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Synthesis and Antitumor Activities of Novel 5-Deazaflavin-Sialic Acid Conjugate Molecules

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Abstract—6-Nitro-5-deazaflavin derivatives bearing *O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α- and β-D-*galacto*-non-2-ulopyranosylonate)alkyl group (sialosylalkyl group) at N(3) or N(10) and 8-amino-5-deazaflavin substituted with the sialosylalkyl group at the amino group were synthesized and their physicochemical properties as well as antitumor effects on KB and L1210 cells have been investigated. The configurations of the glycosides were determined by ^{1}H NMR and rate of hydrolysis of the glycosidic bond. It has been found that these conjugate molecules show significant antitumor activities. Combination of an 8-amino-5-deazaflavin with the sialosylalkyl group have been found to give rise to significant increase in antitumor activities of the compound. Antitumor effects of 6-nitro-5-deazaflavin-sialic acid conjugate molecules were similar or rather weak in comparison with those of the 6-nitro-5-deazaflavin derivatives without sialosylalkyl group. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

Sialic acids[1] are commonly present at the non-reducing terminal positions of carbohydrate chains of glycoproteins and glycolipids on the cell surface and their crucial roles played in biological processes involving cell to cell recognition and interaction,[2] masking effects for cell surface antigen,[3] differentiation of cells,[4] and neoplastic transformation[5] have been well studied. Combination of biologically important compounds with a sialic acid derivative would be anticipated to give rise to selective interaction towards target biological molecules and target tumor cells.[6] From these points of view, Ogura synthesized various sialic acid conjugate molecules, sialosyl cholesterol,[7] sialyl nucleosides,[8] sialyl umbelliferon[9] and sialosylphenyl mitomycin C[10] and reported their remarkable physiological activities. Also, taxol-sialyl conjugate[11] and sialic acid containing 2-nitroimidazole nucleoside analogue[12] as a radiosensitizer for hypoxic cells have been prepared and their biological activities were elucidated.

Recently, we have developed nitro 5-deazaflavin derivatives[13–16] as novel class of nitrohetero-aromatic compounds containing an electrophilic redox coenzyme ring system. It has been found that a series of nitro 5-deazaflavin derivatives shows significant antitumor activities[13] and that 6- and 8-nitro-5-deazaflavin derivatives generating stable one-electron reduction product(s) show marked selective cytotoxicities towards hypoxic cells.[14] It has also been demonstrated that reductively activated 6-nitro-5-deazaflavin derivatives bearing pyrrolecarboxamide(s) as a DNA minor groove binder induce significant oxidative DNA damage under anaerobic conditions.[15] Furthermore, we have first found that reductively activated 6- and 8-nitro-5-deazaflavin derivatives interact specifically with guanine base to give rise to prominent formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) under anaerobic conditions.[16]

To develop more potent (bioreductive) antitumor agents showing a higher selectivity towards tumor cells as well as hypoxic cells, we have designed 6-nitro-5-deazaflavin derivatives **1** and **2** bearing a 2-*O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α- and β-D-*galacto*-non-2-ulopyranosylonate)ethyl group at N(3) position and 6-*O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α- and β-D-*galacto*-non-2-

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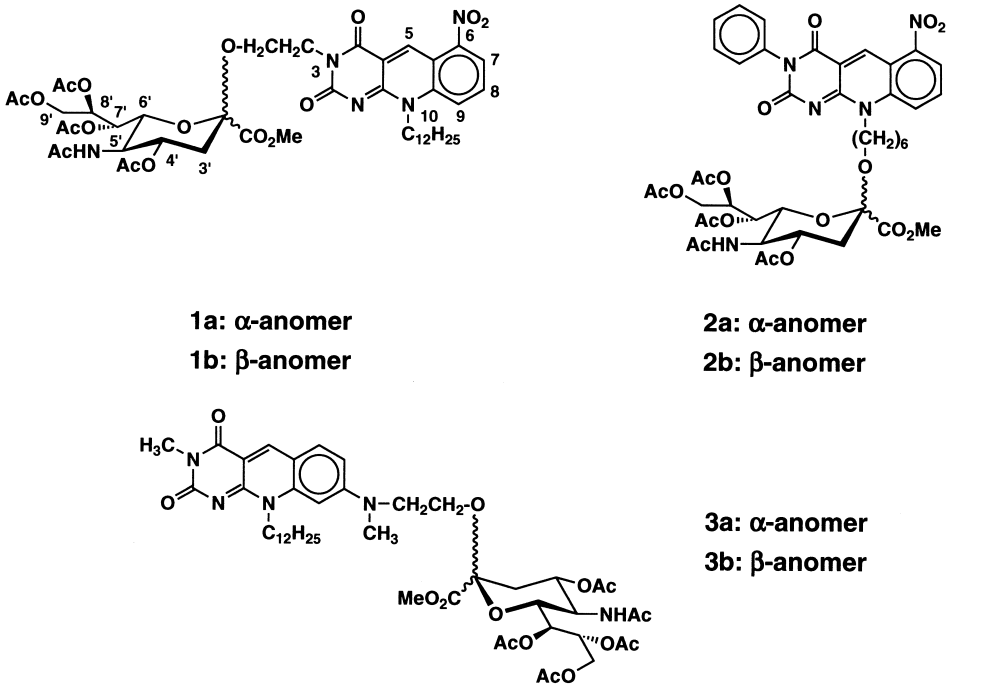
ulopyranosylonate)hexyl group at N(10) position as novel 5-deazaflavin-sialic acid conjugate molecules (Scheme 1). Because among nitro-positional isomer of nitro 5-deazaflavin derivatives, 6-nitro-5-deazaflavin derivatives showed the most potent antitumor activities[13] and the highest selective cytotoxicities towards hypoxic cells,[14] the sialosylalkyl group was introduced into 6-nitro-5-deazaflavin derivatives. Also, as a 5-deazaflavin derivative modified at benzene ring moiety, 8-amino-5-deazaflavin derivatives **3** substituted with 2-[*O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α- and β-D-*galacto*-non-2-ulopyranosylonate)]ethyl group at the amino group have been designed (Scheme 1). In the present paper, we wish to describe synthesis of (nitro) 5-deazaflavin-sialic acid conjugate molecules and their physicochemical properties as well as antitumor activities.

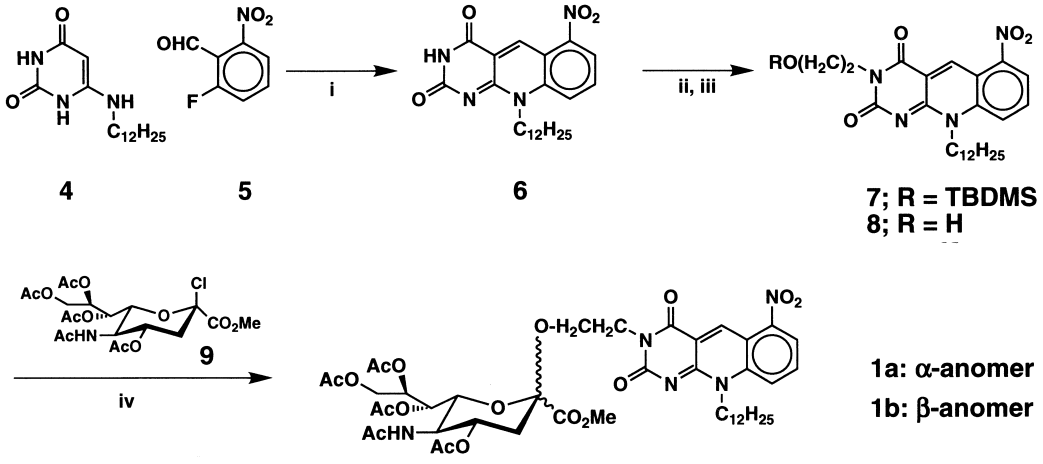
Results and Discussion

Synthesis of 5-deazaflavin-sialic acid conjugate molecules

10-Dodecyl-6-nitro-5-deazaflavin **6** was prepared by condensation of 6-dodecylaminouracil **4** with 2-fluoro-6-nitrobenzaldehyde **5**[17] according to Yoneda's method[13,18] in 82%. Treatment of **6** with 1-(*tert*-butyldimethylsiloxy)-2-iodoethane[19] in the presence of K_2CO_3 in DMF at 60 °C afforded **7** in 82%, which was subjected to hydrolysis with aqueous 12N hydrochloric acid in ethanol to give **8** in 82%. Glycosylation of **8** with methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-non-2-ulopyranosyl chloride)onate **9**[20] under the Koenigs-Knorr like reaction condition[7,8a,8c,21] gave a mixture of glycosides **1a** and **1b**. Chromatographic separation of the anomeric mixture on thin layer silica



Scheme 1.



Scheme 2. Synthesis of compounds **1a** and **1b**: (i) DMF, 120 °C; (ii) $I(CH_2)_2OTBDMS$, K_2CO_3, DMF, 60 °C; (iii) 12 N HCl, EtOH, 50 °C; (iv) AAgOTf, MS 4A, CH_2Cl_2, rt.

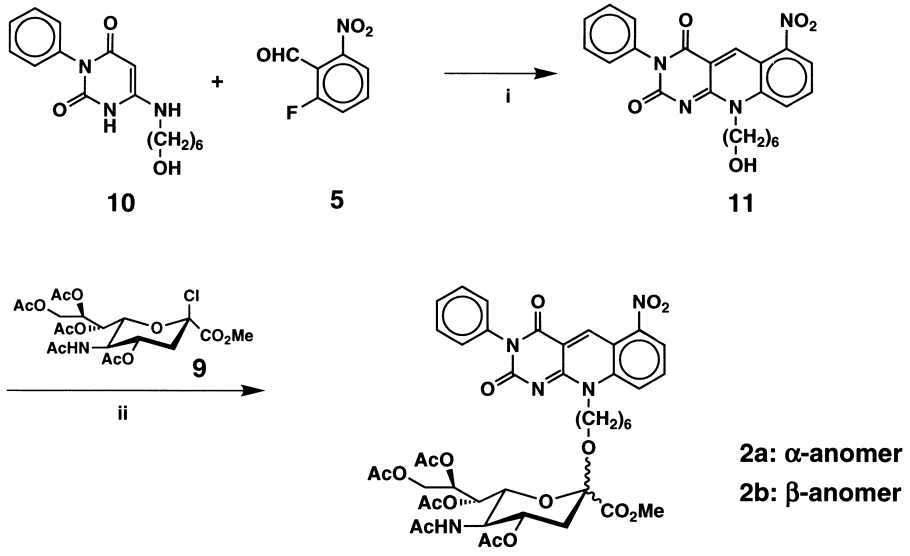
gel plates afforded the respective single anomers in 16 and 26% (Scheme 2).

Also, 10-(6-hydroxyhexyl)-3-phenyl-5-deazaflavin **11** was obtained by condensation of 6-(6-hydroxyhexyl)amino-3-phenyluracil **10** with 2-fluoro-6-nitrobenzaldehyde **5**[17] in 72%. Glycosylation of **11** with **9**[20] and separation of the resulting anomeric mixture of **2a** and **2b** in the similar manner described above gave single anomers in 20 and 20% respectively (Scheme 3).

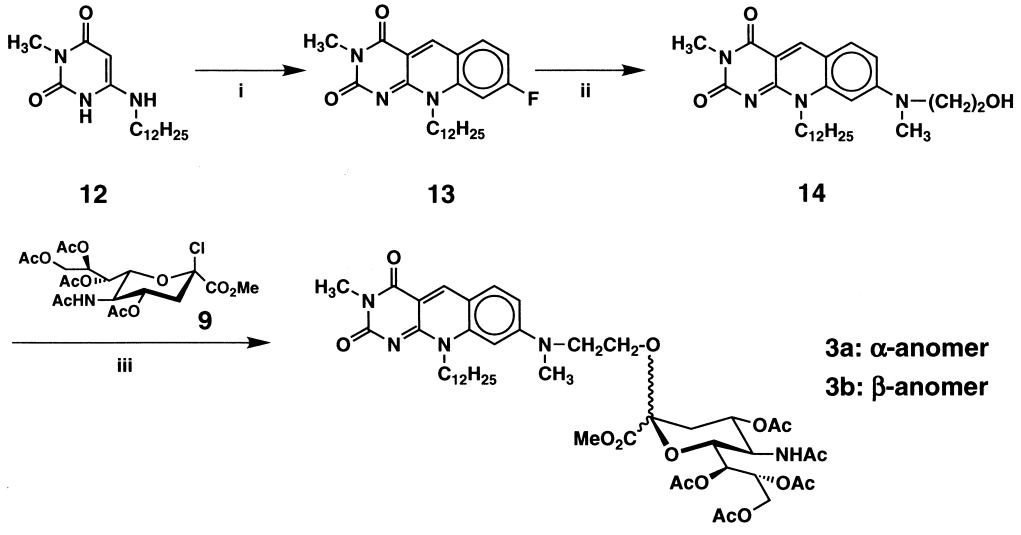
5-Deazaflavin derivatives **3** bearing the *N*-methyl-*N*-(sialosylethyl)amino group at C(8) position were prepared by using 10-dodecyl-8-fluoro-3-methyl-5-deazaflavin **13** which was obtained by condensation of 6-dodecylamino-3-methyluracil **12** with 2,4-difluorobenzaldehyde in 86%. Treatment of **13** with 2-(methylamino)ethanol in DMF at 100 °C gave **14** in 85%.[22] Glycosylation of **14** with **9**[20] and separation of the obtained anomeric mixture of **3a** and **3b** in the above similar manner afforded the respective single anomers in 22 and 34% (Scheme 4).

Configuration of the 5-deazaflavin-sialic acid conjugate molecules

It is well understood that in ^{1}H NMR spectra of sialic acid derivatives, double-doublet peaks for $H_{eq}(3)$ of an α-glycoside appear in lower magnetic field than those of the β-glycoside.[7,21,23] Goto also reported that anomeric configuration of sialic acid derivatives is able to be estimated from the coupling constants between H(7) and H(8) ($J_{H(7),H(8)}$) and the $\Delta\delta|H(9')\text{-}H(9)|$ values in ^{1}H NMR spectra of the glycosides.[24] To consider anomeric configuration of the sialic acid conjugate molecules **1**–**3**, ^{1}H NMR spectra of the anomeric pair of compounds were compared. As Table 1 shows, the chemical shifts for



Scheme 3. Synthesis of compounds **2a** and **2b**: (i) DMF, 120 °C; (ii) AgOTf, MS 4A, CH_2Cl_2, rt.



Scheme 4. Synthesis of compounds **3a** and **3b**: (i) 2,4-difluorobenzaldehyde, DMF, 120 °C; (ii) 2-(methylamino)ethanol, DMF, 100 °C; (iii) AgOTf, MS 4A, CH_2Cl_2, rt.

Table 1. Determination of configuration of 5-deazaflavin-sialic acid conjugate molecules **1–3** by means of ^{1}H NMR spectra[a] and hydrolysis reaction[b]

Compound	Configuration	δ for $H_{eq}(3)$ (ppm)[a]	Hydrolysis rate Conversion (%)[c]
1a	α	2.53, dd, J = 12.9, 4.7 Hz	99
1b	β	2.22, dd, J = 12.7, 5.0 Hz	< 3
2a	α	2.59, dd, J = 12.8, 4.6 Hz	99
2b	β	2.51, dd, J = 12.8, 4.9 Hz	< 3
3a	α	2.56, dd, J = 12.8, 4.6 Hz	98
3b	β	2.43, dd, J = 12.9, 5.0 Hz	< 2

[a]300 MHz, in $CDCl_3$, at 298 K.
[b][Compound] = 5.0×10^{-3} (M), in aqueous 50% acetic acid, at 373 K, 6 h, under nitrogen.
[c]Determined by means of an HPTLC method.

$H_{eq}(3)$ of compounds **1a**, **2a** and **3a** have been found to be larger than those of the other anomers **1b**, **2b** and **3b**. Although the coupling constants between H(7) and H(8) ($J_{H(7),H(8)}$) of compounds **1–3** were not determined due to complexity of the spectra, significant difference of $\Delta\delta|H(9')$- H(9)| values[24,25] has been observed between the anomeric pair of compounds **1–3**.

Furthermore, to confirm the anomeric configuration of compounds **1–3**, the rates of hydrolysis of each pair of glycosides were compared. Ogura reported that an α-anomer of sialic acid derivatives is more susceptible to hydrolysis of glycosidic bond than the corresponding β-anomer.[8c] Thus compounds **1–3** were treated with aqueous 50% acetic acid[26] at 100 °C for 6 h in sealed tubes under nitrogen atmosphere and the reactions were monitored by measuring the increasing concentration of **8**, **11** and **14**. The results of hydrolysis of glycosidic bond of **1a** and **1b** were exemplified in Figure 1. As Table 1 shows, compounds **1a**, **2a**, and **3a** were hydrolyzed almost completely after 6 h, on the other hand, **1b**, **2b** and **3b** were stable under above reaction conditions.

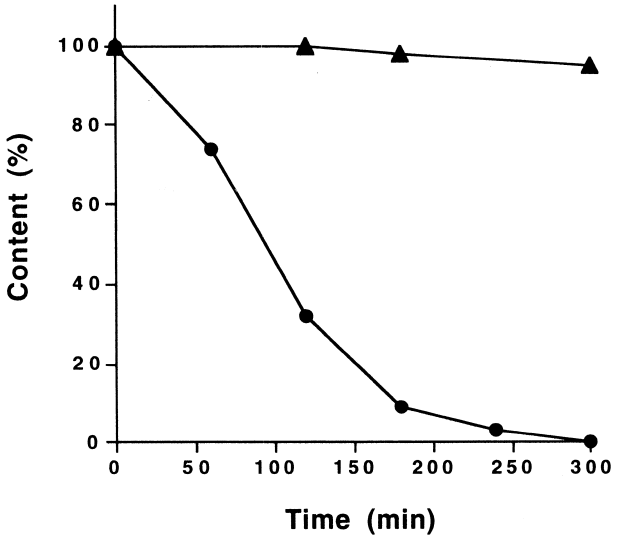


Figure 1. Hydrolysis of glycosidic bond of **1a** (circle) and **1b** (triangle).

From above experimental results of ^{1}H NMR spectra and hydrolysis rates of the compounds, the anomeric configurations of **1a**, **2a** and **3a** were determined to be α and **1b**, **2b** and **3b** to be β.

CD spectra of the 5-deazaflavin-sialic acid conjugate molecules

Circular dichroism (CD) spectral analysis of sialic acid derivatives is also considered to be one of the useful methods for determination of configuration of the glycosides, if other chromophore in the compound does not affect the Cotton effect of carboxyl group at C(1) position due to n-π* transition.[7,21,27] Because (5-deaza)flavin ring system is known as a strong chromophore,[28] interaction of 5-deazaflavin chromophore with sialic acid moiety would affect significantly the CD spectra of the conjugate molecules. UV–vis and CD spectra of **1–3** were measured and shown in Figure 2.

Significant differences of CD spectra between α- and β-anomer of **1–3** have been found. Negative Cotton effect for α-anomer **1a** and **3a** around 220–250 nm due to n-π* transition and positive Cotton effect for β-anomer **1b** and **3b** were observed (Fig. 2(a), and (c)), however, each anomer of **2** has been found to show similar pattern of Cotton effects around 200–250 nm (Fig. 2(b)). And interestingly, marked difference of CD spectra for **1–3** were observed in the visible absorption band characteristic of 5-deazaflavin chromophore. The result suggests that there would be significant stereoselective interaction(s) between sialic acid moiety and 5-deazaflavin ring system, which may affect recognition abilities for biological target of the conjugate molecules.

In vitro antitumor activities of the 5-deazaflavin-sialic acid conjugate molecules

To evaluate antitumor activities of the 5-deazaflavin-sialic acid conjugate molecules and other reference compounds, compounds **1–3**, **8**, **11** and **14** 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.[29,30] Mitomycin C was also employed as reference compound. As Table 2 shows,

Table 2. IC_{50} Values of 5-deazaflavin-sialic acid conjugate molecules on KB and L1210 cells growth in vitro

	IC_{50} (μM)[a]	
	KB cells	L1210 cells
1a	5.1	1.7
1b	2.3	1.9
2a	14.3	20.9
2b	6.4	6.8
3a	5.1	13.8
3b	3.9	10.6
8	0.74	5.7
11	2.0	9.2
14	> 100	> 100
Mitomycin C	0.4	0.6

[a]IC_{50} (μM) value was given as the concentration at 50% inhibition of cell growth.

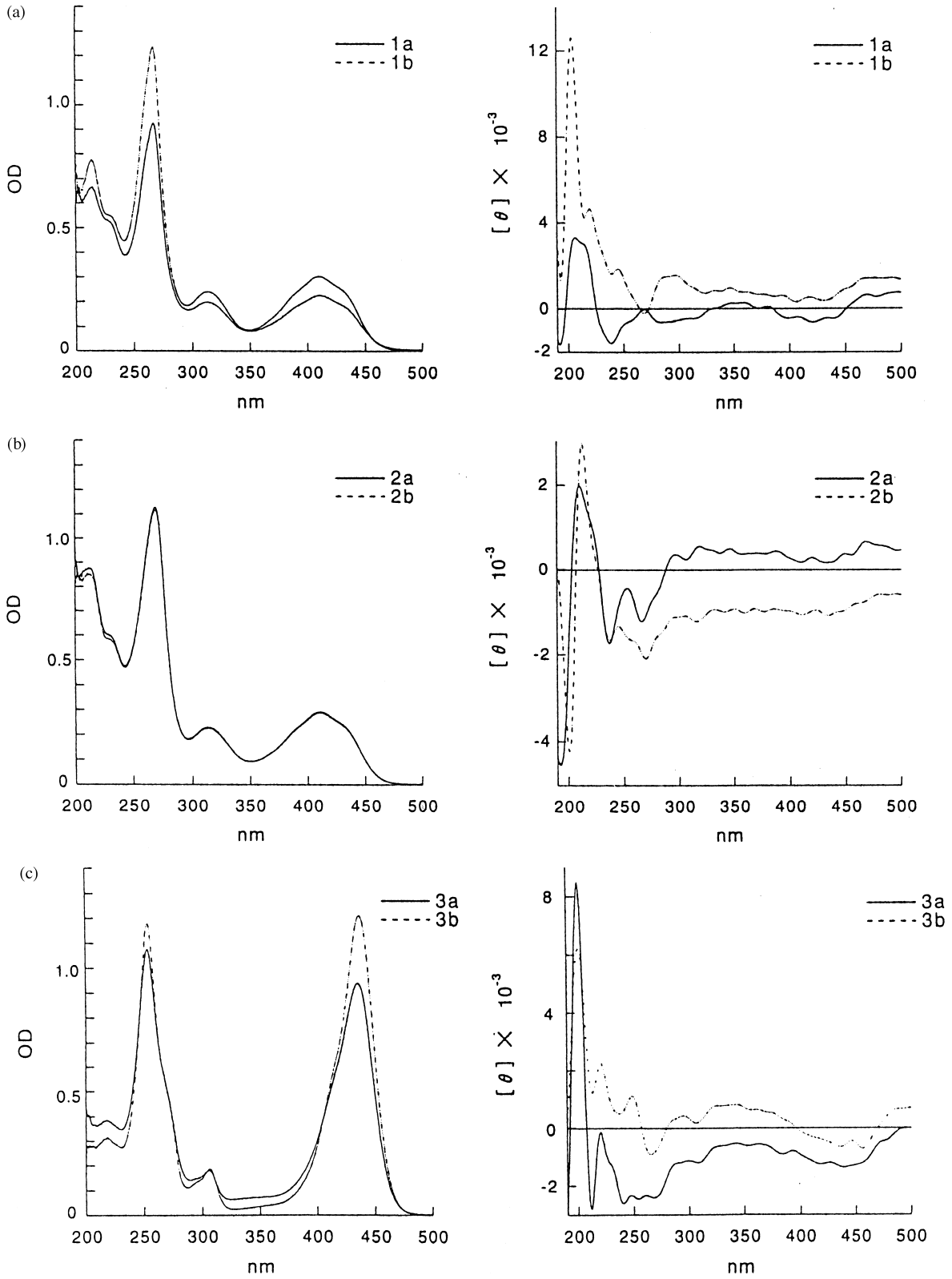


Figure 2. UV–vis and CD spectra of 5-deazaflavin-sialic acid conjugate molecules **1** (a), **2** (b), and **3** (c).

5-deazaflavin-sialic acid conjugate molecules **1**–**3** have been found to show significant antitumor activities.[31] It has been found that β-anomer **2b** shows 2–3 folds more potent antitumor activities than α-anomer **2a** and there appears to be essentially no significant differences of antitumor effects between **1a** and **1b**, and between **3a** and **3b**. Interestingly enough, the conjugate molecules **3** have been found to show much more potent antitumor activities than 8-amino-5-deazaflavin derivative **14** without sialosyl group. The result may suggest that combination of an 8-amino-5-deazaflavin with sialosylalkyl group would give rise to significant effects for antitumor activities of

the compound. Antitumor activities of sialic acid conjugates **1** and **2** were similar or rather weak in comparison with the corresponding 6-nitro-5-deazaflavin derivatives **8** and **11** without sialosyl group.

Conclusion

We have first synthesized 6-nitro- and 8-amino-5-deazaflavin derivatives bearing sialosylalkyl group as novel 5-deazaflavin-sialic acid conjugate molecules and evaluated their antitumor activities. Combination of an 8-amino-5-deazaflavin with a sialosylalkyl group has been found to give rise to significant increase in antitumor activities. Although antitumor effects of 6-nitro-5-deazaflavin-sialic acid conjugate molecules **1** and **2** were similar or rather weak in comparison with 6-nitro-5-deazaflavin without sialosyl group, these compounds may be anticipated to show promoted selective cytotoxicities towards tumor cells as well as hypoxic cells. The detailed biological activities of these unique conjugate molecules as well as biological mechanism for antitumor effects of **3** are under investigation.

Experimental

Melting points were taken using a Mettler thermosystem FP80HT and are uncorrected. Infrared (IR) spectra were recorded on a Shimadzu IR-400 spectrophotometer. Specific rotation values were measured on a JASCO DIP-360 polarimeter. Proton nuclear magnetic resonance (^{1}H NMR) spectra for compounds **1**–**3** were recorded on a Bruker AC-300 (300 MHz) and for other compounds 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)) were recorded on a JEOL JNS-DX303. High-resolution FAB (fast atom bombardment) mass spectra (HRMS (FAB)) were recorded on a JEOL JMS-HX/HX110A. UV–vis spectra were measured on a Shimadzu UV-2100. Circular dichroism (CD) spectra were recorded on a JASCO J-700. Column chromatography was carried out on silica gel (Kieselgel 60, 70-230 mesh, Merck) and on thin layer silica gel plates (Silica Gel 60 F254, HPTLC plate, Merck).

10-Dodecyl-6-nitro-5-deazaflavin (6). A mixture of 6-dodecylaminouracil[18] (**4**; 2.95 g, 10.0 mmol) and 2-fluoro-6-nitrobenzaldehyde[17] (**5**; 1.69 g, 10.0 mmol) in 30 mL of DMF was heated at 120 °C for 2 h under argon atmosphere. The reaction mixture was poured into ice water and deposited precipitate was collected by filtration. The precipitate was washed with water and was dried under reduced pressure. Recrystallization from chloroform-ethanol afforded 3.50 g (82%) of **6** as yellow powder: mp 216–217 °C. IR (KBr) 1711, 1694, 1676, 1622, 1651, 1524 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 0.88 (3H, t, J = 6.6 Hz), 1.10–1.72 (18H, m), 1.75–1.97 (2H, m), 4.62–4.98 (2H, m),7.88–8.02 (2H, m), 8.08 (1H, dd, J = 7.2, 2.0 Hz), 8.76 (1H, s), 9.42 (1H, s). Anal. calcd for $C_{23}H_{30}N_4O_4$: C, 64.77; H, 7.09; N, 13.14. Found: C, 64.88; H, 7.12; N, 13.01.

3-2[(*tert*-Butyldimethylsiloxy)ethyl]-10-dodecyl-6-nitro-5-deazaflavin (7). A mixture of 10-dodecyl-6-nitro-5-deazaflavin (**6**, 2.13 g, 5.0 mmol), 1-(*tert*-butyldimethylsiloxy)-2-iodoethane[19] (2.86 g, 10.0 mmol), and well pulverized K_2CO_3 (3.46 g, 25.0 mmol) in 20 mL of DMF was warmed at 60 °C for 2 h. The reaction mixture was filtered to remove K_2CO_3 and the filtrate was treated with water, and then was extracted with ether. The organic layer was washed successively with water three times and with brine once. The organic layer was dried over $MgSO_4$ and the solvent was evaporated under reduced pressure. The residue was subjected to column chromatography on silica gel (hexane:ethyl acetate = 1:1 as eluent) to afford yellow solid. Recrystallization from hexane-ethyl acetate to afford 2.40 g (82%) of **7** as yellow crystal: mp 119–121 °C. IR (KBr) 1709, 1655, 1622, 1563, 1526 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 0.05 (6H, s), 0.72–0.96 (12H, m), 1.10–1.40 (16H, m), 1.43–1.61 (2H, m), 1.69–1.95 (2H, m), 3.89 (2H, t, J = 6.4 Hz), 4.25 (2H, t, J = 6.4 Hz), 4.77 (2H, br), 7.85–8.09 (3H, m), 9.37 (1H, s). Anal. calcd for $C_{31}H_{48}N_4O_5Si$: C, 63.67; H, 8.27; N, 9.58. Found: C, 63.71; H, 8.38; N, 9.55.

10-Dodecyl-3-(2-hydroxyethyl)-6-nitro-5-deazaflavin (8). To a solution of 3-[2(*tert*-butyldimethylsiloxy)ethyl]-10-dodecyl-6-nitro-5-deazaflavin (**7**, 1.75 g, 3.0 mmol) in 20 mL of ethanol, was added 1 mL of aqueous 12N hydrochloric acid and the mixture was warmed at 50 °C for 30 min. The solvent was evaporated under reduced pressure and the residue was treated with water and then, was extracted with ethyl acetate. The organic layer was washed with brine and was dried over $MgSO_4$. The solvent was evaporated and the obtained yellow solid was recrystallized from hexane-ethyl acetate to afford 1.16 g (82%) of **8** as yellow fine powder: mp 89–91 °C. IR (KBr) 3420, 1707, 1651, 1620, 1561, 1526, 1217 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 0.88 (3H, t, J = 6.4 Hz), 1.10–1.61 (18H, m), 1.72–1.96 (2H, m), 3.87–4.01 (2H, m), 4.28–4.43 (2H, m), 4.78 (2H, br), 7.88–8.04 (2H, m), 8.08 (1H, dd, J = 7.2, 2.0 Hz), 9.43 (1H, s). Anal. calcd for $C_{25}H_{34}N_4O_5$: C, 63.81; H, 7.28; N, 11.91. Found: C, 63.52; H, 7.43; N, 11.75.

6-(6-Hydroxyhexyl)amino-3-phenyluracil (10). A mixture of 6-chloro-3-phenyluracil (2.22 g, 10.0 mmol) and 6-amino-1-hexanol (2.34 g, 20.0 mmol) in 20 mL of 1-butanol was heated to reflux for 3 h. The reaction solution was cooled to room temperature and the deposited precipitate was collected by filtration. The precipitate was washed with ethanol and was dried under reduced pressure to afford 2.31 g (68%) of **10** (obtained as **10**·2H_2O) as white powder: mp 200–202 °C. IR (KBr) 3335, 1721, 1645, 1586, 1566 cm^{-1}. ^{1}H NMR (Me_2SO-d_6) δ 1.18–1.36 (6H, m), 1.37–1.61 (2H, m), 2.68 (1H, t, J = 7.4 Hz, OH), 2.93–3.10 (2H, m), 3.28–3.48 (2H, m), 4.59 (1H, s), 6.83 (1H, br), 7.06–7.20 (2H, m), 7.23–7.49 (3H, m). Anal. calcd for $C_{16}H_{21}N_3O_3$·2H_2O: C, 56.62; H, 7.42; N, 12.38. Found: C, 56.63; H, 7.61; N, 12.33.

10-(6-Hydroxyhexyl)-6-nitro-3-phenyl-5-deazaflavin (11). A mixture of 6-(6-hydroxyhexyl)-amino-3-phenyluracil (**10**·2H_2O, 1.70 g, 5.0 mmol) and 2-fluoro-6-nitrobenzaldehyde (**5**, 0.85 g, 5.0 mmol)[19] in 15 mL of DMF was

heated at 120 °C for 3 h. The solvent was evaporated under reduced pressure and the residue was treated with water and then, was extracted with chloroform. The organic layer was washed with brine and was dried over $MgSO_4$. The solvent was evaporated under reduced pressure and the residue was subjected to column chromatography on silica gel (chloroform : methanol = 10:1 as eluent). The obtained yellow solid was recrystallized from ethanol to afford 1.56 g (72%) of **11** as yellow crystal: mp 185–187 °C. IR (KBr) 3472, 1715, 1655, 1626, 1563, 1530, 1219 cm^{-1}. ^1H NMR (Me_2SO-d_6) δ 1.30–1.62 (6H, m), 1.66–1.91 (2H, m), 3.35–3.51 (2H, m), 4.35 (1H, t, J = 5.1 Hz, OH), 4.62–4.92 (2H, m), 7.20–7.32 (2H, m), 7.36–7.56 (3H, m), 8.04–8.30 (2H, m), 8.39 (1H, d, J = 8.8 Hz), 9.18 (1H, s). Anal. calcd for $C_{23}H_{22}N_4O_5$: C, 63.59; H, 5.10; N, 12.90. Found: C, 63.54; H, 5.20; N, 12.62.

10-Dodecyl-8-fluoro-3-methyl-5-deazaflavin (13). A mixture of 6-dodecylamino-3-methyluracil[18] (**12**, 3.09 g, 10 mmol) and 2,4-difluorobenzaldehyde (1.71 g, 12.0 mmol) in 30 mL of DMF was heated at 120 °C for 2 h. The reaction mixture was poured into ice-water and deposited precipitate was collected by filtration. The precipitate was washed with water and was dried under reduced pressure. Recrystallization from chloroform-ethanol afforded 3.56 g (86%) of **13** as yellow crystal: mp 221–223 °C. IR (KBr) 1703, 1645, 1620, 1572, 1537 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.88 (3H, t, J = 6.4 Hz), 1.14–1.61 (18H, m), 1.71–1.97 (2H, m), 3.48 (3H, s), 4.48–4.87 (2H, m), 7.18–7.38 (2H, m), 7.95 (1H, dd, J = 8.8, 6.2 Hz), 8.86 (1H, s). Anal. calcd for $C_{24}H_{32}N_3O_2F$: C, 69.71; H, 7.80; N, 10.16. Found: C, 69.68; H, 7.65; N, 10.15.

10-Dodecyl-8-[*N*-(2-hydroxyethyl)-*N*-methylamino]-3-methyl-5-deazaflavin (14). A mixture of 10-dodecyl-8-fluoro-3-methyl-5-deazaflavin (**13**, 2.07 g, 5.0 mmol) and 2-(methylamino)ethanol (1.88 g, 25.0 mmol) in 20 mL of DMF was heated at 100 °C for 1 h. The reaction mixture was poured into ice-water and deposited precipitate was collected by filtration. The precipitate was washed with water and was dried under reduced pressure. Recrystallization from ethanol afforded 1.99 g (85%) of **14** as yellow crystal: mp 210 °C. IR (KBr) 3333, 1680, 1632, 1588, 1561, 1524 cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.88 (3H, t, J = 6.4 Hz), 1.18–1.55 (18H, m), 1.60–1.80 (2H, m), 3.22 (3H, s), 3.39 (3H, s), 3.67–3.78 (2H, m), 3.83–3.92 (1H, m, OH), 3.94–4.13 (2H, m), 4.48 (2H, br), 6.53 (1H, s), 6.86 (1H, dd, J = 9.2, 1.8 Hz), 7.45 (1H, d, J = 9.2 Hz), 8.31 (1H, s). Anal. calcd for $C_{27}H_{40}N_4O_3$: C, 69.20; H, 8.60; N, 11.96. Found: C, 69.28; H, 8.52; N, 11.98.

10-Dodecyl-3-[2-*O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α- and β-D-*galacto*-non-2-ulopyranosylonate)ethyl]-6-nitro-5-deazaflavin (1). To a mixture of 10-dodecyl-3-(2-hydroxyethyl)-6-nitro-5-deazaflavin (**8**, 235 mg, 0.5 mmol), methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-non-2-ulopyranosyl chloride)onate[20] (**9**, 255 mg, 0.5 mmol), and well pulverized molecular sieves 4A (1.2 g) in 10 mL of dry dichloromethane, was added silver trifluoromethanesulfonate (128 mg, 0.5 mmol) and the mixture was stirred in the dark at room temperature under argon stream for 1 h. The catalyst and molecular sieves were removed off by filtration and the filtrate was condensed under reduced pressure. The resulting residue was subjected to thin layer chromatography on silica gel (chloroform:acetone = 6:1 as eluent) to afford 80 mg (16%) of **1a** as yellow amorphous powder and 120 mg (26%) of **1b** as yellow amorphous powder: α-Anomer (**1a**): IR (KBr) 1735, 1650, 1620, 1525 cm^{-1}. $[\alpha]_D^{20}$ −4.1° (c = 1, $CHCl_3$). ^1H NMR ($CDCl_3$) δ 0.88 (3H, t, J = 6.6 Hz), 1.02–1.60 (20H, m), 1.84 (3H, s, NAc), 1.94–2.19 (13H, m, OAc + H_{ax}(3′)), 2.53 (1H, dd, J = 12.9, 4.7 Hz, H_{eq}(3′)), 3.59–3.69 (2H, m), 3.79 (3H, s, CO_2Me), 3.92–4.20 (2H, m, H(5′) + H(6′)), 4.25–4.44 (2H, m, H(9′)), 4.60–4.94 (4H, m), 4.82–4.91 (1H, m, H(4′)), 5.13 (1H, d, J = 9.8 Hz, NH), 5.28–5.50 (2H, m, H(7′) + H(8′)), 7.86–8.00 (2H, m), 8.04 (1H, dd, J = 7.5, 1.2 Hz), 9.37 (1H, s). MS (FAB) m/z: 943 [$(M)^+$]. HRMS (FAB) for $C_{45}H_{61}N_5O_{17}$ [$(M)^+$] calcd 943.4046, found 943.4084. β-Anomer (**1b**): IR (KBr) 1745, 1655, 1630, 1535 cm^{-1}. $[\alpha]_D^{20}$ −7.3° (c = 1, $CHCl_3$). ^1H NMR ($CDCl_3$) δ 0.88 (3H, t, J = 6.7 Hz), 1.08–1.63 (20H, m), 1.89 (3H, s, NAc), 1.92–2.15 (13H, m, OAc + H_{ax}(3′)), 2.22 (1H, dd, J = 12.7, 5.0 Hz, H_{eq}(3′)), 3.77 (3H, s, CO_2Me), 3.81–3.98 (2H, m), 4.02–4.23 (2H, m, H(5′) + H(6′)), 4.47 (1H, dd, J = 10.5, 2.3 Hz, H(9′)), 4.50–4.64 (2H, m), 4.67–4.95 (2H, m), 4.80 (1H, dd, J = 12.3, 2.5 Hz, H(9′)), 5.08-5.21 (1H, m, H(4′)), 5.27–5.36 (1H, m, H(8′)), 5.42–5.50 (1H, m, H(7′)), 6.05 (1H, d, J = 10.2 Hz, NH), 7.88–8.01 (2H, m), 8.05 (1H, dd, J = 7.5, 1.4 Hz), 9.52 (1H, s). MS (FAB) m/z: 943 [$(M)^+$]. HRMS (FAB) for $C_{45}H_{61}N_5O_{17}$ [$(M)^+$] calcd 943.4046, found 943.4046.

10-[6-*O*-(Methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-α- and β-D-*glycero*-D-*galacto*-non-2-ulopyranosylonate)hexyl]-6-nitro-3-phenyl-5-deazaflavin (2). To a mixture of 10-(6-hydroxyhexyl)-6-nitro-3-phenyl-5-deazaflavin (11, 1.09 g, 2.5 mmol), methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-non-2-ulopyranosyl chloride)onate[20] (9, 1.27 g, 2.5 mmol), and well pulverized molecular sieves 4A (6.0 g) in 35 mL of dry dichloromethane, was added silver trifluoromethanesulfonate (0.77 g, 3.0 mmol) and the mixture was stirred in the dark at room temperature under argon stream for 1 h. The catalyst and molecular sieves were removed off by filtration and the filtrate was condensed under reduced pressure. The resulting residue was subjected to thin layer chromatography on silica gel (chloroform:acetone = 2:1 as eluent) to afford 456 mg (20%) of **2a** as yellow amorphous powder and 454 mg (20%) of **2b** as yellow amorphous powder: α-Anomer (**2a**): IR (KBr) 1735, 1700, 1660, 1620, 1525 cm^{-1}. $[\alpha]_D^{20}$ −1.7° (c = 1.0, $CHCl_3$). ^1H NMR ($CDCl_3$) δ 1.20–1.31 (4H, m), 1.40–1.70 (4H, m), 1.87 (3H, s, NAc), 1.97–2.20 (13H, m, OAc + H_{ax}(3′)), 2.59 (1H, dd, J = 12.8, 4.6 Hz, H_{eq}(3′)), 3.20–3.31 (2H, m), 3.70–3.83 (1H, m, H(5′)), 3.81 (3H, s, CO_2Me), 3.98–4.08 (2H, m, H(6′) + H(9′)), 4.33 (1H, dd, J = 12.3, 2.6 Hz, H(9′)), 4.62–4.96 (3H, m, H(4′) + CH_2), 5.23 (1H, d, J = 9.5 Hz, NH), 5.28–5.44 (2H, m, H(7′) + H(8′)), 7.28 (1H, d, J = 8.7 Hz), 7.39–7.58 (5H, m), 7.99 (1H, d, J = 4.7 Hz), 8.03–8.12 (1H, m), 9.47 (1H, s). MS (FAB) *m/z*: 907 [$(M)^+$]. HRMS (FAB) for $C_{43}H_{49}N_5O_{17}$ [$(M)^+$] calcd 907.3110, found 907.3132. β-Anomer (**2b**): IR (KBr) 1735, 1700, 1655, 1620, 1525 cm^{-1}. $[\alpha]_D^{20}$ −13.4° (c = 1, $CHCl_3$). ^1H NMR ($CDCl_3$) δ 1.19–1.38 (4H, m), 1.52–1.81 (4H, m), 1.96 (3H, s, NAc),

1.97–2.20 (13H, m, OAc + $H_{ax}(3')$), 2.51 (1H, dd, $J = 12.8$, 4.9 Hz, $H_{eq}(3')$), 3.30–3.42 (2H, m), 3.55–3.68 (1H, m, H(5′)), 3.80 (3H, s, CO_2Me), 3.97–4.20 (2H, m, H(6′) + H(9′)), 4.64–5.09 (2H, m), 4.90 (1H, dd, $J = 12.3$, 2.6 Hz, H(9′)), 5.20–5.41 (3H, m, H(4′) + H(7′) + H(8′)), 6.38 (1H, d, $J = 9.1$ Hz, NH), 7.28 (1H, d, $J = 8.1$ Hz), 7.38–7.58 (5H, m), 7.99 (1H, d, $J = 3.5$ Hz), 8.03–8.18 (1H, m), 9.45 (1H, s). MS (FAB) m/z: 907 [$(M)^+$]. HRMS (FAB) for $C_{43}H_{49}N_5O_{17}$ [$(M)^+$] calcd 907.3110, found 907.3101.

10-Dodecyl-3-methyl-8-[*N*-methyl-*N*-[2-*O*-(methyl 5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-*glycero*-α- and β-D-*galacto*-non-2-ulopyranosylonate)ethyl]amino]-5-deazaflavin (3). To a mixture of 10-dodecyl-8-[*N*-(2-hydroxyethyl)-*N*-methylamino]-3-methyl-5-deazaflavin (**14**, 234 mg, 0.5 mmol), methyl (5-acetamido-4,7,8,9-tetra- *O*-acetyl-3,5-dideoxy-D-*glycero*-β-D-*galacto*-non-2-ulopyranosyl chloride)onate[20] (**9**, 255 mg, 0.5 mmol), and well pulverized molecular sieves, 4A (1.2 g) in 10 mL of dry dichloromethane, was added silver trifluoromethanesulfonate (128 mg, 0.5 mmol) and the mixture was stirred in the dark at room temperature under argon stream for 1 h. The catalyst and molecular sieves were removed off by filtration and the filtrate was condensed under reduced pressure. The resulting residue was subjected to thin layer chromatography on silica gel (chloroform:acetone = 6:1 as eluent) to afford 104 mg (22%) of **3a** as yellow amorphous powder and 160 mg (34%) of **3b** as yellow amorphous powder: α-Anomer (**3a**): IR (KBr) 1740, 1685, 1625, 1590, 1525 cm^{-1}. $[\alpha]_D^{20}$ −0.8° ($c = 1$, $CHCl_3$). ^1H NMR ($CDCl_3$) δ 0.88 (3H, t, $J = 6.6$ Hz), 1.07–1.58 (20H, m), 1.88 (3H, s, NAc), 1.94–2.19 (13H, m, OAc + $H_{ax}(3')$), 2.55 (1H, dd, $J = 12.8$, 4.6 Hz, $H_{eq}(3')$), 3.21 (3H, s), 3.46 (3H, s), 3.63 (3H, s, CO_2Me), 3.68–3.81 (2H, m), 3.86–3.97 (1H, m, H(5′)), 3.98–4.12 (2H, m, H(6′) + H(9′)), 4.19 (1H, d, $J = 13.6$ Hz, H(9′)), 4.42–4.95 (4H, m), 4.78–4.90 (1H, m, H(4′)), 5.23–5.42 (3H, m, H(7′) + H(8′) + NH), 6.54 (1H, s), 6.95 (1H, dd, $J = 9.1$, 2.0 Hz), 7.67 (1H, d, $J = 9.0$ Hz), 8.66 (1H, s). MS (FAB) *m/z*: 941 [$(M)^+$]. HRMS (FAB) for $C_{47}H_{67}N_5O_{15}$ [$(M)^+$] calcd 941.4616, found 941.4650. β-Anomer (**3b**): IR (KBr) 1740, 1685, 1625, 1590, 1525 cm^{-1}. $[\alpha]_D^{20}$ −5.1° ($c = 1$, $CHCl_3$). ^1H NMR ($CDCl_3$) δ 0.88 (3H, t, $J = 6.6$ Hz), 1.17–1.58 (20H, m), 1.97 (3H, s, NAc), 2.00–2.20 (13H, m, OAc + $H_{ax}(3')$), 2.43 (1H, dd, $J = 12.9$, 5.0 Hz, $H_{eq}(3')$), 3.26 (3H, s), 3.42 (3H, s), 3.58–3.72 (2H, m), 3.74 (3H, s, CO_2Me), 3.83 (1H, dd, $J = 10.5$, 2.2 Hz, H(5′)), 3.88–4.06 (2H, m, H(6′) + H(9′)), 4.47–4.68 (4H, m), 5.02 (1H, dd, $J = 12.3$, 2.3 Hz, H(9′)), 5.10–5.25 (3H, m, H(4′) + H(7′) + NH), 5.28–5.32 (1H, m, H(8)), 6.64 (1H, s), 7.08 (1H, dd, $J = 9.2$, 1.9 Hz), 7.72 (1H, d, $J = 9.2$ Hz), 8.56 (1H, s). MS (FAB) *m/z*: 941 [$(M)^+$]. HRMS (FAB) for $C_{47}H_{67}N_5O_{15}$ [$(M)^+$] calcd 941.4616, found 941.4603.

Hydrolysis rate of (nitro) 5-deazaflavin-sialic acid conjugate molecules

Solutions (5 mL) of compounds **1**–**3** (5.0×10^{-3} (M)) in aqueous 50% acetic acid were placed in sealed tubes under nitrogen and were heated at 100 °C. The reaction solutions were analyzed directly by an HPTLC (Merck Kieselgel HPTLC (F254) Plate, Art. 13728) and the concentration of resulting compound **8**, **11**, or **14** was estimated by scanning at 254 nm with CAMAG TLC Scanner 3 (CAMAG Chemie-Erzeugnisse und Adsorptionstechnik AG).

In vitro antitumor activities of (nitro) 5-deazaflavin-sialic acid conjugate molecules

The tetrazolium-based semiautomated colorimetric assay (MTT assay) developed by Carmichael[29] 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.[30]

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25. Estimated $\Delta\delta|H(9')\text{-}H(9)|$ values of the compounds **1**–**3**: **1a**, <0.19 ppm; **1b**, 0.33 ppm; **2a**, 0.25–0.35 ppm; **2b**, 0.70–0.93 ppm; **3a**, 0.07–0.21 ppm; **3b**, 0.96–1.14 ppm.
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31. Antitumor effects of deprotected compounds of **1**–**3** on KB and L1210 cells have been found to be much weaker ($IC_{50} > 100\,\mu M$) than those of **1**–**3**. This result would probably be accounted by lower permeability of the deprotected compounds of **1**–**3** into tumor cells. Similar examples of antitumor activities of protected and deprotected sialosylphenyl mitomycin C derivatives are reported in ref 12.