*=7.6 Hz), 7.59 (1H, s); ^{13}C NMR(125 MHz, CD_3OD) δ ppm: 171.1, 155.4, 144.5, 141.0, 138.6, 129.9, 129.5, 129.4, 128.9, 128.7, 127.5, 123.5, 107.6, 107.1, 89.4, 87.6, 73.1, 69.8, 67.6, 63.8, 42.7, 41.3, 26.6, 26.3, 19.4, 18.9, −4.4, −4.6, −5.2; IR (cm^{-1}): 2925, 2354, 1673, 1563, 1493, 1421; HRMS (ESI) *m/z*: calcd for $C_{43}H_{58}N_4O_8Si_2$ $[M+H]^+$: 815.3866; found 815.3889.

*3.1.11. 3-(3′,5′-Di-O-tert-butyldimethylsilyl-D-ribofuranosyl)-9-(2-N-benzylcarbamyl-ethoxy)-1,3-diaza-7-(1-naphthyl)-2-oxophenoxadine (**8**).* A solution of **6** (40 mg, 0.045 mmol), $Pd(OAc)_2$ (3 mg, 0.012 mmol), SPhos (9 mg, 0.023 mmol), K_3PO_4–nH_2O (36 mg), and 1-naphthalene boronic acid (16 mg, 0.09 mmol) in degassed toluene (4 mL) was heated at 100 °C for 2 h. The reaction mixture was diluted with AcOEt, filtered through a Celite pad, and then the filtrate was evaporated. The residue was purified by column chromatography (silica gel, Hexane/AcOEt=3:2→Hexane/AcOEt=1:2) to give pale yellow form, which was purified by column chromatography (silica gel, $CHCl_3$/acetone=10:1) to give **8** as a pale yellow form (20 mg, 51%). ^1H NMR (400 MHz, CD_3OD) δ ppm: 0.10 (6H, s), 0.11 (3H, s), 0.12 (3H, s), 0.91 (18H, s), 2.07–2.14 (1H, m), 2.30–2.36 (1H, m), 3.51 (2H, t, *J*=4.9 Hz), 3.79 (1H, d, *J*=9.2 Hz), 3.89–3.92 (2H, m), 4.03 (2H, t, *J*=4.9 Hz), 4.44–4.47 (1H, m), 5.10 (2H, s), 6.17 (1H, t, *J*=6.1 Hz), 6.42 (1H, s), 6.64 (1H, s), 7.22–7.34 (6H, m), 7.39 (1H, dd, *J*=7.3, 7.6 Hz), 7.46 (2H, dd, *J*=7.3, 8.2 Hz), 7.60 (1H, s), 7.85 (3H, dd, *J*=7.6, 8.2 Hz); ^{13}C NMR(125 MHz, CD_3OD) δ ppm: 169.8, 159.1, 155.5, 147.8, 144.0, 140.1, 138.4, 138.0, 135.4, 132.6, 129.5, 129.4, 129.1, 128.9, 128.7, 127.7, 127.2, 127.0, 126.4, 126.4, 123.6, 110.9, 110.3, 89.3, 87.5, 72.9, 69.8, 67.6, 63.7, 42.8, 41.3, 26.6, 26.3, 19.3, 18.9, −4.4, −4.6, −5.2, −5.3; IR (cm^{-1}): 2952, 2366, 1696, 1676, 1559, 1493, 1422; HRMS (ESI) *m/z*: calcd for $C_{47}H_{60}N_4O_8Si_2$ $[M+H]^+$: 865.4022; found 865.4010.

*3.1.12. 3-(3′,5′-Di-O-tert-butyldimethylsilyl-D-ribofuranosyl)-9-(2-N-benzylcarbamyl-ethoxy)-1,3-diaza-7-(4-methoxyphenyl)-2-oxophenoxadine (**9**).* A solution of **6** (50 mg, 0.056 mmol), $Pd(OAc)_2$ (3 mg, 0.012 mmol), JohnPhos (9 mg, 0.03 mmol), K_3PO_4 (35 mg, 0.165 mmol), and 4-methoxyphenyl boronic acid (18 mg, 0.118 mmol) in degassed 1,4-dioxane (1.5 mL) was heated at 100 °C for 6 h. The reaction mixture was diluted with AcOEt, filtered through a Celite pad, and then the filtrate was evaporated. The residue was purified by column chromatography (silica gel, Hexane/AcOEt=1:1) to give **9** as a pale yellow form (28 mg, 59%). ^1H NMR (400 MHz, CD_3OD) δ ppm: 0.11 (6H, s), 0.17 (3H, s), 0.19 (3H, s), 0.92 (9H, s), 0.97 (9H, s), 2.06–2.12 (1H, m), 2.32–2.38 (1H, m), 3.55 (2H, t, *J*=4.9 Hz), 3.80 (3H, s), 3.83 (1H, d, *J*=3.1 Hz), 3.91–3.93 (2H, m), 4.11 (2H, t, *J*=4.9 Hz), 4.45–4.49 (1H, m), 5.11 (2H, s), 6.16 (1H, t, *J*=6.1 Hz), 6.51 (1H, s), 6.75 (1H, s), 6.94 (2H, d, *J*=8.8 Hz), 7.23–7.32 (6H, m), 7.42 (2H, d, *J*=8.5 Hz), 7.57 (1H, s); ^{13}C NMR(125 MHz, CD_3OD) δ ppm: 160.9, 159.1, 155.2, 144.4, 138.1, 133.3, 130.0, 129.5, 129.4, 128.9, 128.7, 128.5, 127.6, 123.2, 115.3, 107.0, 106.4, 89.3, 87.5,

73.1, 69.6, 67.6, 63.9, 55.8, 42.7, 41.3, 27.8, 26.7, 26.3, 19.4, 18.9, −4.4, −4.6, −5.2, −5.2; IR (cm^{-1}): 2929, 1717, 1676; HRMS (ESI) *m*/*z*: calcd for $C_{44}H_{60}N_4O_9Si_2$ $[M+H]^+$: 845.3972; found 845.3978.

*3.1.13. 3-(3′,5′-Di-O-tert-butyldimethylsilyl-D-ribofuranosyl)-9-(2-N-benzylcarbamyl-ethoxy)-1,3-diaza-7-(4-cyanophenyl)-2-oxophenoxadine (**10**).* A solution of **6** (25 mg, 0.028 mmol), $Pd(OAc)_2$ (2 mg, 0.0082 mmol), XPhos (7.5 mg, 0.014 mmol), K_3PO_4 (18 mg, 0.084 mmol), and 4-cyanophenyl boronic acid (8 mg, 0.056 mmol) in degassed 1,4-dioxane (0.5 mL) was heated at 100 °C for 17 h. The reaction mixture was diluted with AcOEt, filtered through a Celite pad, and then the filtrate was evaporated. The residue was purified by column chromatography (silica gel, $CHCl_3$/MeOH=49:1) to give **10** as a pale yellow form (10 mg, 43%). ^1H NMR (400 MHz, CD_3OD) δ ppm: 0.11 (6H, s), 0.16 (3H, s), 0.18 (3H, s), 0.92 (9H, s), 0.97 (9H, s), 2.08–2.14 (1H, m), 2.30–2.36 (1H, m), 3.57 (2H, t, *J*=5.2 Hz), 3.83 (1H, d, *J*=11.0 Hz), 3.91–3.94 (2H, m), 4.17 (2H, t, *J*=5.2 Hz), 4.46 (1H, br), 5.10 (2H, s), 6.16 (1H, t, *J*=6.0 Hz), 6.65 (1H, s), 6.92 (1H, s), 7.32–7.23 (5H, m), 7.61 (1H, s), 7.70–7.76 (2H, m); ^{13}C NMR (125 MHz, CD_3OD) δ ppm: 179.2, 170.3, 160.0, 155.4, 145.5, 144.5, 136.2, 133.8, 129.4, 128.9, 128.6, 128.4, 125.0, 109.2, 108.4, 89.3, 87.5, 72.9, 69.7, 67.5, 63.7, 42.8, 41.3, 26.6, 26.3, 19.4, 5.7, −4.5, −4.7, −5.3; IR (cm^{-1}): 2952, 2352, 1718, 1676; HRMS (ESI) *m*/*z*: calcd for $C_{44}H_{60}N_5O_8Si_2$ $[M+H]^+$: 840.3818; found 840.3845.

*3.1.14. 3-(3′,5′-Di-O-tert-butyldimethylsilyl-D-ribofuranosyl)-9-(2-N-benzylcarbamyl-ethoxy)-1,3-diaza-7-(thiophenyl-2-yl)-2-oxophenoxadine (**11**).* A solution of **6** (25 mg, 0.028 mmol), $Pd(OAc)_2$ (2 mg, 0.0082 mmol), cyclohexyl JohnPhos (5 mg, 0.014 mmol), K_3PO_4 (18 mg, 0.084 mmol), and 2-thiophene boronic acid (7 mg, 0.056 mmol) in degassed 1,4-dioxane (2 mL) was heated at 100 °C for 24 h. The reaction mixture was diluted with AcOEt, filtered through a Celite pad, and then the filtrate was evaporated. The residue was purified by column chromatography (silica gel, Hexane/AcOEt=2:1) to give dark yellow oil crude, and purified by preparative thin layer chromatography (silica gel, Hexane/AcOEt=1:1) to give **11** as a pale yellow form (2.7 mg, 12%). ^1H NMR (400 MHz, CD_3OD) δ ppm: 0.05 (6H, s), 0.13 (3H, s), 0.15 (3H, s), 0.87 (9H, s), 0.95 (9H, s), 2.21–2.12 (1H, m), 2.39–2.28 (1H, m), 3.57 (2H, t, *J*=5.2 Hz), 3.86–3.80 (1H, m), 3.97–3.93 (2H, m), 4.16 (2H, t, *J*=5.2 Hz), 4.49 (1H, br), 5.12 (2H, s), 6.19 (1H, t, *J*=6.0 Hz), 6.60 (1H, s), 6.90 (1H, s), 7.04–7.07 (1H, m), 7.17–7.36 (8H, m), 7.70 (1H, s); ^{13}C NMR(125 MHz, $CDCl_3$) δ ppm: 133.4, 131.0, 130.3, 128.9, 128.3, 127.6, 126.8, 126.6, 126.1, 125.5, 124.9, 124.3, 105.5, 105.3, 95.0, 89.8, 88.6, 86.8, 71.1, 68.8, 66.3, 42.1, 40.3, 29.7, 27.5, 26.0, 14.1, −4.6, −4.9, −5.5; IR (cm^{-1}): 2927, 1711, 1561, 1494; HRMS (ESI) *m*/*z*: calcd for $C_{41}H_{56}N_4O_8SSi_2$ $[M+H]^+$: 821.3430; found 821.3470.

3.2. UV and fluorescence spectra, and fluorescence quenching experiments

UV and fluorescent spectra were measured in $CHCl_3$ solution at 25 °C using 3′-*O*- and 5′-*O*-TBDMS protected nucleosides. F_q and F_0 in the fluorescence quenching ratio represent the fluorescence intensity of the 1:1 mixture of 8-oxoGclamp and 8-oxo-dG or dG and the fluorescence intensity of 8-oxoGclamp, respectively. Fluorescence titration was performed by the addition of a stock solution of the 3′-*O*-, 5′-*O*-di-TBDMS protected 2′-deoxynucleoside (0–10 μM final concentration) to a solution of the 8-oxoGclamp analog (1 μM) under the following conditions, A: $CHCl_3$ solution containing 10 mM TEA and 2.7 mM AcOH, B: $CHCl_3$ solution containing 0.33 mM TEA–AcOH, C: $CHCl_3$ solution without addition of AcOH–TEA. The titration curve was analyzed by the non-linear curve fitting method based on the 1:1 complex ratio.

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