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Synthesis and Evaluation of Nitro 5-Deazaflavin-pyrrolecarboxamide(s) Hybrid Molecules as Novel DNA Targeted Bioreductive Antitumor Agents

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Abstract—A series of 6-nitro-5-deazaflavins bearing at N(3) or N(10) position the pyrrolecarboxamide(s) group as DNA minor groove binder has been synthesized. These hybrid molecules show similar redox properties to those of 6-nitro-5-deazaflavins with no pyrrolecarboxamide(s) group, suggesting that they generate stable one- and two-electron reduction product(s). Electrolytic reductions of the hybrid molecules were carried out at a controlled potential under anaerobic conditions in the presence of plasmid pBR322 DNA. Significant conversions of the supercoiled circular pBR322 DNA (form I) to the open circular DNA (form II) have been found by treatment with the reductively activated 6-nitro-5-deazaflavin derivatives. Their DNA damaging effects have been found to be enhanced as the number of pyrrolecarboxamide group as the DNA binder increases. Antitumor activities of the hybrid molecules towards KB and L1210 cells were evaluated in vitro. It has been found that the antitumor effects of the compounds on KB cells slightly change and those on L1210 cells decrease as the number of the pyrrolecarboxamide group increases. These results reveal that the combination of 6-nitro-5-deazaflavin molecule with the pyrrolecarboxamide(s) group increase the DNA binding properties of the compounds, giving rise to promoted DNA damaging effects, and also suggest that the combination would affect the capacity of the compounds to act as the substrate for intracellular reductases and/or the cellular uptake of the compounds.

Introduction

Hypoxic cells[1] present in human tumors represent clinical problems in the chemotherapy of solid tumors because they limit the efficiency of fractionated radiotherapy[2] and are also resistant to many chemotherapeutic drugs.[3] Bioreductive drugs,[4] which are cytotoxic maximally in the absence of oxygen, have attracted considerable attention as selective cytotoxins towards hypoxic cells. Among bioreductive drugs, nitroheteroaromatic compounds[5] have been studied most extensively and used clinically as selective cytotoxins towards hypoxic cells[6] as well as radiosensitizers.[7] It is generally accepted that these drugs undergo enzymatic one-electron reduction[8] and the activated drugs induce DNA damages due to strand breaks[9] and helix destabilization,[10] which subsequently lead to cytotoxicities[11] as well as antimicrobial activities.[12]

Recently, we have developed nitro 5-deazaflavins[13,14] as a novel class of nitrohetero-aromatic compounds containing an electrophilic redox coenzyme ring system. It has been found that a series of nitro 5-deazaflavins shows significant antitumor activities[13] and that 6- and 8-nitro derivatives which generate stable one-electron reduction products show marked selective cytotoxicities towards hypoxic cells.[14] We have also demonstrated that there is significant interaction between a 5-deazaflavin molecule and DNA by using 5-deazaflavin derivatives linked to modified oligonucleotides.[15] From

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these points of view, introduction of DNA binding group into nitro 5-deazaflavin molecule would be anticipated to provide higher concentration of the reductively activated product around DNA as well as to promote the interaction of 5-deazaflavin ring system with DNA, which could give rise to higher antitumor activities. It should be intriguing to investigate DNA interaction and antitumor activities of nitro 5-deazaflavin derivatives bearing a DNA binding group. Oligo(pyrrolecarboxamides) netropsin, distamycin, and their analogues have received considerable attention not solely because of their antitumor and antibiotic activities but also their non-intercalative and non-covalent binding to minor groove of AT rich regions in double stranded B-DNA.[16] And recently, the oligo(pyrrolecarboxamides) derivatives and their analogues have been introduced most popularly as DNA binding group into DNA alkylating reagents,[17] DNA topoisomerase inhibitors,[18] photosensitizers,[19] and radiosensitizers[20] for enhancement of their DNA damaging effects and antitumor activities.[21]

As an extension of bioreductive drug approach, we have designed a series of 6-nitro-5-deazaflavins **2–4** and **6–8** bearing a pyrrolecarboxamide(s) at N(3) position or N(10) position as novel DNA targeted bioreductive antitumor agents (Scheme 1). Since among nitro-positional isomers of nitro 5-deazaflavin derivatives, 6-nitro-5-deazaflavin derivatives show the most potent antitumor activities as well as the highest selective cytotoxicities towards hypoxic cells,[13,14] the DNA binding group was introduced into the 6-nitro derivatives. Considering the cellular uptakes of the compounds,[17c,18b] alkyl (butyl) group was employed as C-terminal group of the pyrrolecarboxamide(s). In the present paper, we wish to describe synthesis, redox properties, DNA interactions, and antitumor activities of the 6-nitro-5-deazaflavin-pyrrolecarboxamide(s) hybrid molecules.

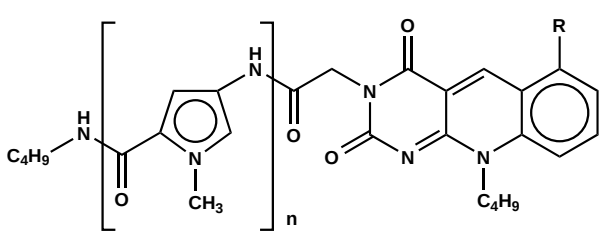
Results and Discussion

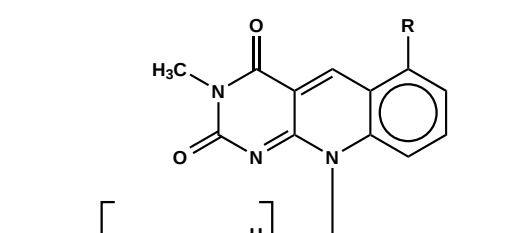
Synthesis of nitro 5-deazaflavin-pyrrolecarboxamide(s) hybrid molecules

10-Butyl-6-nitro-5-deazaflavin **11** was prepared by condensation of 6-butylaminouracil with 2-fluoro-6-nitrobenzaldehyde[22] according to Yoneda's method[13,23] (74%). Treatment of **11** with *tert*-butyl bromoacetate in DMF in the presence of potassium carbonate afforded **12** (88%). Hydrolysis of **12** with hydrochloric acid gave **13** (85%) which was treated with *N*,*N*′-disuccinimidyl carbonate in the presence of pyridine in acetonitrile to afford **14** (78%). Condensations of **14** with the appropriate amines **18b–20b**, which were obtained by hydrogenation of **18a–20a**[24] (Scheme 3), in the presence of DMAP in acetonitrile afforded **2–4** (51–61%) (Scheme 2).

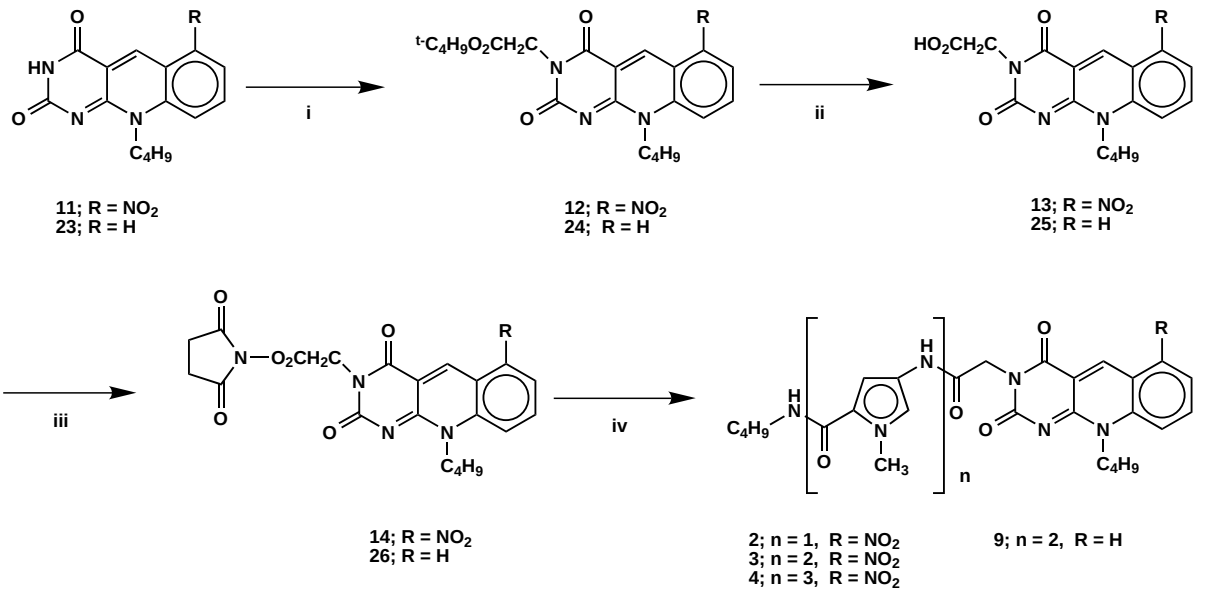
Also 10-(*tert*-butoxycarbonyl)methyl-3-methyl-6-nitro-5-deazaflavin **16** was prepared by condensation of 6-(*tert*-butoxycarbonyl)methylamino-3-methyluracil **15** with 2-fluoro-6-nitrobenzaldehyde[22] (70%). Hydrolysis of **16** with hydrochloric acid gave **17** (88%) and condensations of **17** with the amines **18b–20b**[24] (Scheme 3) in the presence of 1,3-diisopropylcarbodiimide in DMF afforded **6–8** (26–28%) (Scheme 4).

Compounds **1** and **5** (Scheme 1) with no 1-methylpyrrole group were also prepared to compare their antitumor activities with those of **2–4** and **6–8**, by alkylation of **11** with *N*-butylbromoacetoamide in the presence of potassium carbonate in DMF and by condensation of 6-(butylcarbamoyl)methylamino-3-methyluracil **22** and 2-fluoro-6-nitrobenzaldehyde[22] respectively. As reference compounds, compounds **9** (Scheme 2) and **10** (Scheme 4) with no nitro group on the 5-deazaflavin ring and bis(-pyrrolecarboxamide) derivative **21** (Scheme 3) with no (nitro) 5-deazaflavin nucleus were synthesized. These compounds are suitable for comparison with the nitro

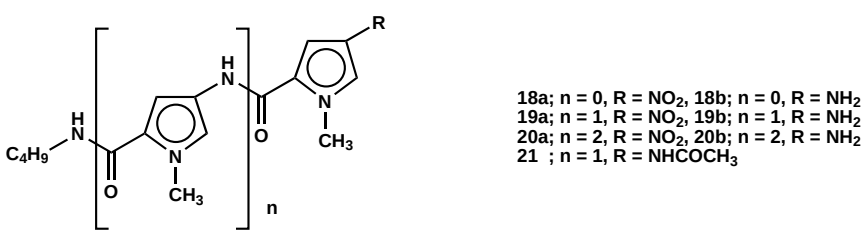




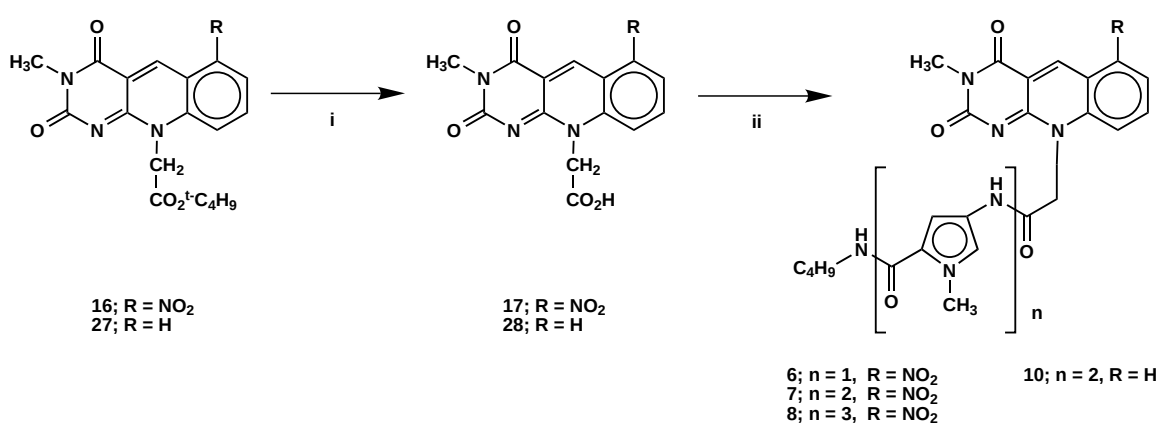
Scheme 1.



Scheme 2. Synthesis of compounds **2–4**, and **9**: i) *tert*-butyl bromoacetate, K_2CO_3, DMF, 60 °C; ii) 12N HCl, 60 °C; iii) *N*,*N*′-Disuccinimidyl carbonate, Pyridine, CH_3CN, rt; iv) **18b–20b**, DMAP, CH_3CN, rt.



Scheme 3.



Scheme 4. Synthesis of compounds **6–8** and **10**: 1) 12N HCl, 60 °C; ii) **18b–20b**, 1,3-Diisopropylcarbodiimide, CH_3CN or DMF, rt.

5-deazaflavin containing hybrid molecules **3** and **7** in terms of antitumor activities.

Redox properties of nitro 5-deazaflavin-pyrrolecarboxamide(s) hybrid molecules

It is well discussed that electron affinity[11,12] and stability of one-electron reduction product[25] of a bioreductive drug affect significantly the antitumor activity as well as the selective cytotoxicity towards hypoxic cells. To investigate the redox properties of the nitro 5-deazaflavin-pyrrolecarboxamide(s) hybrid molecules, the reduction potentials of compounds **1–8** were measured by means of cyclic voltammetry.[14] The cyclic voltammograms of compounds **4** and **8** are exemplified in Figure 1. As Table 1 shows, the reduction potentials of the hybrid molecules **2–4** and **6–8** are similar to those of 6-nitro-5-deazaflavin derivatives **1** and **5** with no pyrrolecarboxamide(s) group. Furthermore, the hybrid molecules have been found to show similar patterns of cyclic voltammograms[14] to those of other 6-nitro-5-deazaflavin derivatives with no pyrrolecarboxamide(s) group, indicating that these hybrid molecules generate stable one- and two-electron reduction product(s). From these results, the hybrid molecules could be anticipated to provide higher concentrations of the stable one-electron reduction products around DNA, which would induce greater DNA damages as well as higher antitumor activities.

Table 1. Reduction potentials (*E*p (V)) of 6-nitro-5-deazaflavin-pyrrolecarboxamide(s) hybrid molecules*

	*E*p (V)	
1	−0.548	−0.880
2	−0.534	−0.870
3	−0.550	−0.880
4	−0.545	−0.870
5	−0.520	−0.834
6	−0.504	−0.822
7	−0.530	−0.855
8	−0.530	−0.840

*All the potentials were measured at 298 K in DMF, [Compound] = 1.0×10^{-3} (M), [Bu_4NClO_4] = 1.0×10^{-1} (M) versus an aqueous Ag/AgCl reference electrode under N_2.

Interaction of nitro 5-deazaflavin-pyrrolecarboxamide(s) hybrid molecules with DNA

It is understood that when a flavin (isoalloxazine) ring system interacts with DNA, hypochromicity and red-shift of visible absorption band characteristic of the flavin chromophore are observed.[26] We also demonstrated that similar spectral changes are found through the interaction of 5-deazaflavin ring system with DNA.[27] To investigate the interactions of (nitro) 5-deazaflavin derivatives **1–10** with DNA, visible spectra of the compounds **1–10** were measured in the presence of calf thymus DNA, poly (dA-dT), and poly (dG-dC), accordingly. Essentially no significant spectral change was found in compounds **1** and **5** with no DNA binding group, however, as the number of the pyrrolecarboxamide group increased, significant spectral changes of compounds **3**, **4**, **7**, **8**, **9**, and **10** were observed. The visible spectra of compounds **4** and **8** in the presence of calf thymus DNA are exemplified in Figure 2. As Figure 2 shows, hypochromicity and red-shift in visible absorption band characteristic of the 5-deazaflavin chromophore have been observed and these spectra have isosbestic points[26] at 464 nm and 460 nm respectively, suggesting that 5-deazaflavin ring system of these

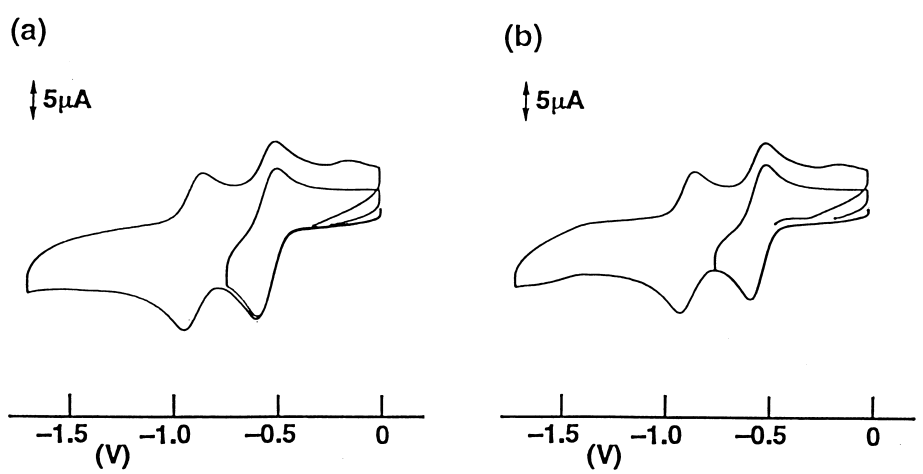


Figure 1. Cyclic Voltammograms of compounds **4** (a) and **8** (b) at 298 K in DMF, [Compound] = 1.0×10^{-3} (M), [Bu_4NClO_4] = 1.0×10^{-1} (M) versus an aqueous Ag/AgCl reference electrode under N_2.

compounds interacts with DNA. Thus, the binding constants of the compounds **3**, **4**, **7**, **8**, **9**, and **10** to calf thymus DNA, poly (dA-dT), and poly (dG-dC) were estimated from the hypochromicity of visible spectra. As Table 2 shows, compounds **3**, **4**, and **9** bearing the pyrrolecarboxamide(s) at N(3) position bind more strongly to poly (dA-dT) than to poly (dG-dC) and higher base pair selectivity has been observed in tris(pyrrolecarboxamides) derivative **4** than in bis(pyrrolecarboxamides) derivatives **3** and **9**. In contrast to this result, compounds **7**, **8**, and **10** possessing the pyrrolecarboxamide(s) at N(10) position bind to poly (dA-dT) as weakly as to poly (dG-dC), showing essentially no base pair selectivity. These results imply that spatial arrangement of (nitro) 5-deazaflavin nucleus and the DNA binding group affects significantly the interaction of 5-deazaflavin ring system with DNA. Compounds **9** and **10** containing a 5-deazaflavin with no nitro group gave similar spectral changes and binding constants to those of compounds **3** and **7**, suggesting that 5-deazaflavin ring system of these compounds interacts similarly with DNA regardless of the nitro group.

Table 2. Estimated apparent binding constants ($K_{app}\times10^5\,M^{-1}$) of compounds **3**, **4**, **7**, **8**, **9**, and **10**

	$K_{app}\times10^5\,M^{-1}$		
	Calf thymus	poly (dA-dT)	poly (dG-dC)
3	0.52	2.1	0.39
4	0.71	3.7	0.52
7	0.62	0.66	0.68
8	0.68	0.72	0.65
9	0.79	2.3	0.74
10	0.70	0.50	0.57

Interaction of reductively activated nitro 5-deazaflavin-pyrrolecarboxamide(s) hybrid molecules with DNA

It has been well studied that the one-electron reduction products[25] of bioreductive drugs induce DNA damages by causing strand breaks[9] and helix destabilization,[10] which subsequently give rise to cytotoxicities. To investigate mechanism of biological actions of the bioreductive drugs, interactions of reduced nitroimidazoles[9,10,28] or 1,2,4-benzotriazine-1,4-di *N*-oxide derivative[29] with DNA under anaerobic conditions have been studied. It is conceivable that nitro 5-deazaflavin derivatives also undergo enzymatic one-electron reduction to give rise to antitumor activities, however, interaction of reductively activated nitro 5-deazaflavins with DNA has remained to be studied.

In order to investigate the interaction of reductively activated nitro 5-deazaflavin derivatives with DNA, compounds **1–8** were reduced electrolytically at a controlled potential[9,10,28] in the presence of plasmid pBR322 DNA under anaerobic conditions. The hybrid molecules **9** and **10** with no nitro group on 5-deazaflavin ring were employed as reference compounds. As Figure 3 shows, marked conversions of supercoiled circular DNA (form I) into the open circular DNA (form II) have been found by electrolytical reductions of nitro 5-deazaflavin derivatives **3**, **4**, **7**, and **8**[30,31]. Essentially no DNA

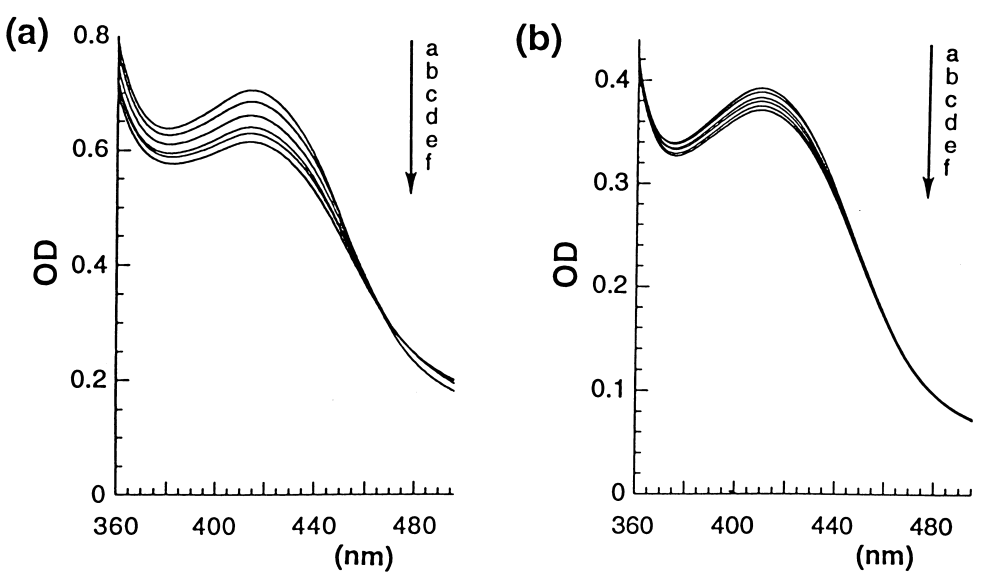


Figure 2. Visible spectra of (a) **4**, and (b) **8** in the presence of calf thymus DNA at 298 K, in 10 mM Tris–HCl buffer (pH 7.0) containing 20% of DMF, [Compound] = 2.1×10^{-5} (M), [calf thymus DNA] = (a) 0, (b) 1.6×10^{-5}, (c) 3.2×10^{-5}, (d) 4.8×10^{-5}, (e) 6.4×10^{-5}, (f) 8.0×10^{-5} (M).

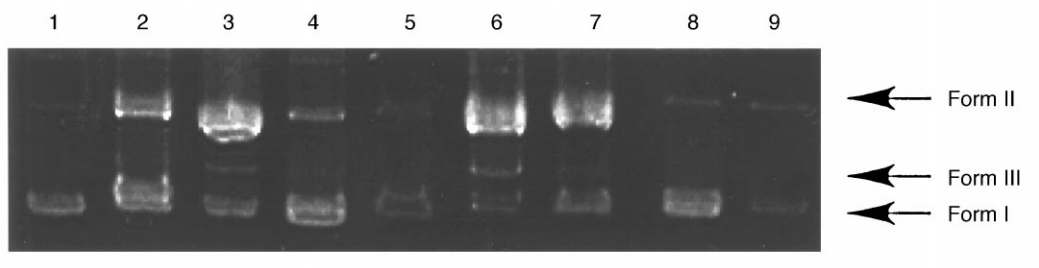


Figure 3. Agarose gel electrophoresis patterns of plasmid pBR322 DNA after treatment of reductivity avtivated nitro 5-deazaflavin derivatives **1–8**. Lane 1–9: Control, **8**, **7**, **6**, **5**, **4**, **3**, **2**, **1**. (The reductive activations of the compounds **1–8** were carried out at a constant potential of −0.95 V versus an aqueous Ag/AgCl under N_2 in 1.5 mM sodium citrate buffer (pH 7.0) containing 25% of DMF). [Compound] = 1.0×10^{-3} (M), [DNA] = 5.0×10^{-5} (M/base pair).

damage was observed in the electrolysis in the absence of the nitro 5-deazaflavin derivatives or even in the presence of the hybrid molecules **9** and **10**.[32] These experimental results strongly support that activation of 6-nitro-5-deazaflavin derivatives by electrolytic reduction leads to DNA damage, suggesting that enzymatic one-electron reduction of 6-nitro-5-deazaflavin derivatives could induce DNA damage. The conversion of form I DNA into form II appears to be enhanced as the number of pyrrolecarboxamide group increases. The above results suggest that the higher concentrations of reductively activated compounds would be provided around DNA owing to the promoted DNA binding properties of the compounds.

In vitro antitumor activities of nitro 5-deazaflavin-pyrrolecarboxamide(s) hybrid molecules

To evaluate antitumor activities of the hybrid molecules and other reference compounds, compounds **1–10** and **21** were tested for in vitro antitumor effects on human oral epidermoid carcinoma KB cells and murine leukemia L1210 cells by the MTT assay developed by Carmichael.[33,34] Mitomycin C and netropsin were also employed as reference compounds. As Table 3 shows, the 6-nitro-5-deazaflavin-pyrrolecarboxamide(s) hybrid molecules **2–4** and **6–8** have been found to show more potent antitumor activities than netropsin and the bis(pyrrolecarboxamides) **21**. The hybrid molecules **9** and **10** composed of 5-deazaflavin with no nitro group and bis(pyrrolecarboxamides) showed no significant antitumor activities. These results suggest that nitro 5-deazaflavin nucleus is more relevant to the antitumor effects of the compounds than the pyrrolecarboxamide(s) group. It has been found that change in the number of the pyrrolecarboxamide of compounds **1–8** affects slightly the antitumor potencies of the hybrid molecules towards KB cells. In contrast, the antitumor effects of compounds **1–8** on L1210 cells decreased as the number of the pyrrolecarboxamide increased.

As compounds **1**, **5**, and other compounds reported in our previous paper[13] show, nitro 5-deazaflavin derivatives with no pyrrolecarboxamide(s) group generally show more potent antitumor activities towards L1210 cells than KB cells (IC_{50}(L1210) < IC_{50}(KB)). Interestingly enough, it has been found that there is a tendency that the hybrid molecules **1–8** maintain similar antitumor activities towards KB cells and show less antitumor effects on L1210 cells as the number of the pyrrolecarboxamide group increases. Considering the results of interaction of reductively activated hybrid molecules with DNA (vide supra), compounds **3**, **4**, **7**, and **8** bearing the increasing number of pyrrolecarboxamide group would be anticipated to show more potent antitumor activities than compounds **1**, **2**, **5**, and **6** bearing only one or no pyrrolecarboxamide group, if the same concentrations of reductively activated compounds are gained around DNA in tumor cells. However, the

Table 3. IC_{50} Values of 6-nitro-5-deazaflavin-pyrrolecarboxamide(s) hybrid molecules and other reference compounds on KB and L1210 cells growth in vitro

	IC_{50} (μM)*	
	KB cells	L1210 cells
1	6.1	1.8
2	10.7	10.3
3	8.4	0.5
4	6.6	45.4
5	6.5	0.4
6	4.3	9.3
7	23.5	73.1
8	8.1	69.2
9	> 100	> 100
10	> 100	> 100
21	> 100	> 100
Mitomycin C	0.4	0.6
Netropsin	> 100	> 100

*IC_{50} (μM) was given as the concentration at 50% inhibition of cell growth.

antitumor activities of the compounds did not heighten despite the increase in the number of the pyrrolecarboxamide group. These results imply that introduction of pyrrolecarboxamide(s) group into 6-nitro-5-deazaflavin molecule would enhance the direct interaction of nitro 5-deazaflavin nucleus with DNA, however, would affect rather adversely cellular uptakes of the compounds and/or capacities of the compounds to act as substrates for intracellular reductases.

Conclusion

We first synthesized 6-nitro-5-deazaflavin-pyrrolecarboxamide(s) hybrid molecules as novel DNA targeted bioreductive antitumor agents and evaluated their redox properties, interactions with DNA, and in vitro antitumor activities. And we first demonstrated the interaction of reductively activated 6-nitro-5-deazaflavin derivatives with DNA. Introduction of DNA binding pyrrolecarboxamide(s) group into 6-nitro-5-deazaflavin molecule enhances DNA binding properties of the compounds, giving rise to marked DNA damage. Although antitumor activities of the present hybrid molecules did not heighten, we consider that further modification of the compounds would promote cellular uptakes as well as reductive activations of the compounds to afford the more potent antitumor agents which would be useful for chemotherapy of solid tumors.

Experimental

Melting points were taken using a Mettler thermosystem FP80HT and are uncorrected. Infrared (IR) spectra were recorded on a Shimadzu IR-400 spectrophotometer. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a JEOL FX200 (200 MHz) spectrometer in $CDCl_3$ or Me_2SO-d_6 with tetramethylsilane (TMS) as an internal standard and chemical shifts are given in ppm. Low-resolution FAB (fast atom bombardment) mass spectra (MS (FAB)) and high-resolution FAB mass spectra (HRMS (FAB)) were recorded on JEOL JMS-HX/HX110A. Column chromatography was carried out on silica–gel (Kieselgel 60, 70–230 mesh, Merck).

10-Butyl-6-nitro-5-deazaflavin (11). A mixture of 6-butylaminouracil[23] (1.83 g, 10.0 mmol) and 2-fluoro-6-nitrobenzaldehyde[22] (1.69 g, 10.0 mmol) in 25 mL of DMF was heated at 120 °C for 2 h under argon atmosphere. The reaction mixture was poured onto 500 mL of ice-water and the precipitate was collected by filtration. The precipitate was dried under reduced pressure at room temperature and recrystallized from chloroform–ethanol to afford 2.33 g (74%) of **11** as yellow powder: mp 253–256 °C. IR (KBr) 1709, 1676 1618, 1524 cm^{-1}. ^{1}H NMR (Me_2SO-d_6) δ 0.99 (3H, t, $J = 7.4$ Hz), 1.38–1.62 (2H, m), 1.62–1.83 (2H, m), 4.71 (2H, m), 8.09 (1H, t, $J = 7.8$ Hz), 8.20 (1H, d, $J = 7.8$ Hz), 8.33 (1H, d, $J = 8.8$ Hz), 9.11 (1H, s), 11.33 (1H, s). Anal. calcd for $C_{15}H_{14}N_4O_4$: C, 57.32; H, 4.49; N, 17.83. Found: C, 57.22; H, 4.47; N, 17.73.

3-(*tert*-Butoxycarbonyl)methyl-10-butyl-6-nitro-5-deazaflavin (12). A mixture of 10-butyl-6-nitro-5-deazaflavin (11; 1.57 g 5.0 mmol), *tert*-butyl bromoacetate (1.95 g, 10.0 mmol), and well pulverized K_2CO_3 (2.07 g, 15.0 mmol) in 15 mL of DMF was warmed at 60 °C for 1 h under argon atmosphere. The solvent was evaporated under reduced pressure and the residue was extracted with chloroform. The organic layer was washed with brine once and was dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the resulting yellow solid was recrystallized from ethyl acetate to afford 1.89 g (88%) of **12** as yellow crystals: mp 208–210 °C. IR (KBr) 1736, 1660, 1624, 1531 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.03 (3H, t, $J = 7.2$ Hz), 1.49 (9H, s), 1.53–1.71 (2H, m), 1.71–1.93 (2H, m), 4.74 (2H, s), 4.80 (2H, br), 7.89–8.03 (2H, m), 8.07 (1H, dd, $J = 6.8$, 2.4 Hz), 9,41 (1H, s). Anal. calcd for $C_{21}H_{24}N_4O_6$: C, 58.87; H, 5.65; N, 13.08. Found: C, 58.84; H, 5.74; N, 12.92.

10-Butyl-6-nitro-5-deazaflavin-3-acetic acid (13). A solution of 3-(*tert*-butoxycarbonyl)methyl-10-butyl-6-nitro-5-deazaflavin (**12**; 0.64 g, 1.5 mmol) in 5 mL of 12 N hydrochloric acid was warmed at 60 °C with vigorous stirring for 1 h under argon atmosphere. The solvent was evaporated to dryness under reduced pressure and the residue was recrystallized from ethanol–water to afford 0.49 g (85%) of **13** (obtained as **13**·0.5 H_2O) as yellow powder: mp 123–125 °C. IR (KBr) 1743, 1711, 1655, 1620, 1531 cm^{-1}. ^{1}H NMR (Me_2SO-d_6) δ 0.98 (3H, t, $J = 7.4$ Hz), 1.38–1.62 (2H, m), 1.62–1.84 (2H, m), 4.57 (2H, s), 4.62–4.88 (2H, m), 8.14 (1H, t, $J = 8.4$ Hz), 8.25 (1H, d, $J = 7.4$ Hz), 8.40 (1H, d, $J = 8.8$ Hz), 9.22 (1H, s). Anal. calcd for $C_{17}H_{16}N_4O_6$·0.5H_2O: C, 53.54; H, 4.49; N, 14.69. Found: C, 53.70; H, 4.64; N, 14.40.

10-Butyl-6-nitro-5-deazaflavin-3-acetic acid *N*-hydroxysuccinimide ester (14). To a suspension of 10-butyl-6-nitro-5-deazaflavin-3-acetic acid (**13**; 372 mg, 1.00 mmol) in 3 mL of acetonitrile was added pyridine (0.16 mL, 2.00 mmol) and *N,N′*-disuccinimidyl carbonate (512 mg, 2.00 mmol) and the reaction mixture was stirred at room temperature under argon atmosphere for 16 h. As the reaction proceeded, the suspension turned into yellow solution. The solvent was evaporated under reduced pressure and the residue was extracted with chloroform. The organic layer was washed with

brine once and was dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the residue was subjected to column chromatography on silica gel (ethyl acetate as eluent). The yellow solid obtained was recrystallized from ethyl acetate–ether to afford 373 mg (78%) of **14** (obtained as **14**·0.5 H_2O) as yellow powder: mp 138–141 °C. IR (KBr) 1743, 1664, 1624, 1531 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.04 (3H, t, $J = 7.2$ Hz), 1.41–1.73 (2H, m), 1.73–1.97 (2H, m), 2.84 (4H, s), 4.81 (2H, br), 5.16 (2H, s), 7.91–8.06 (2H, m), 8.09 (1H, dd, $J = 6.8$, 2.4 Hz), 9.46 (1H, s). Anal. calcd for $C_{21}H_{19}N_5O_8$·0.5H_2O: C, 52.72; H, 4.21; N, 14.64. Found: C, 52.98; H, 4.16; N, 14.43.

6-(*tert*-Butoxycarbonyl)methylamino-3-methyluracil (15). A mixture of 6-chloro-3-methyluracil (3.21 g, 20.0 mmol), glycine *tert*-butyl ester hydrochloride (3.35 g, 20.0 mmol), DBU (3.0 mL, 20.0 mmol), and *N*,*N*-diisopropylethylamine (6.97 mL, 40.0 mmol) in 50 mL of 1-butanol was heated to reflux under argon atmosphere for 3 h. The reaction mixture was cooled at room temperature and deposited crystals were collected by filtration. The crystals were washed with ethanol and were dried under reduced pressure at room temperature to afford 3.62 g (71%) of **15** as white crystals. mp 242–246 °C dec. IR (KBr) 1741, 1709, 1603 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 1.44 (9H, s), 3.04 (3H, s), 3.82 (2H, d, $J = 5.8$ Hz), 4.52 (1H, s), 6.28 (1H, t, $J = 5.8$ Hz), 10.51 (1H, br). Anal. calcd for $C_{11}H_{17}N_3O_4$: C, 51.76; H, 6.71; N, 16.46. Found: C, 51.54; H, 6.74; N, 16.41.

6-(Butylcarbamoyl)methylamino-3-methyluracil (22). A mixture of 6-chloro-3-methyluracil (3.21 g, 20.0 mmol), *N*-butyl-2-aminoacetamide hydrochloride (3.33 g, 20.0 mmol), DBU (3.0 mL, 20.0 mmol), and *N*,*N*-diisopropylethylamine (6.97 mL, 40.0 mmol) in 50 mL of 1-butanol was heated to reflux under argon atmosphere for 3 h. The reaction mixture was cooled at room temperature and deposited crystals were collected by filtration. The crystals were washed with ethanol and were dried under reduced pressure at room temperature to afford 3.76 g (74%) of **22** as white crystals: mp 275–279 °C dec. IR (KBr) 1726, 1618, 1568 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 0.88 (3H, t, $J = 6.6$ Hz), 1.16–1.52 (4H, m), 3.03 (3H, s), 3.10 (2H, d, $J = 6.2$ Hz), 3.65 (2H, d, $J = 3.6$ Hz), 4.42 (1H, s), 6.37 (1H, br), 8.05 (1H, br), 10.41 (1H, br). Anal. calcd for $C_{11}H_{18}N_4O_3$: C, 51.96; H, 7.13; N, 22.03. Found: C, 52.04; H, 6.91; N, 22.27.

10-(*tert*-Butoxycarbonyl)methyl-3-methyl-6-nitro-5-deazaflavin (16). A mixture of 6-(*tert*-butoxycarbonyl)methylamino-3-methyluracil (**15**; 2.55 g, 10.0 mmol) and 2-fluoro-6-nitrobenzaldehyde[22] (1.69 g, 10.0 mmol) in 25 mL of DMF was heated at 120 °C for 2 h under argon atmosphere. The reaction mixture was poured onto 500 mL of ice-water and precipitate appeared was collected by filtration. The precipitate was dried under reduced pressure at room temperature and was recrystallized from chloroform–ethanol to afford 2.70 g (70%) of **16** as yellow crystals: mp 209–212 °C. IR (KBr) 1732, 1659, 1622, 1537 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.49 (9H, s), 3.48 (3H, s), 5.57 (2H, br), 7.62 (1H, d, $J = 8.6$ Hz), 7.93 (1H, t, $J = 8.6$ Hz), 8.07 (1H, d, $J = 7.8$ Hz), 9.44 (1H, s). Anal. calcd for $C_{18}H_{18}N_4O_6$: C, 55.96; H, 4.70; N, 14.50. Found: C, 56.02; H, 4.66; N, 14.38.

3-Methyl-6-nitro-5-deazaflavin-10-acetic acid (17). A suspension of 10-(*tert*-butoxycarbonyl)methyl-3-methyl-6-nitro-5-deazaflavin (**16**; 1.35 g, 3.5 mmol) in 5.6 mL of 12N HCl (70.0 mmol) was warmed at 60 °C with vigorous stirring for 1 h under argon atmosphere. The precipitate was collected by filtration and was washed with ethanol. The precipitate was dried under reduced pressure at room temperature to give 1.02 g (88%) of **17** as yellow powder: mp 226–228 °C. IR (KBr) 1718, 1622, 1597, 1529 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 3.27 (3H, s), 5.58 (2H, s), 8.09 (1H, t, $J = 8.2$ Hz), 8.19–8.33 (2H, m), 9.24 (1H, s). Anal. calcd for $C_{14}H_{10}N_4O_6$: C, 50.92; H, 3.05; N, 16.95. Found: C, 50.94; H, 3.01; N, 16.80.

10-Butyl-5-deazaflavin (23). A mixture of 6-butylaminouracil[23] (1.83 g, 10.0 mmol) and 2-fluorobenzaldehyde (1.24 g, 10.0 mmol) in 25 mL of DMF was heated at 120 °C for 3 h under argon atmosphere. The reaction mixture was cooled and deposited crystals were collected by filtration. Recrystallization from DMF afforded 2.36 g (88%) of **23** as pale yellow crystals: mp 274–276 °C. IR (KBr) 1695, 1666, 1612 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 0.98 (3H, t, $J = 7.2$ Hz), 1.37–1.60 (2H, m), 1.60–1.84 (2H, m), 4.53–4.88 (2H, br), 7.48–7.60 (1H, m), 7.88–8.03 (2H, m), 8.19 (1H, d, $J = 7.6$ Hz), 9.00 (1H, s), 11.10 (1H, br). Anal. calcd for $C_{15}H_{15}N_3O_2$: C, 66.90; H, 5.61; N, 15.60. Found: C, 66.83; H, 5.66; N, 15.55.

3-(*tert*-Butoxycarbonyl)methyl-10-butyl-5-deazaflavin (24). A mixture of 10-butyl-5-deazaflavin (**23**; 2.69 g, 10.0 mmol), *tert*-butyl bromoacetate (3.90 g, 20.0 mmol), and well pulverized K_2CO_3 (4.15 g, 30.0 mmol) in 30 mL of DMF was warmed at 60 °C for 1 h under argon atmosphere. The K_2CO_3 was removed by filtration and the solvent was evaporated under reduced pressure. The residue was extracted with chloroform and the organic layer was washed with brine once. The organic layer was dried over $MgSO_4$ and the solvent was evaporated under reduced pressure to give yellow solid. The solid was recrystallized from chloroform–ethanol to afford 2.99 g (78%) of **24** as yellow crystals: mp 204–206 °C. IR (KBr) 1741, 1703, 1651, 1620 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 1.02 (3H, t, $J = 7.2$ Hz), 1.37–1.67 (2H, m), 1.49 (9H, s), 1.73–1.94 (2H, m), 4.74 (4H, m), 7.49 (1H, t, $J = 7.8$ Hz), 7.67 (1H, d, $J = 8.8$ Hz), 7.84–7.96 (2H,

m), 8.89 (1H, s). Anal. calcd for $C_{21}H_{25}N_3O_4$: C, 65.78; H, 6.57; N, 10.96. Found: C, 65.75; H, 6.39; N, 11.10.

10-Butyl-5-deazaflavin-3-acetic acid (25). A suspension of 3-(*tert*-butoxycarbonyl)methyl-10-butyl-5-deazaflavin (**24**; 1.92 g, 5.0 mmol) in 8.2 mL of 12N HCl (100.0 mmol) was warmed with vigorous stirring at 60 °C for 1 h under argon atmosphere. The reaction solution was cooled at room temperature and the deposited crystals were collected by filtration. The crystals were washed with purified water and were dried under reduced pressure at room temperature to afford 1.48 g (86%) of **25** (obtained as **25**·1.0 H_2O) as yellow crystals: mp 133–136 °C. IR (KBr) 1705, 1641, 1618 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 0.98 (3H, t, $J = 7.2$ Hz), 1.39–1.63 (2H, m), 1.63–1.84 (2H, m), 4.56 (2H, s), 4.62–4.86 (2H, m), 7.52–7.62 (1H, m), 7.93–8.06 (2H, m), 8.24 (1H, d, $J = 7.8$ Hz), 9.09 (1H, s). Anal. calcd for $C_{17}H_{17}N_3O_4 \cdot 1.0H_2O$: C, 59.12; H, 5.55; N, 12.17. Found: C, 59.31; H, 5.34; N, 12.25.

10-Butyl-5-deazaflavin-3-acetic acid *N*-hydroxysuccinimide ester (26). To a suspension of 10-butyl-5-deazaflavin-3-acetic acid (**25**; 655 mg, 2.00 mmol) in 3 mL of acetonitrile was added pyridine (0.49 mL, 6.00 mmol) and *N,N′*-disuccinimidyl carbonate (1025 mg, 4.00 mmol) and the mixture was stirred at room temperature under argon atmosphere for 3 h. As the reaction proceeded, the suspension turned into a yellow solution. The solvent was evaporated under reduced pressure and the residue was extracted with chloroform. The organic layer was washed with brine once and was dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the residue was recrystallized from ethyl acetate to afford 679 mg (80%) of **26** as yellow crystals: mp 218–221 °C. IR (KBr) 1743, 1709, 1654, 1614, 1529 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.03 (3H, t, $J = 7.4$ Hz), 1.45–1.73 (2H, m), 1.73–1.95 (2H, m), 2.84 (4H, s), 4.79 (2H, br), 5.19 (2H, s), 7.52 (1H, t, $J = 7.8$ Hz), 7.70 (1H, d, $J = 9.2$ Hz), 7.86–7.99 (2H, m), 8.95 (1H, s). Anal. calcd for $C_{21}H_{20}N_4O_6$: C, 59.43; H, 4.75; N, 13.20. Found: C, 59.24; H, 4.95; N, 12.97.

10-(*tert*-Butoxycarbonyl)methyl-3-methyl-5-deazaflavin (27)

A mixture of 6-(*tert*-butoxycarbonyl)methylamino-3-methyluracil (**15**; 2.55 g, 10.0 mmol) and 2-fluorobenzaldehyde (1.37 g, 11.0 mmol) in 25 mL of DMF was heated at 120 °C for 3 h under argon atmosphere. The reaction mixture was cooled at room temperature and deposited crystals were collected by filtration. The crystals were washed with ethanol and were dried under reduced pressure at room temperature to afford 2.97 g (87%) of **27** as yellow crystals: mp 235–237 °C. IR (KBr) 1741, 1701, 1653, 1620, 1535 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 1.45 (9H, s), 3.27 (3H, s), 5.52 (2H, s), 7.57 (1H, t, $J = 7.6$ Hz), 7.81 (1H, d, $J = 8.8$ Hz), 7.96 (1H, t, $J = 7.6$ Hz), 8.26 (1H, d, $J = 7.6$ Hz), 9.14 (1H, s). Anal. calcd for $C_{18}H_{19}N_3O_4$: C, 63.33; H, 5.61; N, 12.31. Found: C, 63.18; H, 5.47; N, 12.47.

3-Methyl-5-deazaflavin-10-acetic acid (28). To a suspension of 10-(*tert*-butoxycarbonyl)methyl-3-methyl-5-deazaflavin (**27**; 2.73 g, 8.0 mmol) in 20 mL of ethanol was added 6.58 mL of 12N HCl (80.0 mmol) dropwise and the mixture was warmed at 50 °C with vigorous stirring for 1 h under argon atmosphere. The reaction mixture was cooled at room temperature and the precipitate appeared was collected by filtration. The precipitate was washed with purified water and ethanol and was dried under reduced pressure at room temperature to give 2.15 g (95%) of **28** as pale yellow powder: mp 225–228 °C. IR (KBr) 1745, 1687, 1621, 1535 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 3.26 (3H, s), 5.52 (2H, s), 7.56 (1H, t, $J = 7.8$ Hz), 7.80–8.01 (2H, m), 8.25 (1H, d, $J = 7.8$ Hz), 9.13 (1H, s). Anal. calcd for $C_{14}H_{11}N_3O_4$: C, 58.95; H, 3.89; N, 14.73. Found: C, 58.75; H, 3.96; N, 14.66.

***N*-Butyl-1-methyl-4-nitropyrrole-2-carboxamide (18a).** To a solution of 1-methyl-4-nitro-2-trichloroacetylpyrrole[24] (10.0 g, 36.8 mmol) in 50 mL of THF, was added *n*-butylamine (2.96 g, 40.5 mmol) dropwise at 0 °C. The solution was stirred at room temperature for 1 h under argon atmosphere. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography on silica–gel (ethyl acetate as eluent) to afford 8.1 g (98%) of **18a** as pale yellow needles: mp 100–103 °C. IR (KBr) 1639, 1543, 1309 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.92 (3H, t, $J = 7.2$ Hz), 1.29–1.48 (2H, m), 1.48–1.68 (2H, m), 3.39 (2H, dd, $J = 12.9$, 6.9 Hz), 3.99 (3H, s), 6.22 (1H, br), 7.10 (1H, d, $J = 1.8$ Hz), 7.57 (1H, d, $J = 1.8$ Hz). Anal. calcd for $C_{10}H_{15}N_3O_3$: C, 53.32; H, 6.71; N, 18.66. Found: C, 53.38; H, 6.63; N, 18.71.

***N*-Butyl-1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamide (19a).** A solution of *N*-butyl-1-methyl-4-nitropyrrole-2-carboxamide (**18a**; 1.0 g, 4.4 mmol) in 10 mL of methanol was hydrogenated at atmospheric pressure at room temperature in the presence of 0.1 g of 10% Pd-C for 1 h. The catalyst was removed by filtration and the solvent was evaporated under reduced pressure to give 0.86 g of *N*-butyl-4-amino-1-methylpyrrole-2-carboxamide **18b**. To a solution of **18b** (0.86 g, 4.4 mmol) and triethylamine (1.23 mL, 8.8 mmol) in 10 mL of THF, was added 1-methyl-4-nitro-2-trichloroacetylpyrrole[24] (1.15 g, 4.22 mmol) at 0 °C. The solution was stirred at room temperature for 16 h under argon atmosphere. The solvent was evaporated under reduced pressure and the

residue was recrystallized from methanol to afford 1.15 g (78%) of **19a** as yellow powder: mp 222–224 °C. IR (KBr) 1657, 1628, 1533, 1304 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 0.90 (3H, t, $J = 7.2$ Hz), 1.21–1.39 (2H, m), 1.39–1.57 (2H, m), 3.39 (2H, dd, $J = 12.8$, 6.8 Hz), 3.81 (3H, s), 3.97 (3H, s), 6.85 (1H, d, $J = 2.0$ Hz), 7.19 (1H, d, $J = 2.0$ Hz), 7.58 (1H, d, $J = 2.0$ Hz), 8.00 (1H, t, $J = 5.6$ Hz), 8.16 (1H, d, $J = 2.0$ Hz), 10.21 (1H, s). Anal. calcd for $C_{16}H_{21}N_5O_4$: C, 55.32; H, 6.09; N, 20.16. Found: C, 55.22; H, 6.18; N, 20.04.

***N*-Butyl-1-methyl-4-[1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]pyrrole-2-carboxamide (20a).** A solution of *N*-butyl-1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamide (**19a**; 0.70 g, 2.0 mmol) in 10 mL of methanol was hydrogenated at atmospheric pressure at room temperature in the presence of 0.1 g of PtO_2 for 1 h. The catalyst was removed by filtration and the solvent was evaporated under reduced pressure to give 0.63 g of *N*-butyl-1-methyl-4-(4-amino-1-methylpyrrole-2-carboxamido)pyrrole-2-carbox- amide **19b**. To a solution of **19b** (0.63 g, 2.0 mmol) and triethylamine (0.28 mL, 4.0 mmol) in 10 mL of acetonitrile, was added 1-methyl-4-nitro-2-trichloroacetylpyrrole[24] (0.50 g, 1.8 mmol) at 0 °C. The solution was stirred at room temperature for 16 h under argon atmosphere. The solvent was evaporated under reduced pressure and the residue was recrystallized from methanol to afford 0.53 g (62%) of **20a** as yellow powder: mp 215–218 °C. IR (KBr) 1639, 1589, 1535, 1311 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 0.91 (3H, t, $J = 7.4$ Hz), 1.18–1.39 (2H, m), 1.39–1.58 (2H, m), 3.18 (2H, dd, $J = 12.8$, 6.6 Hz), 3.81 (3H, s), 3.88 (3H, s), 3.98 (3H, s), 6.87 (1H, d, $J = 1.8$ Hz), 7.05 (1H, d, $J = 1.8$ Hz), 7.19 (1H, d, $J = 1.8$ Hz), 7.28 (1H, d, $J = 1.8$ Hz), 7.61 (1H, d, $J = 1.8$ Hz), 7.97 (1H, t, $J = 5.4$ Hz), 8.17 (1H, d, $J = 1.8$ Hz), 9.93 (1H, s), 10.28 (1H, s). Anal. calcd for $C_{22}H_{27}N_7O_5$: C, 56.28; H, 5.80; N, 20.88. Found: C, 56.11; H, 5.88; N, 20.68.

3-(Butylcarbamoyl)methyl-10-butyl-6-nitro-5-deazaflavin (1). A mixture of 10-butyl-6-nitro-5-deazaflavin (**11**; 0.63 g 2.0 mmol), *N*-butylbromoacetamide (0.78 g, 4.0 mmol), and well pulverized K_2CO_3 (0.83 g, 6.0 mmol) in 10 mL of DMF was warmed at 60 °C for 1 h under argon atmosphere. The solvent was evaporated under reduced pressure and the residue was extracted with chloroform. The organic layer was washed with brine once and was dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the resulting yellow solid was recrystallized from chloroform-ethanol to afford 0.64 g (75%) of **1** as yellow crystals: mp 264–267 °C. IR (KBr) 1709, 1657, 1624, 1529 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 0.92 (3H, t, $J = 7.0$ Hz), 1.07 (3H, t, $J = 7.2$ Hz), 1.20–1.73 (6H, m), 1.73–1.96 (2H, m), 3.29 (2H, dd, $J = 13.0$, 6.6 Hz), 4.72 (2H, s), 4.79 (2H, br), 5.87 (1H, br), 7.88–8.03 (2H, m), 8.07 (1H, dd, $J = 6.8$, 2.4 Hz), 9.41 (1H, s). Anal. calcd for $C_{21}H_{25}N_5O_5$: C, 59.01; H, 5.89; N, 16.38. Found: C, 59.04; H, 5.85; N, 16.23.

***N*-Butyl-1-methyl-4-[(10-butyl-6-nitro-5-deazaflavin-3-yl)-methylcarboxamido]pyrrole-2-carboxamide (2).** A solution of *N*-butyl-1-methyl-4-nitropyrrole-2-carboxamide (**18a**; 676 mg, 3.0 mmol) in 10 mL of methanol was hydrogenated at atmospheric pressure at room temperature in the presence of 30 mg of PtO_2 for 1 h. The catalyst was removed by filtration and the solvent was evaporated under reduced pressure to give 952 mg of *N*-butyl-4-amino-1-methylpyrrole-2-carboxamide **18b**. To a solution of **18b** (952 mg, 3.0 mmol) in 10 mL of acetonitrile, was added 10-butyl-6-nitro-5-deazaflavin-3-acetic acid *N*-hydroxysuccinimide ester (**14**; 1408 mg, 3.0 mmol) and DMAP (366 mg, 3.0 mmol) and the mixture was stirred at room temperature under argon atmosphere for 16 h. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography on silica gel (chloroform: methanol = 10:1 as eluent) to afford 1038 mg (61%) of **2** (obtained as **2**·1.0 H_2O) as yellow powder: mp 223–226 °C. IR (KBr) 1705, 1650, 1624, 1531 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 0.88 (3H, t, $J = 7.1$ Hz), 0.98 (3H, t, $J = 7.4$ Hz), 1.20–1.62 (6H, m), 1.63–1.85 (2H, m), 3.15 (2H, dd, $J = 12.2$, 6.6 Hz), 3.77 (3H, s), 4.64 (2H, s), 4.78 (2H, br), 6.68 (1H, d, $J = 1.0$ Hz), 7.06 (1H, d, $J = 1.0$ Hz), 7.95 (1H, br), 8.16 (1H, dd, $J = 9.0$, 7.8 Hz), 8.26 (1H, d, $J = 7.8$ Hz), 8.41 (1H, d, $J = 9.0$ Hz), 9.21 (1H, s), 10.02 (1H, s). Anal. calcd for $C_{27}H_{31}N_7O_6 \cdot 1.0H_2O$: C, 57.13; H, 5.86; N, 17.27. Found: C, 57.20; H, 5.76; N, 17.08.

***N*-Butyl-1-methyl-4-[1-methyl-4-[(10-butyl-6-nitro-5-deazaflavin-3-yl)methylcarboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamide (3).** A solution of *N*-butyl-1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)-pyrrole-2-carboxamide (**19a**; 987 mg, 2.84 mmol) in 10 mL of methanol was hydrogenated at atmospheric pressure at room temperature in the presence of 90 mg of PtO_2 for 1 h. The catalyst was removed by filtration and the solvent was evaporated under reduced pressure to give 900 mg of *N*-butyl-1-methyl-4-(4-amino-1-methylpyrrole-2-carboxamido)pyrrole-2-carboxamide **19b**. To a solution of **19b** (900 mg, 2.84 mmol) in 10 mL of acetonitrile, was added 10-butyl-6-nitro-5-deazaflavin-3-acetic acid *N*-hydroxysuccinimide ester (**14**; 1330 mg, 2.84 mmol) and DMAP (346 mg, 2.84 mmol) and the mixture was stirred at room temperature under argon atmosphere for 16 h. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography on silica gel (chloroform:methanol = 10:1 as eluent) to afford 1030 mg (54%) of **3** as yellow powder: mp 164–168 °C. IR (KBr) 1707, 1640, 1626, 1531 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 0.89 (3H, t,

$J = 7.3$ Hz), 0.99 (3H, t, $J = 7.3$ Hz), 1.28 (2H, m), 1.34 (2H, m), 1.44 (2H, m), 1.49 (2H, m), 3.17 (2H, t, $J = 6.6$ Hz), 3.79 (3H, s), 3.82 (3H, s), 4.66 (2H, br), 4.78 (2H, s), 6.83 (1H, s), 6.89 (1H, s), 7.12 (1H, s), 7.16 (1H, s), 7.97 (1H, br), 8.14 (1H, dd, $J = 8.6$, 7.9 Hz), 8.25 (1H, d, $J = 8.6$ Hz), 8.40 (1H, d, $J = 7.9$ Hz), 9.21 (1H, s), 9.84 (1H, s), 10.09 (1H, s). MS (FAB): *m/z*: 672 [$(M+H)^+$]. HRMS (FAB) for $C_{33}H_{38}N_9O_7$ [$(M+H)^+$] calcd 672.2897, found 672.2912.

***N*-Butyl-1-methyl-4-[1-methyl-4-[1-methyl-4-[(10-butyl-6-nitro-5-deazaflavin-3-yl)methylcarboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamide (4).** A solution of *N*-butyl-1-methyl-4-[1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]pyrrole-2-carboxamide (**20a**; 1.4 g, 2.98 mmol) in 15 mL of methanol was hydrogenated at atmospheric pressure at room temperature in the presence of 140 mg of PtO_2 for 1 h. The catalyst was removed by filtration and the solvent was evaporated under reduced pressure to give 1.3 g of *N*-butyl-1-methyl-4-[1-methyl-4-(4-amino-1-methylpyrrole-2-carboxamido) pyrrole-2-carboxamido]pyrrole-2-carboxamide **20b**. To a solution of **20b** (1.3 g, 2.98 mmol) in 15 mL of acetonitrile, was added 10-butyl-6-nitro-5-deazaflavin-3-acetic acid *N*-hydroxysuccinimide ester (**14**; 1.4 g, 2.98 mmol) and DMAP (363 mg, 2.98 mmol) and the mixture was stirred at room temperature under argon atmosphere for 16 h. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography on silica–gel (chloroform : methanol = 10:1 as eluent) to afford 1.2 g (51%) of **4** as yellow powder: mp 211–213 °C. IR (KBr) 1707, 1651, 1618, 1560, 1527 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 0.89 (3H, t, $J = 7.3$ Hz), 0.99 (3H, t, $J = 7.3$ Hz), 1.28 (2H, m), 1.34 (2H, m), 1.44 (2H, m), 1.49 (2H, m), 3.17 (2H, t, $J = 6.6$ Hz), 3.79 (3H, s), 3.83 (3H, s), 3.84 (3H, s), 4.66 (2H, br), 4.77 (2H, s), 6.85 (1H, s), 6.92 (1H, s), 7.03 (1H, s), 7.13 (1H, s), 7.17 (1H, s), 7.23 (1H, s), 7.97 (1H, br), 8.13 (1H, dd, $J = 8.6$, 7.9 Hz), 8.25 (1H, d, $J = 8.6$ Hz), 8.40 (1H, d, $J = 7.9$ Hz), 9.21 (1H, s), 9.88 (1H, s), 9.91 (1H, s), 10.10 (1H, s). MS (FAB) *m/z*: 794 [$(M+H)^+$]. HRMS (FAB) for $C_{39}H_{44}N_{11}O_8$ [$(M+H)^+$] calcd 794.3378, found 794.3369.

10-(Butylcarbamoyl)methyl-3-methyl-6-nitro-5-deazaflavin (5). A mixture of 6-(butylcarbamoyl)methylamino-3-methyluracil (**22**; 0.51 g, 2.0 mmol) and 2-fluoro-6-nitrobenzaldehyde[22] (0.34 g, 2.0 mmol) in 10 mL of DMF was heated at 120 °C for 2 h under argon atmosphere. The precipitate appeared was collected by filtration and was washed with ethanol. The precipitate was dried under reduced pressure at room temperature to afford 0.55 g (75%) of **5** (obtained as 5·0.5 H_2O) as yellow powder: mp 267–271 °C. IR (KBr) 1709, 1655, 1620, 1539 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 0.91 (3H, t, $J = 7.2$ Hz), 1.19–1.52 (4H, m), 3.11 (2H, dd, $J = 12.2$, 6.4 Hz), 3.28 (3H, s), 5.48 (2H, br), 8.02–8.14 (2H, m), 8.22 (1H, d, $J = 8.8$ Hz), 8.34 (1H, t, $J = 5.6$ Hz), 9.23 (1H, s). Anal. calcd for $C_{18}H_{19}N_5O_5 \cdot 0.5H_2O$: C, 54.82; H, 5.11; N, 17.76. Found: C, 54.73; H, 4.88; N, 17.65.

***N*-Butyl-1-methyl-4-[(3-methyl-6-nitro-5-deazaflavin-10-yl)methylcarboxamido]pyrrole-2-carboxamide (6).** A solution of *N*-butyl-1-methyl-4-nitropyrrole-2-carboxamide (**18a**; 225 mg, 1.0 mmol) in 3 mL of methanol was hydrogenated at atmospheric pressure at room temperature in the presence of 20 mg of PtO_2 for 1 h. The catalyst was removed by filtration and the solvent was evaporated under reduced pressure to give 195 mg of *N*-butyl-4-amino-1-methylpyrrole-2-carboxamide **18b**. To a solution of **18b** (225 mg, 1.0 mmol) in 3 mL of acetonitrile, was added 3-methyl-6-nitro-5-deazaflavin-10-acetic acid (**17**; 330 mg, 1.0 mmol) and 1,3-diisopropylcarbodiimide (126 mg, 1.00 mmol) and the mixture was stirred at room temperature under argon atmosphere for 16 h. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography on silica gel (chloroform : methanol = 10:1 as eluent) to give yellow solid. Recrystallization from chloroform–ethanol afforded 134 mg (26%) of **6** (obtained as **6**·0.5H_2O) as yellow powder: mp 169–172 °C. IR (KBr) 1701, 1653, 1618, 1564, 1533 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 0.92 (3H, t, $J = 7.2$ Hz), 1.18–1.36 (2H, m), 1.36–1.53 (2H, m), 3.03–3.22 (2H, m), 3.28 (3H, s), 3.76 (3H, s), 5.67(2H, br), 6.73 (1H, d, $J = 1.8$ Hz), 7.06 (1H, d, $J = 1.8$ Hz), 7.97 (1H, t, $J = 5.6$ Hz), 8.09 (1H, d, $J = 8.4$ Hz). Anal. calcd for $C_{24}H_{25}N_7O_6 \cdot 0.5H_2O$: C, 55.81; H, 5.07; N, 18.98. Found: C, 55.88; H, 4.99; N, 19.02.

***N*-Butyl-1-methyl-4-[1-methyl-4-[(3-methyl-6-nitro-5-deazaflavin-10-yl)methylcarboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamide (7).** A solution of *N*-butyl-1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamide (**19a**; 1000 mg, 2.88 mmol) in 10 mL of methanol was hydrogenated at atmospheric pressure at room temperature in the presence of 100 mg of PtO_2 for 1 h. The catalyst was removed by filtration and the solvent was evaporated under reduced pressure to give 910 mg of *N*-butyl-1-methyl-4-(4-amino-1-methylpyrrole-2-carboxamido) pyrrole-2-carboxamide **19b**. To a solution of **19b** (910 mg, 2.88 mmol) in 10 mL of acetonitrile, was added 3-methyl-6-nitro-5-deazaflavin-10-acetic acid (**17**; 950 mg, 2.88 mmol) and 1,3-diisopropylcarbodiimide (435 mg, 3.45 mmol) and the mixture was stirred at room temperature under argon atmosphere for 16 h. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography on silica gel (chloroform:methanol = 10:1 as eluent) to afford 507 mg (28%) of **7** as yellow powder: mp 213–216 °C. IR (KBr) 1702, 1641, 1626 cm^{-1}.

^{1}H NMR (Me_2SO-d_6) δ 0.89 (3H, t, $J = 7.3$ Hz), 1.23 (2H, m), 1.46 (2H, m), 3.10 (3H, s), 3.16 (2H, t, $J = 6.6$ Hz), 3.79 (3H, s), 3.81 (3H, s), 5.96 (2H, br), 6.81 (1H, s), 6.93 (1H, s), 7.13 (1H, s), 7.16 (1H, s), 7.97 (1H, br), 8.07 (1H, dd, $J = 8.6$, 7.9 Hz), 8.22 (1H, d, $J = 8.6$ Hz), 8.27 (1H, d, $J = 7.9$ Hz), 9.25 (1H, s), 9.84 (1H, s), 10.53 (1H, s). MS (FAB) m/z: 630 [$(M+H)^+$]. HRMS (FAB) for $C_{30}H_{32}N_9O_7$ [$(M+H)^+$] calcd 630.2428, found 630.2417.

***N*-Butyl-1-methyl-4-[1-methyl-4-[1-methyl-4-[(3-methyl-6-nitro-5-deazaflavin-10-yl)methylcarboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamide (8).** A solution of *N*-butyl-1-methyl-4-[1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]pyrrole-2-carboxamide (**20a**; 1000 mg, 2.13 mmol) in 10 mL of methanol was hydrogenated at atmospheric pressure at room temperature in the presence of 75 mg of PtO_2 for 1 h. The catalyst was removed by filtration and the solvent was evaporated under reduced pressure to give 935 mg of *N*-butyl-1-methyl-4-[1-methyl-4-(4-amino-1-methylpyrrole-2-carboxamido)pyrrole-2-carboxamido]pyrrole-2-carboxamide **20b**. To a solution of **20b** (935 mg, 2.13 mmol) in 10 mL of acetonitrile, was added 3-methyl-6-nitro-5-deazaflavin-10-acetic acid (**17**; 700 mg, 2.12 mmol) and 1,3-diisopropylcarbodiimide (403 mg, 3.20 mmol) and the mixture was stirred at room temperature under argon atmosphere for 16 h. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography on silica gel (chloroform:methanol = 10:1 as eluent) to afford 415 mg (26%) of **8** as yellow powder: mp 261–265 °C. IR (KBr) 1702, 1637, 1626, 1560 cm^{-1}. ^{1}H NMR (Me_2SO-d_6) δ 0.90 (3H, t, $J = 7.3$ Hz), 1.32 (2H, m), 1.46 (2H, m), 3.08 (3H, s), 3.16 (2H, t, $J = 6.6$ Hz), 3.79 (3H, s), 3.83 (3H, s), 3.84 (3H, s), 5.95 (2H, br), 6.84 (1H, s), 6.85 (1H, s), 6.96 (1H, s), 7.02 (1H, s), 7.16 (1H, s), 7.23 (1H, s), 7.96 (1H, br), 8.07 (1H, dd, $J = 8.6$, 7.9 Hz), 8.24 (1H, d, $J = 8.6$ Hz), 8.27 (1H, d, $J = 7.9$ Hz), 9.26 (1H, s), 9.87 (1H, s), 9.89 (1H, s), 10.56 (1H, s). MS (FAB) m/z: 752 [$(M+H)^+$]. HRMS (FAB) for $C_{36}H_{38}N_{11}O_8$ [$(M+H)^+$] calcd 752.2908, found 752.2875.

***N*-Butyl-1-methyl-4-[1-methyl-4-[(10-butyl-5-deazaflavin-3-yl)methylcarboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamide (9)**. A solution of *N*-butyl-1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamide (**19a**; 347 mg, 1.00 mmol) in 3 mL of methanol was hydrogenated at atmospheric pressure at room temperature in the presence of 30 mg of PtO_2 for 1 h. The catalyst was removed by filtration and the solvent was evaporated under reduced pressure to give 317 mg of *N*-butyl-1-methyl-4-(4-amino-1-methylpyrrole-2-carboxamido)pyrrole-2-carboxamide **19b**. To a solution of **19b** (317 mg, 1.00 mmol) in 5 mL of acetonitrile, was added 10-butyl-5-deazaflavin-3-acetic acid *N*-hydroxysuccinimide ester (**26**, 424 mg, 1.00 mmol) and DMAP (122 mg, 1.00 mmol) and the mixture was stirred at room temperature under argon atmosphere for 16 h. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography on silica gel (chloroform : methanol = 10:1 as eluent) to afford 426 mg (68%) of **9** as yellow powder: mp 179–182 °C. IR (KBr) 1690, 1641, 1618, 1568, 1529 cm^{-1}. ^{1}H NMR (Me_2SO-d_6) δ 0.89 (3H, t, $J = 7.4$ Hz), 0.99 (3H, t, $J = 7.2$ Hz), 1.20–1.36 (2H, m), 1.36–1.62 (4H, m), 1.62–1.86 (2H, m), 3.16 (2H, dd, $J = 12.8$, 6.6 Hz), 3.79 (3H, s), 3.83 (3H, s), 4.66 (2H, s), 4.74 (2H, br), 6.83 (1H, d, $J = 1.8$ Hz), 6.90 (1H, d, $J = 1.6$ Hz), 7.13 (d, 1H, $J = 1.8$ Hz), 7.16 (1H, d, $J = 1.6$ Hz), 7.50–7.63 (1H, m), 7.90–8.08 (3H, m), 8.25 (1H, d, $J = 7.6$ Hz), 9.10 (1H, s), 9.84 (1H, s), 10.06 (1H, s). MS (FAB) m/z 627 [$(M+H)^+$]. HRMS (FAB) for $C_{33}H_{39}N_8O_5$ [$(M+H)^+$] calcd 627.3047, found 627.3051.

***N*-Butyl-1-methyl-4-[1-methyl-4-[(3-methyl-5-deazaflavin-10-yl)methylcarboxamido]pyrrole-2-carboxamido]pyrrole-2-carboxamide (10).** A solution of *N*-butyl-1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamide (**19a**; 347 mg, 1.00 mmol) in 3 mL of methanol was hydrogenated at atmospheric pressure at room temperature in the presence of 30 mg of PtO_2 for 1 h. The catalyst was removed by filtration and the solvent was evaporated under reduced pressure to give 317 mg of *N*-butyl-1-methyl-4-(4-amino-1-methylpyrrole-2-carboxamido) pyrrole-2-carboxamide **19b**. To a solution of **19b** (317 mg, 1.00 mmol) in 3 mL of DMF, was added 3-methyl-5-deazaflavin-10-acetic acid (**28**; 282 mg, 1.00 mmol) and 1,3-diisopropylcarbodiimide (126 mg, 1.00 mmol) and the mixture was stirred at room temperature under argon atmosphere for 16 h. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography on silica–gel (chloroform : methanol = 10:1 as eluent) to afford 117 mg (20%) of **10** as yellow powder: mp 192–195 °C. IR (KBr) 1689, 1640, 1620, 1570, 1533 cm^{-1}. ^{1}H NMR (Me_2SO-d_6) δ 0.89 (3H, t, $J = 7.0$ Hz), 1.18–1.38 (2H, m), 1.38–1.59 (2H, m), 2.88–3.20 (2H, m), 3.27 (3H, s), 3.79 (3H, s), 3.82 (3H, s), 5.61 (2H, br), 6.82 (1H, s), 6.93 (1H, s), 7.12 (1H, s), 7.16 (1H, s), 7.55 (1H, t, $J = 7.4$ Hz), 7.86 (1H, t, $J = 8.6$ Hz), 7.91–8.05 (2H, m), 8.26 (1H, d, $J = 7.8$ Hz), 9.15 (1H, s), 9.84 (1H, s), 10.44 (1H, s). MS (FAB) m/z 585 [$(M+H)^+$]. HRMS (FAB) for $C_{30}H_{33}N_8O_5$ [$(M+H)^+$] calcd 585.2577, found 585.2567.

***N*-Butyl-1-methyl-4-(1-methyl-4-acetamido-pyrrole-2-carboxamido)pyrrole-2-carboxamide (21)**. A solution of *N*-butyl-1-methyl-4-(1-methyl-4-nitropyrrole-2-carboxamido)pyrrole-2-carboxamide (**19a**; 0.70 g, 2.0 mmol) in 10 mL of methanol was hydrogenated at atmospheric

pressure at room temperature in the presence of 0.1 g of PtO_2 for 1 h. The catalyst was removed by filtration and the solvent was evaporated under reduced pressure to give 0.63 g of *N*-butyl-1-methyl-4-(4-amino-1-methylpyrrole-2-carboxamido) pyrrole-2-carboxamide **19b**. To a solution of **19b** (0.63 g, 2.0 mmol), pyridine (0.16 mL, 2.0 mmol), and triethylamine (0.28 mL, 4.0 mmol) in 10 mL of acetonitrile, was added acetic anhydride (204 mg, 2.0 mmol) at 0 °C. The solution was stirred at room temperature under argon atmosphere for 1 h. The solvent was evaporated under reduced pressure and the residue was recrystallized from methanol to afford 0.56 g (74%) of **21** (obtained as **21**·1.0 H_2O) as pale yellow powder: mp 110–112 °C. IR (KBr) 1641, 1583, 1535 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 0.90 (3H, t, $J = 7.4$ Hz), 1.19–1.39 (2H, m), 1.39–1.59 (2H, m), 1.97 (3H, m), 3.17 (q, 2H, $J = 6.2$ Hz), 3.80 (3H, s), 3.83 (3H, s), 6.84 (1H, s), 6.85 (1H, s), 7.15 (1H, d, $J = 1.6$ Hz), 7.17 (1H, d, $J = 1.6$ Hz), 7.97 (1H, t, $J = 5.6$ Hz), 9.82 (1H, s), 9.84 (1H, s). Anal. calcd for $C_{18}H_{25}N_5O_3 \cdot 1.0H_2O$: C, 57.28; H, 7.21; N, 18.55. Found: C, 57.22; H, 7.18; N, 18.47.

Redox potentials of the compounds

The reduction peak potentials (*E*p) of the compounds were determined in DMF solutions (1.0×10^{-3} M) relative to the Ag/AgCl electrode using cyclic voltammetry (Hokuto Denko HB-104 voltage scanner and Hokuto Denko HA-301 potentiostat using glassy carbon and platinum wire as the working and auxiliary electrodes and tetrabutylammonium perchlorate (1.0×10^{-1} M) as a supporting electrolyte, scan rate $\nu = 100$ mVs^{-1}) at 298 K in the dark under argon atmosphere.

DNA-titration by UV-VIS spectra

The UV-VIS spectra of the compounds (2.1×10^{-5} (M)) were measured on Shimadzu UV-3100PC spectrophotometer in the presence of $[poly\ (dA\text{-}dT)]_2$ (Sigma), $[poly\ (dG\text{-}dC)]_2$ (Sigma), or calf thymus DNA (Sigma) in 10 mM Tris-HCl buffer (pH 7.0) containing 20% of DMF. The apparent binding constants of the compounds (K_{app}) were determined according to the method as was described in ref. 26.

Reductive activation of the compounds and plasmid DNA-cleaving assay

Controlled potential electrolysis was performed using a three electrode configuration involving a platinum (5 mmϕ) working electrode, platinum wire counter electrode, and Ag/AgCl reference electrode.[9] The working and counter electrode were separated each other with sintered glass filter to prevent the reoxidation of reductively activated compounds.

Electrolytic reductions of the compounds in the presence of plasmid DNA (pBR322 (Sigma), 5×10^{-5} M/bp) were carried out at 298 K under argon atmosphere in the dark at a constant potential of −950 mV for compounds **1–8** and −1100 mV for compounds **9** and **10** in 1.5 mM sodium citrate buffer containing 20% DMF. The electrons involved in the reduction process of the compounds were monitored on Yanaco V10-CM coulometer. After the complete reduction of the compounds, the reaction solution was incubated at room temperature overnight. The degradation of form I of pBR322 DNA was analyzed by means of electrophoresis (1% agarose gel) with standard procedure.

In vitro antitumor activities of nitro 5-deazaflavin-pyrrolecarboxamide(s) hybrid molecules

The tetrazolium-based semiautomated colorimetric assay (MTT assay) developed by Carmichael[33] was modified and used for the in vitro assay. 2000 Cells (human oral epidermoid carcinoma KB cells or murine leukemia L1210 cells) in 180 µL of RPML-1640 medium were seeded in a 96-well flat bottom microtest plate and 20 µL of drug solutions with graded concentrations were simultaneously added in triplicate to each well. The plate was incubated for 3 days at 37 °C in a humidified atmosphere of 5% CO_2. To each well was added 25 µl of MTT reagent (2 mg/mL in Dulbecco's phosphate buffered saline without calcium and magnesium). After another 4 h incubation at 37 °C, the microplate was centrifuged at 3000 rev./min for 10 min and the medium was removed by aspiration. To each well was added 0.2 mL of dimethyl sulfoxide and each well was mixed thoroughly with a mechanical plate mixer to solubilize the resulting MTT-formazan. Absorbance at 540 nm (OD_{540}) was measured with a ImmunoReader NJ-2000 (InterMed Japan, Tokyo Japan). By using the OD_{540} of each well the 50% inhibitory drug concentration (IC_{50} value) was determined as was described previously.[34]

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30. Because of the limited solubility of compound **8** in the reaction solvent employed, the DNA damage induced by the reductively activated **8** appeared to be not so effective as that by reductively activated **7**. In separate experiments where **8** was dissolved completely, reductively activated **8** induced as great DNA damage as **7**.

31. Reductive activation of higher concentrations of compounds **1**, **2**, **5**, and **6** afforded markedly form II DNA.

32. Essentially no DNA damage was found in the presence of compounds **1–8** without electrolytical reductions.

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