Synthesis of 6-formylpterin nucleoside analogs and their ROS generation activities in the presence of NADH in the dark

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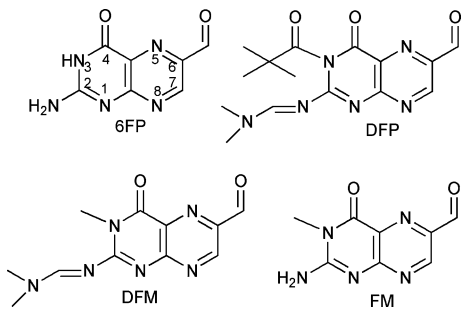
We demonstrated previously that 3-position-modified 6-formylpterin (6FP) derivatives produce reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) from oxygen in the presence of NADH in the dark. It has been shown that 6FP derivatives markedly generate ROS, which gives rise to their particular physiological activities, such as induction of apoptosis in cellular and living systems, suggesting that such compounds provide a hint for the design of a ROS controlling agent *in vivo*. However, it is not well understood why such unique activities appear on chemical modification. In the present study, in order to see the effect on ROS generation activity in the dark by the modification of the 1-position in 6FP, we have developed a new synthetic procedure for nucleoside analogs of 6FP and prepared 1-(β-D-ribofuranosyl)-2-(*N*,*N*-diethylaminomethyleneamino)-6-formylpteridin-4-one (RDEF) and 1-(β-D-ribofuranosyl)-2-(piperidine-1-ylmethyleneamino)-6-formylpteridin-4-one (RPIF) in which the 1-position of 6FP is glycosylated. At pH 7.4, NADH was spontaneously oxidized to NAD^+ in the presence of RDEF in the dark. Using electron paramagnetic resonance analysis coupled with the spin trapping technique, we show that O_2 was converted to H_2O_2 *via* superoxide anion radical ($^{\bullet}O_2^-$) during this reaction. The modification of the 1-position of 6FP did not cancel ROS generation activities, which were demonstrated in 3-position-modified 6FPs. Since the 6FP derivatives developed in the present study have a ribose moiety, these compounds can be subjected to further derivatization, such as incorporation into oligonucleotides, oligosaccharides, proteins, or any other compounds that recognize and interact with specific biomolecules, and therefore would be useful in pharmaceutical investigations that need generation of appropriate and controllable amounts of ROS *in vivo*.

Introduction

6-Formylpterin (6FP, Scheme 1) is a pteridine derivative produced from folic acid by photooxidation in the presence of O_2.[1] *In vivo*, 6FP has been shown to be produced from folic acid in some pathological conditions, such as carcinoma.[2] Furthermore, in the past two decades, 6FP has been investigated in the field of photochemistry.[3] Under UV irradiation, for instance, 6FP activates O_2, generates singlet oxygen, and also forms H_2O_2 from O_2.[4] However, detailed reaction mechanisms for both examples remain to be defined.



Scheme 1 Structures of 6FP and 6FP derivatives.

In previous studies, we have shown that reactive oxygen species (ROS), such as H_2O_2, are generated by 6FP and that it induced apoptosis in HL-60 cells, inhibited Fas-mediated apoptosis in Jurkat cells, and suppressed activation of NF-κB, cytokine production, and cell proliferation in PanC-1 and human blood T cells.[5] In addition, 6FP showed neuroprotective effects against transient ischemia-reperfusion injury (IRI) in gerbils[6] and similar effects for retinal IRI in rats;[7] however, the mechanism of such physiological activities has not been elucidated to date. IRI occurs in conditions, including cardiac infarction, brain infarction, and organ transplantation, that result in apoptotic cell death and, therefore, protective agents against IRI have been one of the most important research targets in medicinal and pharmaceutical studies. Although 6FP shows notable physiological activities, it has not been used clinically, partly because of its poor water solubility at neutral pH.[8]

To overcome this drawback, 6FP derivatives with improved water solubility at neutral pH have been developed and assessed for their chemical activities *in vitro*.[8,9] It is demonstrated that 6FP

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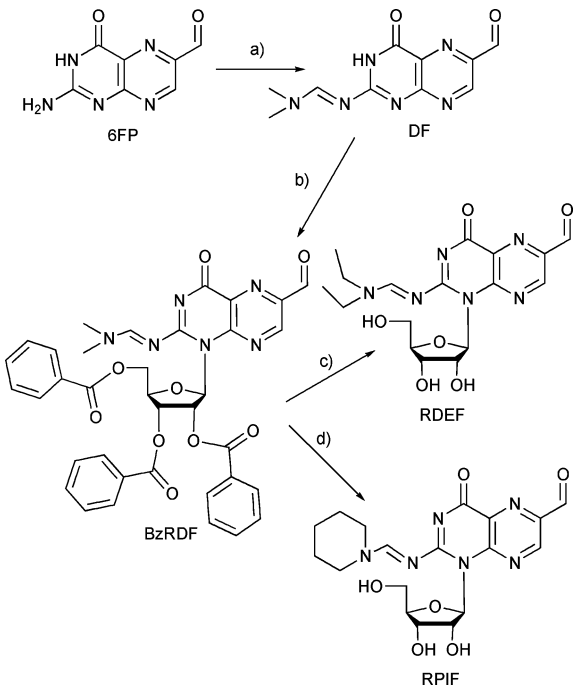
derivatives, such as 2-(*N*,*N*-dimethylaminomethyleneamino)-6-formyl-3-pivaloylpteridin-4-one (DFP), 2-(*N*,*N*-dimethylaminomethyleneamino)-6-formyl-3-methylpteridin-4-one (DFM), and 2-amino-6-formyl-3-methylpteridin-4-one (FM) (Scheme 1), with a modified 3-position can generate H_2O_2 through NADH to NAD^+ oxidation in the dark in phosphate-buffered saline (PBS) at pH 7.4 without the aid of any biological systems.[10] It is important to explore ROS generation by 6FP derivatives in the absence of light because most biological events occur in the dark. In addition, DFP, one of the 3-position modified 6FP derivatives in which hydrogen atoms on the 2- and 3-positions of 6FP are replaced by *N*,*N*-dimethylaminomethylene and pivaloyl groups, respectively, showed suppressed proliferation of PanC-1 cells.[11] Although the mechanism is not clearly understood yet, we speculate that ROS generated by DFP in the presence of NADH is involved in the mechanism.

It is now widely known that ROS are not only involved in cell death but are also involved in the modulation of a variety of cell functions,[12] thus, it is important to understand the relationship between ROS activity and biological events. 6FP derivatives that can generate ROS in the dark through NADH oxidation at neutral pH in the absence of any biological systems, can be potent physiological compounds that generate appropriate amounts of ROS in living systems. In addition, it is possible that by using these compounds the above-mentioned physiological activities of 6FP can be introduced in most biological systems. In the present study, in order to determine the factors responsible for such activities, we have developed a synthetic procedure for nucleoside analogs of 6FP in which the 1-position is modified. It should be noted that in photochemistry, pteridine nucleoside analogs, other than the 6FP derivatives, have been developed and used for analytical purposes due to their unique fluorescence properties.[13]

Recently, a number of homing devices that direct physiological compounds to specific cells or biomolecules, *i.e.*, specific drug delivery systems,[14] have been developed. Nucleoside analogs of 6FP derivatives developed in the present work contain a ribose on the pteridine ring and, therefore, can be incorporated into homing devices such as oligonucleotides, oligosaccharides, proteins, or any other compounds. These compounds could be directly introduced into specific cellular systems to recognize and interact with specific biomolecules and generate appropriate and controllable amounts of ROS in some specific position *in vivo* because NADH, a central intermediate in oxidative catabolism that acts as a convenient source of readily transferable electrons in cells,[15] is abundant.

Results and discussion

When 6FP was reacted with *tert*-butoxybis(dimethylamino)-methane in *N*,*N*-dimethylformamide (DMF) at 60 °C for 20 min, 2-(*N*,*N*-dimethylaminomethyleneamino)-6-formylpteridin-4-one (DF)[9] was obtained in a yield of 95%, as shown in Scheme 2. Then the glycosylation reaction of DF was conducted. When DF was reacted with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose in the presence of 1,8-diazabicyclo[5,4,0]undec-7-ene and trimethylsilyl trifluoromethanesulfonate in acetonitrile at 60 °C for 14 h, glycosylation reaction[16] occurred at the 1-position of DF and 1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-2-(*N*,*N*-dimethylaminomethyleneamino)-6-formylpteridin-4-one (BzRDF) was obtained in a yield of 68% (Scheme 2). Next, the deprotection



Scheme 2 Chemical synthesis of RDEF and RPIF: (a) synthesis of DF from 6FP using *tert*-butoxybis(dimethylamino)methane in DMF at 60 °C for 20 min, 95%; (b) glycosylation of DF using 1,8-diazabicyclo-[5,4,0]undec-7-ene, trimethylsilyl trifluoromethanesulfonate, and 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose in CH_3CN at 60 °C for 14 h, 68%; (c) deprotection of BzRDF in MeOH containing 25% diethylamine at rt for 24 h, 49%; (d) deprotection of BzRDF in MeOH containing 25% piperidine at rt for 24 h, 25%.

reaction of BzRDF was carried out. When BzRDF was stirred in methanol containing 25% diethylamine solution at room temperature for 24 h, 1-(β-D-ribofuranosyl)-2-(*N*,*N*-diethylaminomethyleneamino)-6-formylpteridin-4-one (RDEF) was obtained in a yield of 49% as a nucleoside analog of a 6FP derivative (Scheme 2). RDEF shows over 100 mM solubility in PBS at pH 7.4 because of the hydrophilic ribose moiety. In this reaction, the dimethylaminomethyleneamino group on the 2-position was substituted by *N*,*N*-diethylaminomethyleneamino. To determine if this occurred generally, BzRDF was stirred in methanol containing 25% piperidine solution at room temperature for 24 h, and it was found that 1-(β-D-ribofuranosyl)-2-(piperidine-1-ylmethyleneamino)-6-formylpteridin-4-one (RPIF) was produced in a yield of 25% (Scheme 2).

We have demonstrated previously that DFP suppresses proliferation of PanC-1 cells without photo irradiation.[11] We speculate that the observation of DFP-induced suppression of proliferation in PanC-1 cells may be due to ROS generation through NADH oxidation by DFP. ROS generation in the presence of NADH in the dark is one of the most prominent physiological activities attributed to the 6FP derivatives because it is related to the physiological activities of 6FP.[5] Therefore, the ROS generation activity through NADH oxidation by RDEF in the dark was investigated.

PBS solutions (pH 7.4) containing 2 mM RDEF and 2 mM NADH were stirred in the dark in an open system at room temperature and the time-dependent concentration change of each

component was analyzed by reversed-phase high performance liquid chromatography (RP-HPLC). During the reaction, a decrease in NADH concentration and generation of NAD^+ was observed, as shown in Fig. 1. In this oxidation reaction, RDEF concentration also gradually decreased, probably because of the decomposition caused by ROS generated in this system. In the absence of NADH, RDEF remained constant over 100 h (data not shown). In addition, in the absence of RDEF, NADH remained constant over 100 h (data not shown). From these results, we conclude that RDEF, a 6FP derivative in which the 1-position is glycosylated, oxidized NADH to NAD^+ in the dark.

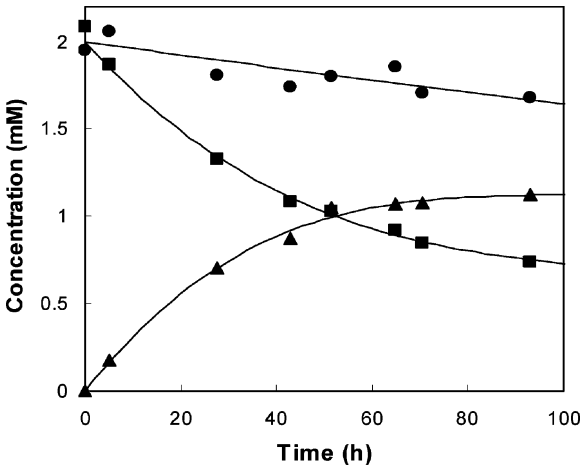


Fig. 1 Oxidation reaction of NADH by RDEF in the dark. The time course of the change in RDEF (●), NADH (■), and NAD^+ (▲) concentrations in the dark in an open system was measured by RP-HPLC. A PBS solution containing 2 mM RDEF and 2 mM NADH was stirred in the dark at pH 7.4. In the absence of RDEF, NADH is constant over the 100 h of this experiment.

To identify the ROS generated in this reaction, electron paramagnetic resonance (EPR) spectroscopy coupled with the spin trapping technique, using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a spin trap, was employed. PBS solutions (pH 7.4) containing 2 mM RDEF and 2 mM NADH were incubated for 48 h and diethylenetriaminepentaacetic acid (DETAPAC) (500 μM), $FeSO_4$ (250 μM), and DMPO (100 mM) were added to the reaction mixture prior to EPR analysis. It should be noted that in the presence of ferrous ion (Fe^{2+}), hydroxyl radical ($^{\bullet}OH$) is generated from H_2O_2 *via* the iron-catalyzed Fenton reaction.[17] The results are summarized in Fig. 2. The solutions containing either RDEF or NADH did not show EPR signals over 48 h, as represented in Fig. 2(a) and 2(b). The solution containing RDEF and NADH showed a quartet with the hyperfine splitting constant (HFSC) of $A_N = 1.49$ mT and $A_H = 1.49$ mT (Fig. 2(c)). This signal was quenched by adding catalase (10 000 units ml^{-1}), which eliminates H_2O_2, to the crude reaction solution (Fig. 2(d)). Based on these observations, the quartet was identified as a DMPO adduct of $^{\bullet}OH$ (DMPO–OH), indicating that H_2O_2 was generated in the dark in the solutions containing RDEF and NADH.

To identify the exact ROS species directly generated in the reaction of RDEF and NADH, further EPR spectroscopy coupled with the spin trapping technique, using a newly developed spin trap, 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline-*N*-oxide (CYPMPO),[18] was employed. CYPMPO has

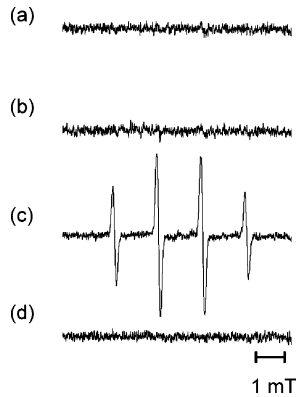


Fig. 2 EPR spectra obtained for PBS (pH 7.4) containing (a) 2 mM RDEF, (b) 2 mM NADH, (c) 2 mM RDEF and 2 mM NADH, and (d) 2 mM RDEF and 2 mM NADH. The reactions were performed for 48 h in the dark in an open system. After the reaction, 500 μM DETAPAC, 250 μM $FeSO_4$, and 100 mM DMPO were added to the reaction mixtures, which were diluted to 1/10 immediately prior to the EPR measurement. Catalase (10 000 units ml^{-1}) was also added to (d).

been shown to demonstrate excellent ability to trap $^{\bullet}O_2^-$ and $^{\bullet}OH$ as compared with DMPO.[18] PBS solutions (pH 7.4) containing 4 mM RDEF, 4 mM NADH, and 20 mM CYPMPO were incubated at room temperature for 1 h in the dark in an open system and measured using EPR spectroscopy. The results are summarized in Fig. 3. The solutions containing either RDEF or NADH and CYPMPO did not show EPR signals (data not shown). The solutions containing RDEF, NADH, and CYPMPO showed a prominent EPR spectrum pattern of a mixture of $^{\bullet}O_2^-$ and $^{\bullet}OH$ adducts of CYPMPO[18] (Fig. 3(a)). These signals were completely quenched by superoxide dismutase (SOD), which converts $^{\bullet}O_2^-$ to H_2O_2 in the presence of a proton source (Fig. 3(b)). Fig. 3(c) is a computer simulated spectrum of a mixture of $^{\bullet}O_2^-$ and $^{\bullet}OH$ adducts of CYPMPO. Both $^{\bullet}O_2^-$ and $^{\bullet}OH$ adducts are composed of two diastereomers respectively. The computer-simulated spectrum showed good coincidence with the previously reported spectrum of the mixture of $^{\bullet}O_2^-$ and $^{\bullet}OH$ adducts of CYPMPO,[18] and also with the spectrum in Fig. 3(a) in this study.

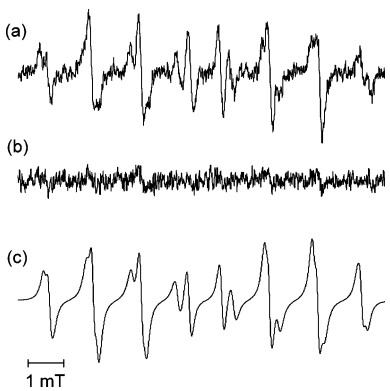


Fig. 3 EPR spectra obtained for PBS (pH 7.4) containing (a) 4 mM RDEF, 4 mM NADH and 20 mM CYPMPO, (b) 4 mM RDEF, 4 mM NADH, 20 mM CYPMPO and 2000 units ml^{-1} SOD. The reactions were performed for 1 h at room temperature in the dark in an open system and measured using EPR spectroscopy. (c) Computer-simulated spectrum for a mixture of $^{\bullet}O_2^-$ and $^{\bullet}OH$ adducts of CYPMPO.

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Table 1 HFSCs of $^{\bullet}O_2^-$ and $^{\bullet}OH$ adducts of CYPMPO used for computer-simulation

	$^{\bullet}O_2^-$ adduct	$^{\bullet}OH$ adduct
HFSC (A_N, A_H, A_P)/mT	1.26, 1.11, 5.25 1.27, 1.04, 5.10	1.37, 1.37, 4.98 1.35, 1.23, 4.88

HFSCs used in this simulation are listed in Table 1. These results clearly indicate that O_2 was converted into $^{\bullet}O_2^-$ in the presence of RDEF and NADH.

To further confirm $^{\bullet}O_2^-$ generation in the reaction, a chemiluminescence method with the $^{\bullet}O_2^-$ specific probe 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazine-3-one (CLA)[19] was also employed. After mixing 2 mM RDEF and 100 μM CLA with or without 700 units ml^{-1} SOD in PBS solution, the luminescence was monitored with a luminescence reader. It should be noted that if $^{\bullet}O_2^-$ is generated in the reaction of RDEF and NADH, $^{\bullet}O_2^-$ reacts with CLA and chemiluminescence is observed. When 2 mM NADH was added to the mixture containing RDEF and CLA, enhancement of the luminescence was observed (Fig. 4(a)). When NADH was added to the mixture containing RDEF, CLA, and SOD, the luminescence was suppressed (Fig. 4(b)). These results also indicate that $^{\bullet}O_2^-$ was generated in the presence of RDEF and NADH.

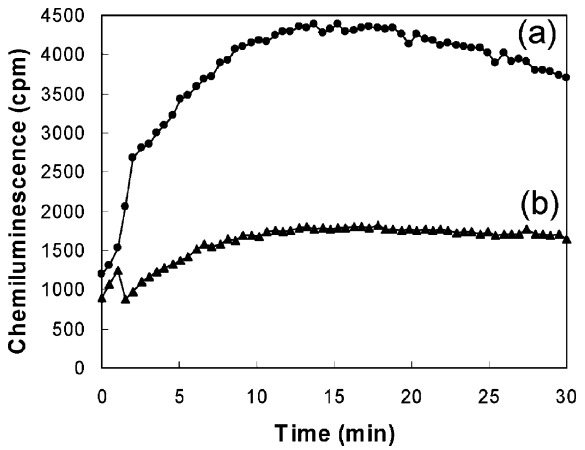


Fig. 4 Detection of $^{\bullet}O_2^-$ in PBS solutions containing RDEF and NADH by the chemiluminescence method. PBS solutions containing (a) 2 mM RDEF and 100 μM CLA, (b) 2 mM RDEF, 100 μM CLA, and 700 units ml^{-1} SOD were incubated and the luminescence intensities (● for (a), and ▲ for (b)) were monitored every 30 s for 30 min. 2 mM NADH was added to the solutions 90 s after the measurement was started.

In summary, we have shown that in the solutions containing RDEF and NADH in the dark, RDEF oxidized NADH to NAD^+ and simultaneously O_2 was converted to $^{\bullet}O_2^-$. The generated $^{\bullet}O_2^-$ was finally converted to H_2O_2. The oxidation reaction of NADH by RDEF was shown to be essential for $^{\bullet}O_2^-$ and H_2O_2 generation. In previous studies, it has been reported that biopterin, neopterin, and 6-(hydroxymethyl)pterin activate O_2 to H_2O_2 under UV irradiation,[4] and reaction mechanisms that involve an intermediate such as 6-formyl-5,8-dihydropterin, a reduced form of 6FP, have been proposed. As evidence, such an intermediate was produced from 6-(hydroxymethyl)pterin by UV irradiation, which activated O_2 to H_2O_2 and simultaneously converted 6FP by a thermal reaction in the absence of light.[4] Based on the present results and previously reported data, it is possible that NADH was oxidized by RDEF to NAD^+ by hydride transfer,[20] and then the reduced form of RDEF thus generated would reduce O_2 to a ROS such as H_2O_2, through $^{\bullet}O_2^-$ formation. It should be emphasized that all these reactions occurred in the dark without any biological systems.

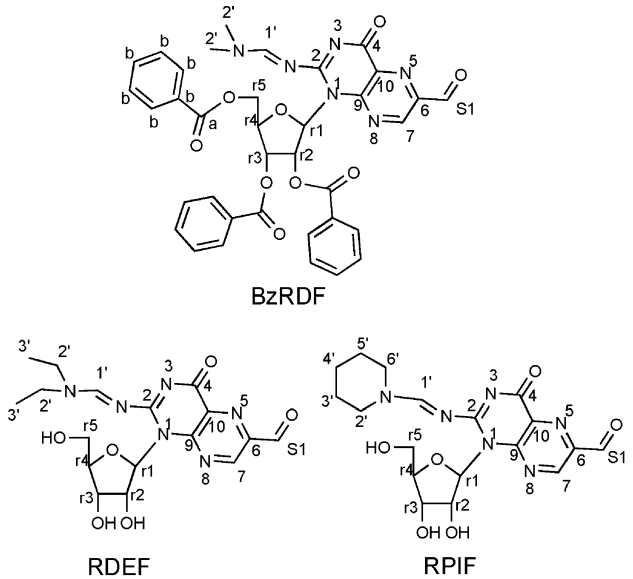
Conclusions

We synthesized novel nucleoside analogs of 6FP. RDEF, which has a ribose on the 1-position of the pteridin ring, showed $^{\bullet}O_2^-$ and H_2O_2 generation activity through the oxidation of NADH to NAD^+ in the dark at pH 7.4 without any biological systems. This indicates that, in addition to the modification at the 3-position, a modification at the 1-position also introduces the activity of ROS generation in the presence of NADH in the dark. The compounds developed in the present study have a ribose moiety, and would therefore be of help in designing physiologically active molecules such as oligonucleotides, oligosaccharides, proteins, or any other compounds that recognize and interact with specific biomolecules in specific cells in living systems and generate appropriate and controllable amounts of ROS. Further applications of these nucleoside analogs of 6FP derivatives are now under investigation.

Experimental

Materials and methods

6FP (Scheme 1) was obtained from Sankyo Kasei Kogyo (Hiratsuka, Japan); *tert*-butoxybis(dimethylamino)methane, Sigma Chemical Co. (St. Louis, MO, USA); *N*,*N*-dimethylformamide (DMF), 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose, and acetonitrile, Wako (Osaka, Japan); 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU), trimethylsilyl trifluoromethanesulfonate, and catalase, Sigma-Aldrich Co. (St. Louis, USA); NADH, Oriental Yeast (Tokyo, Japan); 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), Labotec Co. (Tokyo, Japan); $FeSO_4 \cdot 7H_2O$ ($FeSO_4$), Kantokagaku (Tokyo, Japan), diethylenetriaminepentaacetic acid (DETAPAC), Nacalai Tesque (Kyoto, Japan), 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazine-3-one (CLA), Tokyo Kasei (Tokyo, Japan), and 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline-*N*-oxide (CYPMPO), Radical Research Inc. (Tokyo, Japan). Column chromatography was performed using silica gel 60 from Nacalai Tesque (Kyoto, Japan). RDEF and RPIF, were isolated using a reversed-phase high performance liquid chromatography (RP-HPLC) system consisting of a CCPM pumping system, and a PX-8010 system controller (TOSOH; Tokyo, Japan): Column, ULTRON VX-ODS, Shinwa Chemical Industries (Kyoto, Japan); column and particle size, 20.0 × 250 mm and 5 μm, respectively; eluting with H_2O–CH_3CN (80 : 20). ^1H and ^{13}C NMR spectra were recorded on a JEOL ECA-600 NMR spectrometer (JEOL Ltd.; Tokyo, Japan) operating at 600 MHz and 150 MHz, respectively. All chemical shifts are reported in ppm downfield from tetramethylsilane. Peak multiplicities are denoted by singlet (s), broad singlet (br s), doublet (d), broad doublet (br d), triplet (t), and multiplet (m), with coupling constants (*J*) given in Hz. Numberings of BzRDF, RDEF, and RPIF were as described in Scheme 3. Mass spectra were recorded on a JEOL DX-300 at



Scheme 3 The numbering of the 6FP nucleoside analogs synthesized in this study.

30 eV for electron impact (EI) or fast atom bombardment (FAB) conditions. The reaction in the dark was performed in a vessel covered completely with foil. The EPR spectra were recorded on a Model JES-TE300 spectrometer (JEOL Ltd.; Tokyo, Japan).

Synthesis

2-(*N*,*N*-Dimethylaminomethyleneamino)-6-formylpteridin-4-one (DF). DF was synthesized from 6FP according to the procedure reported previously.[9] 6FP (570.0 mg, 3.0 mmol) and *tert*-butoxybis(dimethylamino)methane (930 µl, 4.5 mmol) were mixed in DMF (6 ml) with stirring under nitrogen for 20 min at 60 °C. During this time, the suspended solid 6FP dissolved. The mixture was evaporated *in vacuo* and the resultant orange-tan oil was purified by column chromatography, eluting with chloroform–methanol (90 : 10), to give the title compound as a yellow solid (700.5 mg, 95%). δ_H (600 MHz; $CDCl_3$–DMSO-d_6 = 4 : 1) 11.89 (1H, br s, NH), 10.17 (1H, s, CHO), 9.24 (1H, s, 7), 9.02 (1H, s, NCHN), 3.28 (3H, s, CH_3), and 3.24 (3H, s, CH_3). δ_C (150 MHz; $CDCl_3$–DMSO-d_6 = 4 : 1) 191.1, 162.0, 160.3, 160.2, 159.1, 148.2, 142.3, 130.9, 41.8, and 35.7. MS(EI) *m*/*z* M^+, 246.

1-(2,3,5-Tri-*O*-benzoyl-β-D-ribofuranosyl)-2-(*N*,*N*-dimethylaminomethyleneamino)-6-formylpteridin-4-one (BzRDF). Trimethylsilyl trifluoromethanesulfonate (1450 µl, 8.9 mmol) was added slowly with ice cooling to a solution of 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (1006.5 mg, 2.24 mmol), DF (550.2 mg, 2.24 mmol), and DBU (900 µl, 6.0 mmol) in acetonitrile (10 ml). After stirring under nitrogen at 60 °C for 14 h, the mixture was evaporated *in vacuo* and the resultant brown oil was purified by column chromatography, eluting with chloroform–methanol (98 : 2), to give the title compound as a yellow solid (939.3 mg, 68%). By heteronuclear multiple bond correlation (HMBC) NMR analysis, it was observed that H at C(r1) of the ribose ring has correlations with C(2) and C(9) of the pterin ring. δ_H (600 MHz; $CDCl_3$) 10.25 (1H, s, S1), 9.08 (1H, s, 7), 9.01 (1H, s, 1′), 7.97 (4H, b), 7.88 (2H, b), 7.56–7.53 (3H, b), 7.37–7.33 (6H, b), 7.46 (1H, d, *J* 2.4, r1), 6.43 (1H, br s, r2), 6.26 (1H, br s, r3), 4.86 (1H, br d, r5), 4.73 (1H, m, r4), 4.64 (1H, dd, *J* 12.0, 4.8, r5), 3.21 (3H, s, 2′), 3.12 (3H, br s, 2′). δ_C (150 MHz; $CDCl_3$) 191.2 (S1), 166.6 (4), 166.2 (a), 165.4 (b), 165.3 (b), 160.9 (1′), 159.7 (2), 149.9 (9), 144.2 (7), 133.6 (6), 133.4 (b), 130.9 (10), 129.7–128.4 (b), 89.6 (r1), 78.2 (r4), 73.4 (r2), 70.6 (r3), 63.0 (r5), 42.2 (2′), 36.3 (2′). MS(FAB) *m*/*z* ($M + H^+$), 691.2151.

1-(β-D-Ribofuranosyl)-2-(*N*,*N*-diethylaminomethyleneamino)-6-formylpteridin-4-one (RDEF). BzRDF (69.1 mg, 0.1 mmol) was allowed to stir in methanol (containing 25% diethylamine, 8 ml) under nitrogen at room temperature for 24 h. The mixture was evaporated *in vacuo*. Then, H_2O (10 ml) was added and neutralized by 0.6 N HCl. The product was isolated by RP-HPLC. The fraction was evaporated *in vacuo* and the title compound was obtained as a yellow solid (19.9 mg, 49%). δ_H (600 MHz; $CDCl_3$) 10.14 (1H, s, S1), 9.05 (1H, s, 7), 8.90 (1H, s, 1′), 7.11 (1H, d, *J* 5.5, r1), 5.04 (1H, t, *J* 1.5, r2), 4.71 (1H, t, *J* 6.2, r3), 4.09 (1H, m, r4), 3.87 (2H, ddd, *J* 76.6, 12.0, 2.8, r5), 3.71 (2H, ddd, *J* 6.9, 2′), 3.55 (2H, ddd, *J* 6.9, 2′), 1.37 (3H, t, *J* 6.9, 3′), 1.31 (3H, t, *J* 7.6, 3′). δ_C (150 MHz; $CDCl_3$) 190.9 (s1), 166.4 (4), 161.0 (2), 159.8 (1′), 150.1 (9), 144.1 (7), 143.8 (6), 130.5 (10), 91.6 (r1), 84.7 (r4), 71.4 (r2), 67.0 (r3), 62.4 (r5), 48.1 (2′), 42.0 (2′), 14.4 (3′), 12.4 (3′). MS(FAB) *m*/*z* ($M + H^+$) 407.1691.

1-(β-D-Ribofuranosyl)-2-(piperidine-1-ylmethyleneamino)-6-formylpteridin-4-one (RPIF). BzRDF (138.0 mg, 0.2 mmol) was allowed to stir in methanol (containing 25% piperidine, 10 ml) under nitrogen at room temperature for 24 h. The mixture was evaporated *in vacuo*. Then, H_2O (10 ml) was added and neutralized by 0.6 N HCl. The product was isolated by RP-HPLC. The fraction was evaporated *in vacuo* and the title compound was obtained as a yellow solid (20.9 mg, 25%). δ_H (600 MHz; $CDCl_3$) 10.11 (1H, s, S1), 9.05 (1H, s, 7), 8.78 (1H, s, 1′), 7.06 (1H, d, *J* 4.8, r1), 5.12 (1H, br, r2), 4.69 (1H, t, *J* 5.7, r3), 4.09 (1H, m, r4), 3.87 (2H, ddd, *J* 74.6, 12.1, 2.5, r5), 3.83 (2H, br d, 2′), 3.65 (2H, br, 6′), 1.80 (2H, br, 4′), 1.78 (2H, br, 5′), 1.75 (2H, br, 3′). δ_C (150 MHz; $CDCl_3$) 190.9 (s1), 166.3 (4), 161.0 (2), 158.9 (1′), 150.1 (9), 144.3 (7), 143.7 (6), 130.1 (10), 91.5 (r1), 84.7 (r4), 71.1 (r2), 69.9 (r3), 62.3 (r5), 53.0 (6′), 45.9 (2′), 26.5 (4′), 25.3 (3′), 23.9 (5′). MS(FAB) *m*/*z* ($M + H^+$) 419.1664.

Oxidation reaction of NADH by RDEF in the dark

PBS solutions (4 ml, pH 7.4) containing 2 mM RDEF and 2 mM NADH were prepared. These solutions were kept in the dark and stirred in an open system at room temperature. The time-dependent concentration change of each component in the sample solutions was analyzed by RP-HPLC consisting of a DP-8020 pumping system, a CO-8020 column oven, and a PX-8020 system controller (TOSOH; Tokyo, Japan): Column, ULTRON VX-ODS, Shinwa Chemical Industries; (Kyoto, Japan); column and particle size, 4.6 × 150 mm and 5 µm, respectively; eluent, 10 mM TEAA buffer (pH 7.0); gradient, CH_3CN concentration in the liner gradient mode (0 min: 0%; 30 min: 50%); flow rate, 1.0 ml min^{-1}; temperature, 37 °C. NAD^+ in the RP-HPLC peak was identified by UV absorption spectra, ^{1}H- and ^{13}C-NMR analysis, and RP-HPLC was used for its quantitative analysis.

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EPR spectra measurement in PBS solutions containing RDEF and NADH in the dark

PBS solutions (pH 7.4) containing 2 mM RDEF and 2 mM NADH were stirred for 48 h at room temperature in the dark and measured using electron paramagnetic resonance (EPR) spectroscopy combined with spin trapping with DMPO. The reaction mixtures were diluted to 1/10, and 100 mM DMPO, 500 μM DETAPAC, and 250 μM $FeSO_4$ were added before the EPR measurement. The EPR settings were as follows: microwave power, 5 mW; field, 329.4 ± 5 mT (9.2533 GHz); modulation, 0.079 mT; time constant, 0.03 s; amplitude, 500; and sweep time, 1 min. An Mn^{2+} marker was used as a reference.

PBS solutions (pH 7.4) containing 4 mM RDEF and 4 mM NADH with 20 mM CYPMPO were incubated for 1 h at room temperature in the dark in an open system and measured using EPR spectroscopy. The EPR settings were as follows: microwave power, 8 mW; field, 335.0 ± 7.5 mT (9.3949 GHz); modulation, 0.10 mT; time constant, 0.10 s; amplitude, 1000; and sweep time, 4 min. Sample solutions were placed in a flat quartz EPR aqueous cell fixed in the cavity of the EPR spectrometer. Computer EPR simulation was carried out using WIN-RAD software package (Radical Research Inc.).

Detection of $^{\bullet}O_2^-$ in PBS solutions containing RDEF and NADH by chemiluminescence method

The $^{\bullet}O_2^-$ generation activity of RDEF in the presence of NADH was examined using chemiluminescence with an $^{\bullet}O_2^-$ specific probe, CLA.[19] After mixing 2 mM RDEF and 100 μM CLA with or without 700 units ml^{-1} SOD in PBS solutions, the mixture was mounted on a luminescence reader (Aloka BLR-301) and the luminescence was monitored every 30 s for 30 min. 2 mM NADH was added to the mixture 90 s after the measurement was started.

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