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Synthesis and properties of double-stranded RNA-bindable oligodiaminogalactose derivatives conjugated with vitamin E

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ABSTRACT

RNA interference (RNAi) is a gene-regulating system that is controlled by external short interfering RNAs (siRNAs). Sequence selective gene silencing by siRNA shows promise in clinical research. However, there have been few efficient methods for delivering siRNAs to target cells. In this study, we propose a novel type of RNA duplex-bindable molecule with an oligodiaminosaccharide structure. These 2,6-diamino-2,6-dideoxy-(1-4)-β-D-galactopyranose oligomers (oligodiaminogalactoses; ODAGals) conjugated with α-tocopherol (vitamin E; VE) or a VE analog were designed as novel siRNA-bindable molecules that can be utilized to deliver RNAi drugs to the liver. Among these compounds, the VE analog-bound ODAGal was suggested to bind to RNA duplexes without inhibiting RNAi activity.

1. Introduction

Since the discovery of RNA interference (RNAi),[1] short interfering RNAs (siRNAs) have been receiving a lot of attention as candidates for next-generation drugs.[2–4] SiRNAs are composed of double-stranded RNA. Because they target complementary sequences of mRNAs, siRNAs promise high target specificity. Furthermore, RNA molecules are smaller than antibody drugs and can cross cell membranes using a suitable drug delivery system (DDS). In principle, therefore, siRNAs can be delivered to any tissue and target mRNA in any cell. However, DDSs for nucleic acid drugs are far from established.

For the effective transfection and DDS of siRNAs, a variety of methods have previously been reported, including viral and non-viral delivery methods. Viral delivery is much effective, but there are problems of cytotoxicity and immunological response.[5] Non-viral delivery strategies have included the use of a variety of RNA-conjugates and carriers, such as cationic lipids, polymers, and other molecules.[6–8] Among these strategies, cationic carriers are one of the dominant methods, and cationic polymers, represented by Lipofectamine™, have been widely used for in vivo experiments. However, these cationic carriers generally need to be used in large doses to form nanoparticles, and suffer from cytotoxicity because of the increased quantities of cations.[9–11]

We previously reported that 'oligodiaminosaccharides,' which have amino groups at the 2- and 6-positions of α-1-4 linked oligo-D-glucose, exhibit A-type nucleic acid duplex-binding properties.[12] These 'oligodiaminoglucoses (ODAGlcs)' can specifically interact with RNA duplexes rather than B-type DNA duplexes. The 4mer of an ODAGlc can bind to the 12mer of an RNA duplex in approximately a stoichiometric ratio. Thus, these RNA duplex-specific-binding oligodiaminosaccharides can be useful as a component of siRNA carriers.

Herein we report the synthesis of a novel type of oligodiaminosaccharide conjugated with vitamin E or its analogs that can be useful as a carrier of siRNA drugs. Vitamin E (VE), known as α-tocopherol, is a fat-soluble vitamin. Such lipophilic compounds are transported to liver cells with chylomicrons after absorption from the small intestine. The liver-endocytosed vitamin E is drawn to the cytosol of liver cells by α-tocopherol transfer protein (α-TTP).[13]

Previously, a vitamin E-conjugated siRNA was reported to be efficiently transported to mouse liver cells. Importantly, vitamin E had very low cytotoxicity,[14] and the vitamin E-conjugated siRNA did not show any side effects.[15]

In the present study, we attempted to construct vitamin E-siRNA conjugates through noncovalent interaction with oligodiaminosaccharides. The noncovalent approach can prevent vitamin E

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from the sterically hindering the RNAi process if the binding molecules become disassociated from the siRNA in cytoplasm. Conjugates of vitamin E and its analogs with 2,6-diamino-2,6-dideoxy-β-D-oligogalactopyranoside are expected to have similar structures and functions as those of ODAGlcs. We thus propose these 'oligodiaminogalactoses (ODAGals)' as novel RNA duplex-bindable molecules.

It has been previously reported that vitamin E is recognized by α-TTP at the chroman ring, rather than at the alkyl chain.[16,17] Thus, we synthesized two types of ODAGal derivatives: one ODAGal derivative was conjugated with native vitamin E, whose phenolic hydroxy group was used to covalently bind vitamin E to the ODAGal moiety, and the other ODAGal derivative was conjugated with a VE analog whose phenolic hydroxyl group was preserved.

2. Results and discussion

2.1. Preparation of the glycosyl donor

To construct the β-linked oligodiaminogalactose structure via glycosylation, an adequately designed glycosyl donor was synthesized from the known galactosamine derivative **1**, which was synthesized by the procedure described in the literature.[18] Next, the 6-OH group of **1** was converted to a phthalimide group via the Mitsunobu reaction to afford **2.**[19] The 3-OH group of **2** was then selectively protected with a *p*-methoxybenzyl (PMB) group using dibutyltin oxide to obtain **3**.[20] Finally, the 4-OH group of **3** was chloroacetylated to afford the glycosyl donor **4** (Scheme 1).

2.2. Elongation of sugar chains

In the glycosylation reaction, commonly used *N*-iodosuccinimide and trifluoromethanesulfonic acid were employed for activation of the thiophenyl glycoside **4**. However, when using dichloromethane or dichloromethane–diethyl ether (1:1, v/v) as the solvent, partial removal and iodination of the PMB group were observed (Scheme 3). Furthermore, the iodinated PMB group was more stable and difficult to remove than the unmodified PMB group under acidic conditions (data not shown). In this study, these side reactions were inhibited using dichloromethane-diethyl ether (1:3, v/v) as the solvent, and the glycosylation reaction proceeded in good yield. Dechloroacetylation was then carried out under weak basic conditions, as shown in Scheme 1. When the 4-O-acetylated glycosyl donor was used, however, the acetyl group could not be selectively removed under basic, acidic, or enzymatic conditions. Thus, by repeating the synthetic cycle including glycosylation and dechloroacetylation, the trimer of ODAGal **7** was obtained (Scheme 2).

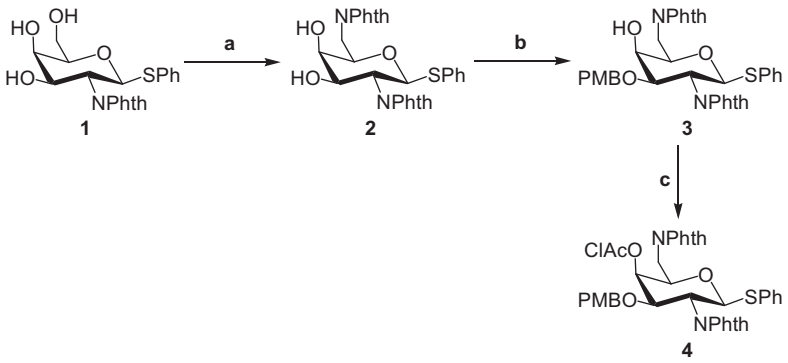
The PMB groups of trisaccharide **7** were removed in good yield under acidic conditions using trifluoroacetic acid, although a minor amount of cleavage of the glycoside bond occurred as a side reaction. It is noteworthy that in the case of 3-*O*-benzyl trisaccharide, the benzyl groups were not efficiently removed. In reductive reactions, such as catalytic reduction using palladium on carbon, the reaction rate was very slow, and two of the benzyl groups remained even after one day. Under acidic conditions, the desired compound was not obtained because nealy all of the glycoside bonds were cleaved.

As shown in Schemes 4–6, the trisaccharide bromide **8** was azidated and conjugated with a propargylated vitamin E (**11**), vitamin E analog (**16**[17]), and the 4-methoxytriphenylmethyl (MMTr) group (**19**) via the Huisgen reaction[21] to obtain triazole-linked trisaccharides **13**, **17** and **20**, respectively. The phthalimide groups of **13** and **17** were then removed by treatment with hydrazine monohydrate and the products were purified by reverse-phase HPLC to afford the VE and VE analog conjugated ODAGal derivatives **14** and **18**. Separately, the MMTr group was removed following dephthaloylation of **20** to obtain the non-conjugated ODAGal **21**.

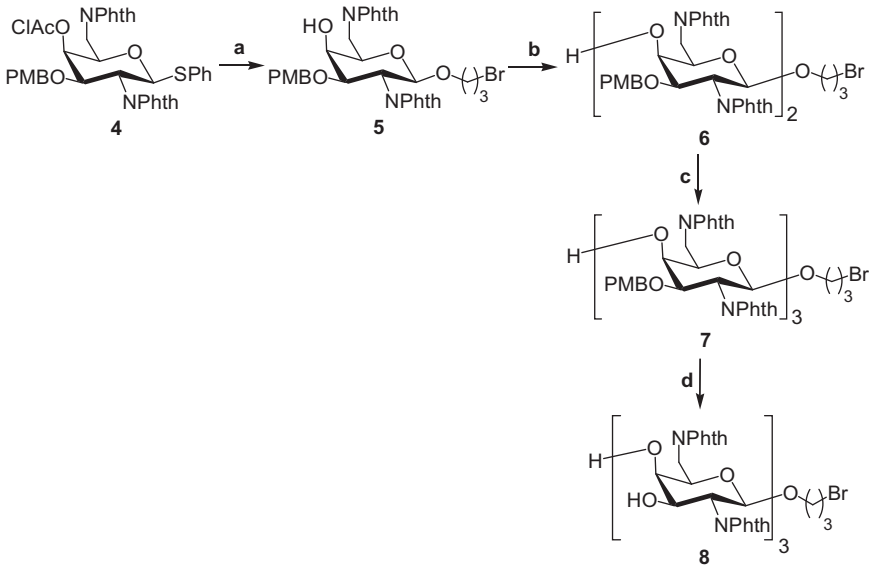
2.3. Evaluation of the interactions between the ODAGal derivatives and RNA–RNA duplexes

To evaluate whether ODAGals can interact with and induce structural changes or thermodynamic stabilization of RNA duplexes, UV melting, CD spectrometry, and fluorescence anisotropy measurements were carried out. All of the experiments were performed under near to physiological conditions with a 10 mM phosphate buffer containing 100 mM NaCl at pH 7.0. Figures 1 and 2 present the results of the UV melting analyses of the non-conjugated ODAGal **21** complexed with the RNA duplexes (5′-rCGCGAAUUCGCG-3′)$_2$ (RNA-I) and (5′-rAAAAAAUUUUUU-3′)$_2$ (RNA-II) (1 equiv of **21** was added to the RNA duplex solution) respectively. The melting temperatures (T_m) for the two systems increased by 2.0 °C and 2.2 °C, respectively (Table 1). These results suggest that the ODAGal moiety did interact with the RNA duplexes, and thermodynamically stabilized them. On the other hand, when 4 equiv of **21** were added to the RNA duplex solutions, the T_m values were slightly decreased. These results suggest that an excess amount of ODAGal thermodynamically destabilizes the duplexes, or aggregation of **21** occurs at such a high concentration.

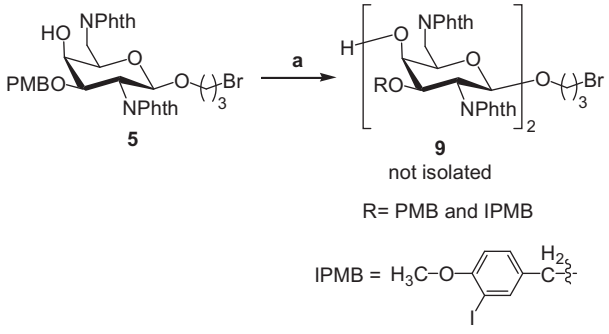
The UV melting curves for the complexes of VE-bound ODAGal **14** with RNA-I and RNA-II are shown in Figures 3 and 4, respectively. Although the curves for both RNA-I and RNA-II were significantly changed, an increase in the T_m values was not observed in either case. In addition, when 4 equiv of **14** were added, the T_m values for RNA-I decreased and the UV melting curve showed an



Scheme 1. Synthesis of the glycosyl donor **4**. Reagents and conditions: (a) DIAD, phthalimide, PPh3, THF, rt, 1 h, 71%; (b) (i) Bu2SnO, toluene, reflux, 12.5 h, (ii) PMBCl, TBAI, toluene, reflux, 5.5 h, 93% over two steps; (c) ClAcCl, pyridine, 0 °C, 30 min, 93%.



Scheme 2. Synthesis of the protected tri-diaminogalactose **8**. Reagents and conditions: (a) (i) NIS, TfOH, 3-bromo-1-propanol, CH_2Cl_2, 0 °C, 20 min, (ii) NaOMe, CH_2Cl_2–MeOH, 0 °C, 12.5 h, 85% over 2 steps; (b) (i) **4**, NIS, TfOH, CH_2Cl_2–Et_2O, 0 °C, 1.5 h, (ii) NaOMe, CH_2Cl_2–MeOH, 0 °C, 13 h, 79% over two steps; (c) (i) **4**, NIS, TfOH, CH_2Cl_2–Et_2O, 0 °C, 1.5 h, (ii) NaOMe, CH_2Cl_2–MeOH, 0 °C, 14 h, 77% over two steps; (d) 10%TFA in CH_2Cl_2, 0 °C, 2 h, 71%.



Scheme 3. Side reaction in glycosylation reaction.

abnormal shape, while the T_m value for RNA-II could not be determined. These results indicate that, unlike the non-conjugated ODAGal **21**, the VE-bound **14** did not bind efficiently to the RNA duplexes.

In contrast, the VE analog-bound ODAGal **18** stabilized the RNA duplexes. As shown in Figures 5 and 6, although the addition of 1 equiv of **18** induced no significant change in the UV melting curves, an increase in the melting temperatures was observed when 4 equiv of **18** were added to the RNA solutions. In this case, the T_m values increased by 2.7 °C for RNA-I and 9.4 °C for RNA-II. These results suggest that the VE analog-bound ODAGal **18** interacts with the RNA duplexes in a manner similar to that of ODAGlc and non-conjugated ODAGal. However, for RNA-II, a second flexion point was observed at 50–60 °C. This phenomenon can be attributed to other events rather than dissociation of the duplex.

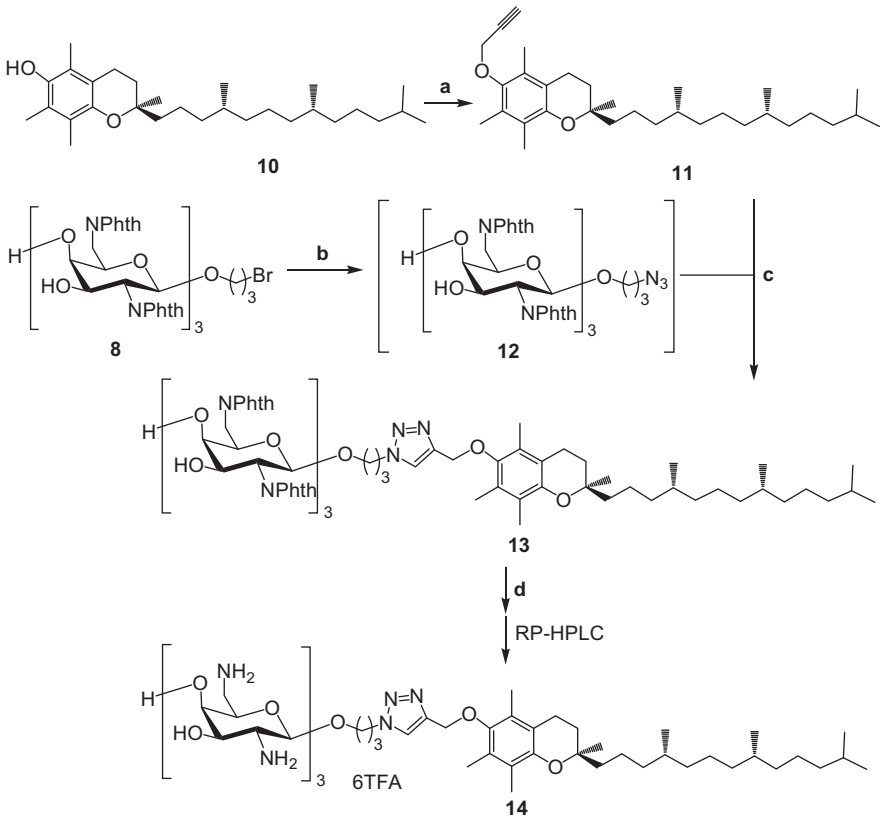
This difference in the properties of the VE-bound ODAGal **14** and VE analog-bound ODAGal **18** likely result from steric hindrance near the oligodiaminogalactose moiety. In the case of VE-bound ODAGal **14**, the methyl groups on the aromatic ring are in proximity to the ODAGal moiety and prevent it from interacting with the RNA duplexes. On the other hand, the VE analog-bound ODAGal **18** is less sterically hindered and advantageous for RNA binding.

Next, to detect the structural changes in the RNA duplexes, CD spectra were measured for RNA-I and VE analog-bound ODAGal **18**, which possibly binds to the duplex. Figure 7 shows the CD spectra of RNA-I in the presence and absence of **18**. Upon addition of VE analog-bound ODAGal **18**, changes in the spectra of the RNA–RNA duplex were observed. The positive peak near 265 nm shifted 1–2 nm to a longer wavelength, its peak intensity increased, and the molar ellipticity near 230 nm continuously changed. These changes in the peak at 265 nm are very similar to those observed in our previous study upon addition of ODAGlcs to RNA duplexes.[12] On the basis of the combined results for UV melting and CD analyses, it can be concluded that the VE analog-bound ODAGal **18** interacts with RNA duplexes in a manner similar to that for ODAGlcs.

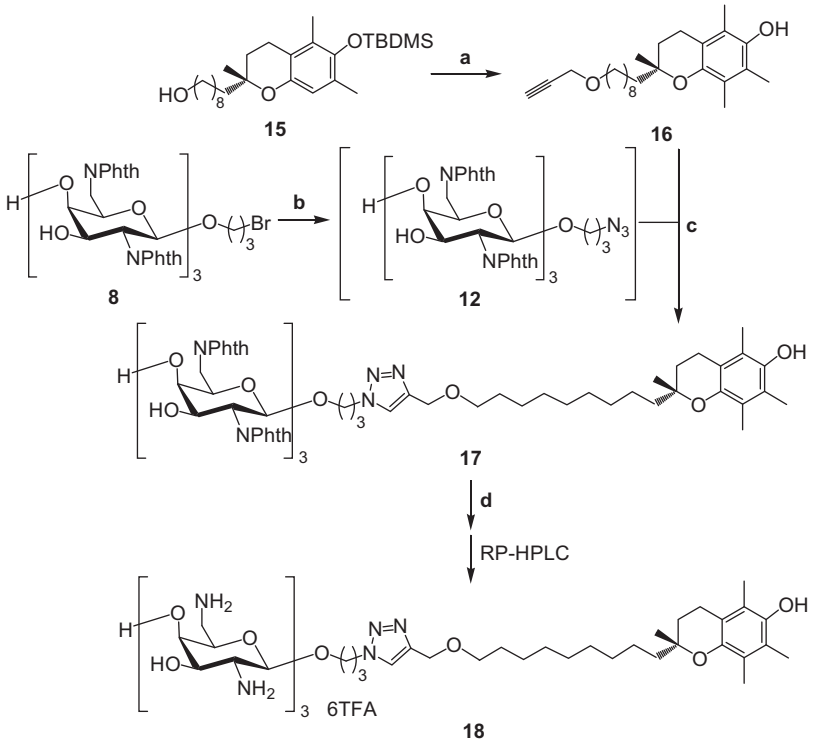
Finally, we measured the binding affinity of the VE derivative-bound ODAGals for RNA duplexes using direct fluorescence anisotropy titration. A fluorophore-labeled RNA duplex (5′-FAM-CGCGAAUUCGCG)$_2$ was used in this experiment. Figure 8 clearly shows that the VE analog-bound ODAGal **18** binds to the RNA duplex with K_d 3.8 ± 1.2 ($\times 10^{-8}$ M). On the other hand, the affinity of VE-bound ODAGal **14** for the the RNA duplex could not be confirmed from the titration (see SI). In addition, with **14**, the observed values did not converge and anisotropy could not be measured at more than 0.5 μM **14**.

2.4. Evaluation of RNAi activity in the presence of ODAGal derivatives

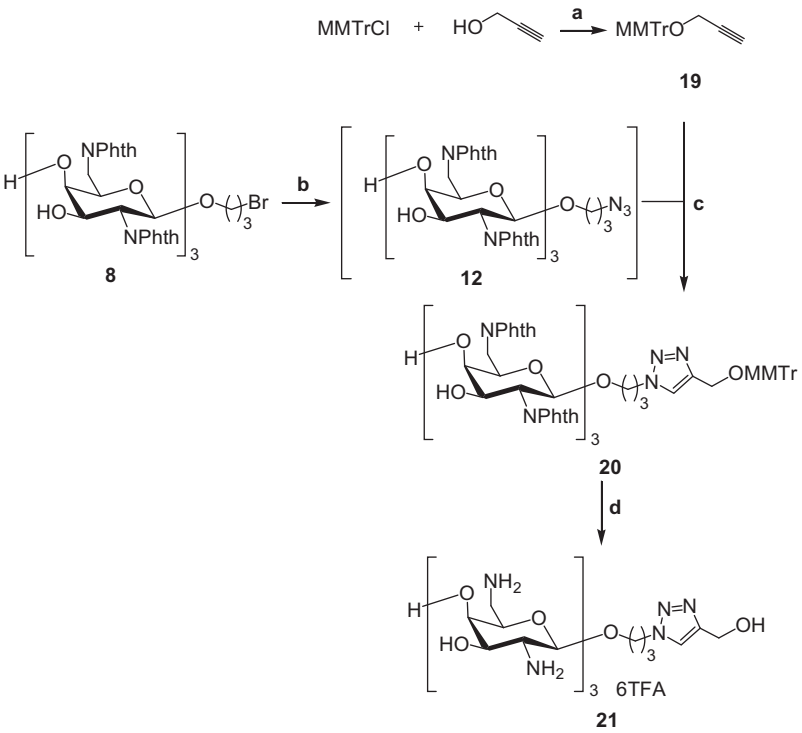
On the basis of the above results, it can be concluded that at least a portion of the ODAGal derivatives interact and form complexes with RNA duplexes. Therefore, experiments designed to evaluate RNA interference in the presence of the ODAGal derivatives were performed using an siRNA that targets apoB1 mRNA.[22] In these experiments, only the VE analog-bound ODAGal **18** was used because it was determined that the VE-bound ODAGal **14** had no or only a weak binding ability with RNA duplexes. Before the RNAi experiments were conducted, UV melting analyses of the siRNA r(5′-GUCAUCACACUGAAUACCAAU-3)-r(3′-CACAGUAGU GUGACUUAUGGU-5′) with added **18** were carried out. Interestingly, it was observed that the T_m value of the RNA was only slightly increased upon addition of **18** (see SI). These results are likely to derive from the high T_m value of the siRNA (71 °C). It has been previously shown that the oligodiaminosaccharides



Scheme 4. Synthesis of the VE bound ODAGal **14**. Reagents and conditions: (a) 3-bromopropyne, NaH, DMF, rt, 20 min, 82%; (b) NaN_3, DMF, 80 °C, 15 h; (c) Cu powder, *t*-BuOH–water, 80 °C, 19 h, 66% over two steps from **8**; (d) 3%$NH_2NH_2{\cdot}H_2O$, EtOH, 80 °C, 4.5 h, 29%.



Scheme 5. Synthesis of the VE analog bound ODAGal **18**. Reagents and conditions: (a) (i) 3-bromopropyne, NaH, THF, 60 °C, 6 h, (ii) 1 M TBAF, THF, rt, 30 min, 75% over two steps; (b) NaN_3, DMF, 80 °C, 12 h; (c) Cu powder, *t*-BuOH–water, 80 °C, 3.5 h, 91% over two steps from **8**; (d) 3%$NH_2NH_2{\cdot}H_2O$, EtOH, 90 °C, 4.5 h, 59%.



Scheme 6. Synthesis of the VE unbound ODAGal **21**. Reagents and conditions: (a) pyridine, rt, 3 d, 82%; (b) NaN_3, DMF, 80 °C, 12 h; (c) Cu powder, *t*-BuOH–water, 80 °C, 3.5 h, quant over two steps from **8**; (d) (i) 3%$NH_2NH_2{\cdot}H_2O$, EtOH, 90 °C, 4 h, (ii) 80%AcOHaq, 11% over two steps.

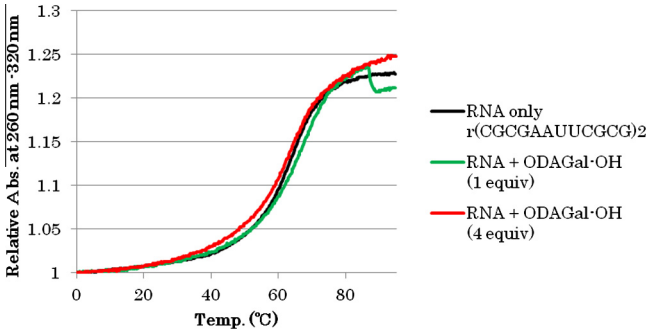


Figure 1. UV melting curves of (5′-rCGCGAAUUCGCG-3′)2 in the presence of VE unbound ODAGal **21**.

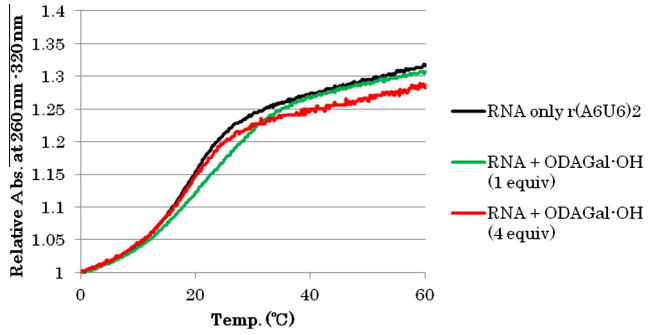


Figure 2. UV melting curves of (5′-rAAAAAAUUUUUU-3′)2 in the presence of VE unbound ODAGal **21**.

increase the T_m values of RNA duplexes that have low T_m values, and not high T_m values.[12] Next, RNAi activity in the presence and absence of the ODAGal derivative **18** was determined, and the results are shown in Figure 9.

Table 1
Melting temperatures of RNA duplexes in the presence of ODAGal derivatives

		RNA-I		RNA-II	
		T_m (°C)	ΔT_m (°C)	T_m (°C)	ΔT_m (°C)
	RNA only	19.7	—	63.2	—
21	1 equiv	21.9	2.2	65.2	2.0
	4 equiv	18.8	−0.9	62.4	−0.8
14	1 equiv	19.8	0.1	62.5	−0.7
	4 equiv	17.6	−2.1	n.d.	—
18	1 equiv	20.1	0.4	63.8	0.6
	4 equiv	29.1	9.4	65.9	2.7

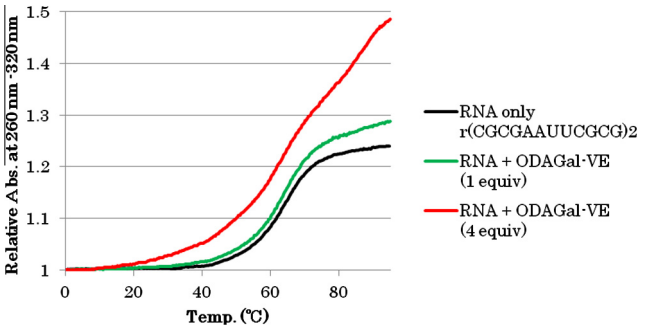


Figure 3. UV melting curves of (5′-rCGCGAAUUCGCG-3′)2 in the presence of VE bound ODAGal **14**.

The level of apoB1 mRNA was evaluated using a quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). The cell lines showed nearly the same RNAi activity regardless of the addition of the ODAGal derivative. This result confirms that the ODAGal did not affect the RNAi activity. Although there is room for further discussion as to whether the ODAGal derivative **18**

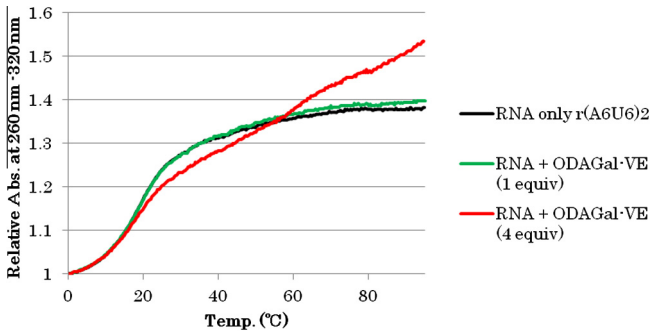


Figure 4. UV melting curves of (5′-rAAAAAAUUUUUU-3′)2 in the presence of VE unbound ODAGal **14**.

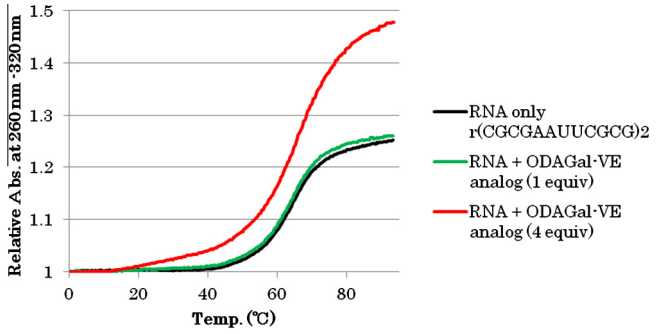


Figure 5. UV melting curves of (5′-rCGCGAAUUCGCG-3′)2 in the presence of VE analog bound ODAGal **18**.

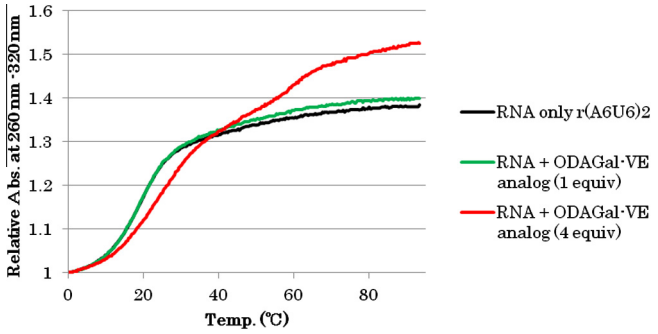


Figure 6. UV melting curves of (5′-rAAAAAAUUUUUU-3′)2 in the presence of VE analog bound ODAGal **18**.

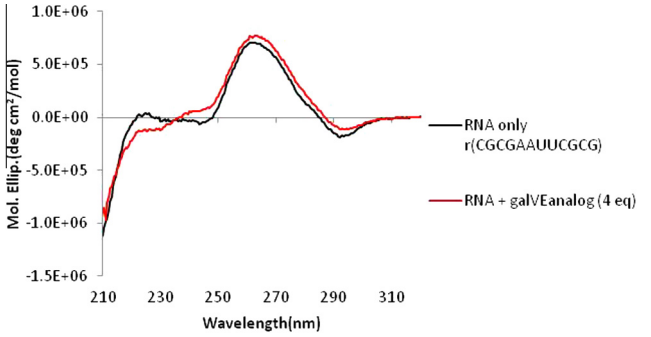


Figure 7. UV melting curves of (5′-rCGCGAAUUCGCG-3′)2 in the presence of VE analog bound ODAGal **18**.

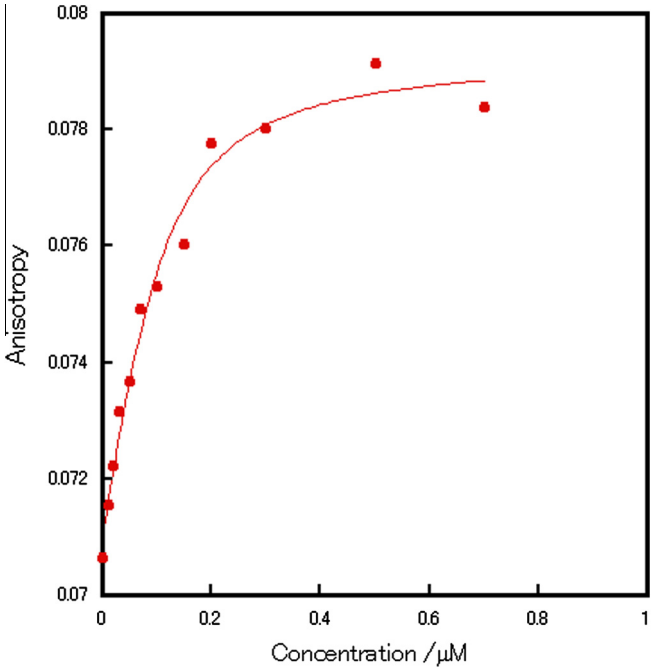


Figure 8. Fluorescence anisotropy of 100 nM of 5′-FAMlabeled RNA duplex was titrated by increaseing concentration of VE analog-bound ODAGal **18** at 20 °C. The formation of the **18**-RNA complex is reflected an increase in the observed anistropy values.

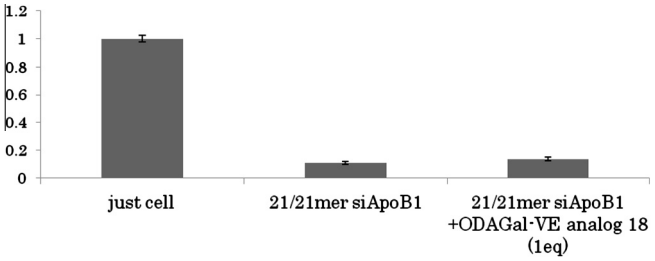


Figure 9. Evaluation of siRNA activity in the presence of ODAGal derivatives; Cell line name: McA-RH7777; Transfection reagent: Lipofectamine 2000 in culture cells of McA-RH7777. m ± SEM Sense Sequence: 5′-GUCAUCACACUGAAUACCAAU-3′ Antisense Sequence: 3′-CACAGUAGUGUGACUUAUGGU-5′.

binds to siRNAs, it appears that the compound does not inhibit the process of RNA interference, such as RISC formation.

3. Conclusion

Novel oligodiaminosaccharides, 2,6-diamino-2,6-dideoxy-β-D-oligogalactopyranoside derivatives conjugated with VE and its analog, were synthesized. The analysis of UV melting and CD spectral observations suggests that, like ODAGlcs, the ODAGal moiety efficiently interacts with and thermodynamically stabilizes RNA duplexes with small structural changes, although steric hindrance likely affects their binding ability. RNA interference experiments also demonstrated that the addition of the VE analog-bound ODAGal **18** to an siRNA did not affect the RNAi activities. This VE analog has recognition sites for α-TTP, and thus, the ODA derivative **18** may have potential as a useful carrier of RNAi drugs with the ability to bind to RNA duplexes without inhibiting RNAi activity. Further in vitro and in vivo experiments, including siRNA delivery to liver cells are now in progress.

4. Experimental section

4.1. General methods and materials

All reactions were carried out under an atmosphere of argon. ^{1}H NMR spectra were obtained at 300 MHz on a Varian MERCURY 300 spectrometer with tetramethylsilane (TMS) as an internal standard in $CDCl_3$. ^{13}C NMR spectra were obtained at 75.45 MHz on a Varian MERCURY 300 spectrometer with $CDCl_3$ as an internal standard (δ 77.0) in $CDCl_3$. Mass spectra were recorded on Voyager System 4327 (Applied Biosystem). Analytical TLC was performed on Merck Kieselgel 60-F254 plates. Melting curves of nucleic acid duplexes were recorded on a UV-1650PC UV–visible spectrophotometer (Shimadzu). CD spectra were measured on a J-725 spectropolarimeter (JASCO). Fluorescence anisotropies were recorded on a FP-6500 spectrofluorometer (JASCO). Silica gel column chromatography was carried out using Silica gel 60 N (63–210 μm or 40–50 μm). Reversed phase HPLC was carried out using a μBondasphere 5-μm C4 or C18, 100 Å, 4 mm × 150 mm (Waters) with a gradient of 0–100% acetonitrile in water at 25 °C at a rate of 0.5 mL/min and acetonitrile and water is buffered by TFA (trifluoroacetic acid) with 0.05% v/v. Organic solvents were purified and dried by the appropriate procedure. RNA oligomers were purchased from Hokkaido System Science Co., Ltd and Japan Bio Services Co., Ltd. The yields of compounds were calculated from their dry weights except for **14**, **18** and **21**. The yields of **14** and **18** were estimated from absorbance compared with that of corresponding vitamin E derivatives. The yield of **21** was estimated from absorbance of $MMTr^+$ generated by deprotection.

4.1.1. Conditions for the UV melting analyses

Absorbance versus temperature profile measurements were carried out with eight-sample cell changer, in quarts cells of 1 cm pathlength. The variation of the difference of UV absorbance at between 260 nm and 320 nm with temperature was monitored. The temperature was scanned between 0 °C and 95 °C, and the rate of temperature increase was 0.2 °C/min. Oligonucleotides were annealed after addition of amino sugars. The samples containing oligonucleotides and amino sugars were first rapidly heated to 95 °C, left for 20 min, and then allowed to cool slowly to room temperature. These samples were furthermore cooled to 0 °C and left for 1 h, and then the dissociation was recorded by heating to 95 °C at rate of 0.2 °C/min.

4.1.2. Conditions for the CD spectrometry

All of the CD spectra were recorded at 25 °C. The following instrument settings were used: resolution, 0.1 nm; sensitivity, 10 mdeg; response, 4 s; speed, 10 nm/min; accumulation, 6.

4.1.3. Conditions for the fluorescence anisotropy measurement

The all titrations were measured at 20 °C. The following instrument settings were used: Ex/Em = 490 nm/520 nm; response, 2 s; Band width (Ex), 5 nm; Band width (Em), 5 nm; PMT voltage, 450 V; No. of cycle, 4;

4.2. Phenyl 2,6-dideoxy-2,6-diphthalimimde-1-thio-β-D-galactopyranoside (2)

Compound **1** (6.74 g, 16.8 mmol), triphenylphosphine (8.81 g, 33.6 mmol, 2.0 equiv), and phthalimide (4.94 g, 33.6 mmol, 2.0 equiv) were dissolved in dry tetrahydrofurane (168 mL) and cooled to 0 °C. To this mixture, diisopropyl azodicarboxylate (6.5 mL, 33.6 mmol, 2.0 equiv) was added dropwise over 20 min. After 40 min, further diisopropyl azodicarboxylate (3.0 mL) was added. After 30 min, methanol (20 mL) was added to the mixture and the solvent was evaporated. The crude product was recrystallized from methanol (50 mL) and then from ethyl acetate-hexane (1:5, v/v, 180 mL), and **2** was obtained as a colorless solid (6.28 g, 11.8 mmol, 71%).

^{1}H NMR ($CDCl_3$) δ 7.91–7.69 (m, 8H, N*Phth*), 7.39–7.34 (m, 2H, S*Ph*), 7.20–7.10 (m, 3H, S*Ph*), 5.59 (d, J = 10.2, 1H, H-1), 4.48–4.32 (m, 2H, H-2, H-3), 4.19–4.12 (m, 1H), 4.00–3.83 (m, 3H, 4-H), 3.62 (d, J = 5.1, 1H, 4-OH), 2.68 (d, J = 10.8, 1H, 3-OH).

^{13}C NMR ($CDCl_3$) δ 168.7, 134.6, 134.1, 132.7, 132.0, 131.8, 131.7, 131.6, 128.8, 127.9, 123.8, 123.7, 123.3, 84.0, 75.1, 68.6, 67.8, 53.1, 37.3.

MALDI-TOF MS: m/z calcd for $C_{36}H_{30}N_2NaO_8S$ $[M+Na]^+$: 553.10 Found: 553.11.

4.3. Phenyl 2,6-dideoxy-2,6-diphthalimimde-3-*O*-*p*-methoxybenzyl-1-thio-β-D-galactopyranoside (3)

Compound **2** (6.28 g, 11.8 mmol) was coevaporated with dry toluene (10 mL × 4) and dissolved in dry toluene (200 mL). Dibutyltin oxide (3.25 g, 13.0 mmol, 1.1 equiv) was added to the solution and it was refluxed for 12.5 h. Tetrabutylammonium iodide (4.81 g, 13.0 mmol, 1.1 equiv) and *p*-methoxybenzyl chloride (1.55 mL, 13.0 mmol, 1.1 equiv) was added. After 5.5 h, the mixture was cooled to rt and concentrated to dryness. The crude product was purified by silica gel column chromatography (dichloromethane (1.5% methanol)). The purified product was dissolved in toluene–dichloromethane–ethyl acetate (8:1:1, v/v/v, 500 mL) and washed with water (200 mL × 3). The product was further purified by silica gel column chromatography (dichloromethane (1.5% methanol)) and **3** was obtained as a colorless foam (7.35 g, 11.0 mmol, 93%).

^{1}H NMR ($CDCl_3$) δ 7.88–7.63 (m, 8H, N*Phth*), 7.23–7.16 (m, 2H, Ar), 6.97–6.82 (m, 5H, Ar), 6.45 (d, J = 8.7, 2H, Ar), 5.36 (d, J = 8.7, 1H, H-1), 4.57–4.50 (m, 2H, ArCH_2-a, H-2), 4.43–4.36 (m, 1H, H-5), 4.27–4.21 (m, 2H, ArCH_2-b, H-3), 4.08 (s, 1H, 4-H), 4.01–3.97 (m, 1H, H-6a), 3.82–3.76 (m, 1H, H-6b), 3.65 (s, 3H, CH_3OPh), 2.74 (d, J = 0.9, 1H, 4-OH).

^{13}C NMR ($CDCl_3$) δ 168.1, 167.9, 167.3, 159.1, 134.0, 133.9, 133.8, 132.9, 131.9, 131.8, 131.6, 131.5, 129.6, 129.0, 128.3, 127.6, 123.4, 123.1, 113.6, 83.7, 75.0, 74.9, 71.0, 66.0, 54.9, 50.7, 38.9.

MALDI-TOF MS: m/z calcd for $C_{36}H_{30}N_2NaO_8S$ $[M+Na]^+$: 673.16 Found: 673.08.

4.4. Phenyl 4-*O*-chloroacetyl-2,6-dideoxy-2,6-diphthalimimde-3-*O*-*p*-methoxybenzyl-1-thio-β-D-galactopyranoside (4)

Compound **3** (7.35 g, 11.0 mmol) was dissolved in a mixed solvent of pyridine-dichloromethane (1:20, v/v, 126 mL) and cooled to 0 °C. To the solution was added chloroacetyl chloride (1.75 mL, 22.0 mmol, 2.0 equiv). After 25 min, the solution was diluted with toluene and the solvent was evaporated. The crude product was purified by silica gel column chromatography (chloroform (0.5% ethanol)) and **4** was obtained as a colorless foam (7.39 g, 10.2 mmol, 93%).

^{1}H NMR($CDCl_3$) δ 7.88–7.60 (m, 8H, N*Phth*), 7.29–7.26 (m, 2H, Ar), 7.07–6.94 (m, 3H, Ar), 6.83 (d, J = 7.2, 2H), 6.38 (d, J = 8.4, 2H), 5.54 (d, J = 2.7, 1H, 4-H), 5.46 (d, J = 10.5, 1H, H-1), 4.45–4.23 (m, 6H, ClAc, ArCH_2-a, H-2, H-3, H-5), 4.12–3.98 (m, 2H, ArCH_2-b, H-6a), 3.88–3.81 (m, 1H, H-6b), 3.64 (s, 3H, CH_3OPh).

^{13}C NMR ($CDCl_3$) δ 167.9, 167.6, 167.4, 167.2, 159.1, 134.2, 133.9, 133.8, 133.0, 131.7, 131.5, 131.4, 129.8, 128.9, 128.5, 127.9, 123.5, 123.4, 123.1, 113.5, 83.9, 73.4, 72.8, 70.9, 68.0, 54.9, 51.1, 41.1, 38.1.

MALDI-TOF MS: *m*/*z* calcd for $C_{38}H_{31}ClN_2NaO_9S$ $[M+Na]^+$: 749.13 Found: 749.12.

4.5. Compound 5

Compound **4** (0.1452 g, 200 μmol) was coevaporated with dry toluene (2 mL × 4). *N*-Iodosuccinimide (0.1127 g, 500 μmol, 2.5 equiv) and 3-bromo-1-propanol (36 μL, 400 μmol, 2.0 equiv) were added and the mixture was dissolved in dichloromethane (1.0 mL). The solution was cooled and then 0.2% trifluoromethanesulfonic acid in diethyl ether (1.0 mL) was added. After stirring for 20 min, a saturated aqueous solution of $NaHCO_3$ (1 mL) was added and the mixture was warmed to rt. The solution was diluted with chloroform (30 mL), washed with a saturated aqueous solution of $NaHCO_3$ (30 mL) and 10% $Na_2S_2O_3$aq (30 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated. After the crude product was cooled to 0 °C, 7.5 mM sodium methoxide solution in methanol (10 mL) and dichloromethane (5 mL) was added to the mixture. After 12.5 h, Dowex 50 W × 8 (2 g) was added, and stirred for 30 min and the Dowex resin was removed by filtration. The crude product was purified by silica gel column chromatography (ethyl acetate–toluene (1:4, v/v)) to afford the pure β-glycoside **5** as a colorless oil (0.1156 g, 170 μmol, 85%).

^{1}H NMR ($CDCl_3$) δ 7.91–7.65 (m, 8H, N*Phth*), 6.95 (d, *J* = 8.7, 2H, Ar), 6.46 (d, *J* = 8.4, 2H, Ar), 5.04 (d, *J* = 8.4, 1H, H-1), 4.54 (d, *J* = 12.6, 1H), 4.48–4.41 (m, 1H), 4.32–4.22 (m, 3H), 4.01–3.92 (m, 3H), 3.73–3.65 (m, 4H), 3.48–3.40 (m, 1H), 3.18–3.14 (m, 1H), 2.83 (s, 1H), 1.95–1.74 (m, 2H).

^{13}C NMR ($CDCl_3$) δ 168.2, 167.7, 159.0, 134.1, 133.8, 133.7, 131.8, 131.5, 129.4, 129.1, 123.4, 123.3, 122.9, 113.4, 98.4, 74.3, 71.5, 71.1, 66.5, 65.6, 54.9, 52.0, 38.5, 32.1, 30.2.

4.6. Compound 6

A mixture of **5** (50.4 mg, 71 μmol) and **4** (77.6 mg, 106 μmol, 1.5 equiv) was coevaporatated with dry toluene (2 mL × 4). *N*-Iodosuccinimide (59.9 mg, 265 μmol, 3.75 equiv) was added and the mixture was dissolved in dichloromethane (0.5 mL). The solution was cooled, and then 0.04% trifluoromethanesulfonic acid in diethyl ether (1.5 mL) was added to the solution. After 1.5 h, a saturated aqueous solution of $NaHCO_3$ (1 mL) was added and the mixture was warmed to rt. The solution was diluted with chloroform (30 mL), washed with a saturated aqueous solution of $NaHCO_3$ (30 mL) and 10% $Na_2S_2O_3$aq (30 mL). The organic layer was dried over Na_2SO_4, filtered, and concentrated. After the crude product was cooled to 0 °C, 7.5 mM sodium methoxide solution in methanol (10 mL) and dichloromethane (5 mL) was added to the mixture. After 13 h, Dowex 50 W × 8 (1 g) was added, stirred for 30 min and the Dowex resin was removed by filtration. The crude product was purified by silica gel column chromatography (ethyl acetate–toluene (7:13, v/v)) to afford the pure β-glycoside **6** as a colorless oil (68.7 mg, 56.3 μmol, 79%).

^{1}H NMR ($CDCl_3$) δ 8.10 (d, *J* = 7.8, 1H, Ar), 7.88–7.00 (m, 17H, Ar),.6.51–6.17 (m, 6H, Ar), 5.24 (d, *J* = 8.1, 1H, H-1), 4.74 (d, *J* = 8.4, H-1), 4.68–4.58 (m, 2H), 4.47–4.25 (m, 4H), 4.15–3.45 (m, 18H), 3.33–3.25 (m, 1H), 3.11–3.06 (m, 2H), 2.84 (d, *J* = 3.0, 1H), 1.87–1.67 (m, 2H).

^{13}C NMR ($CDCl_3$) δ 168.8, 168.1, 168.0, 167.8, 167.4, 166.1, 159.1, 158.6, 133.9, 133.8, 133.3, 133.2, 132.9, 132.7, 132.2, 131.8, 131.6, 131.4, 129.5, 129.3, 124.5, 123.5, 123.3, 123.2, 122.7, 122.5, 122.0, 113.6, 113.4, 113.1, 99.8, 97.7, 75.1, 74.4, 73.8, 72.1, 71.6, 71.0, 66.2, 65.4, 55.0, 54.8, 52.0, 51.4, 40.1, 39.4, 32.3, 30.6.

MALDI-TOF MS: *m*/*z* calcd for $C_{93}H_{79}BrN_6NaO_{25}$ $[M+Na]^+$: 1241.26 Found: 1241.26.

4.7. Compound 7

The same procedure for the synthesis of **6** was applied to that of **7**, except for using **6** (0.0942 g, 77 μmol) and **4** (0.1056 g, 146 μmol, 1.9 equiv) as starting materials, and ethyl acetate–toluene (9:11, v/v) as eluting solvent in silica gel column chromatography. Compound **7** was obtained as a colorless oil (0.1044 mg, 59 μmol, 77%).

^{1}H NMR ($CDCl_3$) δ 8.35 (d, *J* = 7.2, 1H, Ar), 7.91–7.05 (m, 27H, Ar), 6.52–6.07 (m, 8H, Ar), 5.24 (d, *J* = 8.7, 1H, H-1), 4.94 (d, *J* = 7.8, 1H, H-1), 4.76–4.51 (m, 4H), 4.37–4.00 (m, 10H), 3.93–3.43 (m, 21H), 3.31–3.08 (m, 4H), 1.95–1.73 (m, 2H).

^{13}C NMR ($CDCl_3$) δ 169.2, 168.4, 168.1, 168.0, 167.9, 167.6, 167.3, 166.1, 165.8, 159.0, 158.5, 158.4, 133.9, 133.7, 133.6, 133.3, 133.1, 133.0, 132.8, 132.4, 132.2, 132.0, 131.8, 131.6, 131.3, 129.7, 129.6, 129.4, 129.1, 129.1, 125.2, 123.8, 123.3, 123.2, 123.1, 122.6, 122.3, 122.0, 121.7, 113.5, 113.0, 112.9, 99.6, 99.5, 97.5, 75.1, 74.3, 74.0, 73.8, 72.8, 71.7, 71.6, 71.0, 70.8, 70.6, 66.4, 65.3, 55.0, 54.8, 54.7, 52.1, 51.8, 51.3, 40.5, 40.4, 39.4, 32.4, 30.8.

MALDI-TOF MS: *m*/*z* calcd for $C_{93}H_{79}BrN_6NaO_{25}$ $[M+Na]^+$: 1781.42 Found: 1781.39.

4.8. Compound 8

Compound **7** (21.9 mg, 12.5 μmol) was dissolved in dichloromethane (5 mL) and cooled to 0 °C. To the solution, 20% trifluoroacetic acid in dichloromethane (5 mL) was added and stirred for 2 h. The reaction was quenched by addition of a saturated aqueous solution of $NaHCO_3$ (5 mL) and diluted with dichloromethane (60 mL). The solution was washed with a saturated aqueous solution of $NaHCO_3$ (100 mL) and the aqueous layer was back extracted with dichloromethane (20 mL × 3). The organic layer was dried over Na_2SO_4, filtered and concentrated. The crude product was purified by silica gel column chromatography (dichloromethane (3% methanol)) and **8** was obtained as a colorless oil (12.4 mg, 8.9 μmol, 71%).

^{1}H NMR ($CDCl_3$) δ 8.03–7.60 (24H, N*Phth*), 5.69 (d, *J* = 8.1, 1H, H-1), 5.46 (d, *J* = 7.8, 1H, H-1), 4.81 (d, *J* = 8.1, 1H, H-1), 4.62–4.56 (m, 1H), 4.34–3.59 (m, 18H), 3.51–3.44 (m, 1H), 3.25–3.17 (m, 2H), 3.04–2.93 (m, 4H), 1.95–1.73 (m, 2H).

^{13}C NMR ($CDCl_3$) δ 168.5, 168.2, 167.9, 134.4, 133.9, 133.7, 132.2, 131.9, 131.7, 131.6, 124.1, 123.8, 123.5, 123.1, 99.3, 99.1, 98.5, 75.4, 75.3, 72.6, 72.4, 72.3, 71.2, 69.6, 68.9, 68.5, 66.7, 54.4, 54.2, 53.6, 39.8, 37.6, 31.9, 30.3.

MALDI-TOF MS: *m*/*z* calcd for $C_{69}H_{55}BrN_6NaO_{22}$ $[M+Na]^+$: 1421.24 Found: 1420.91.

4.9. Compound 11

α-Tocopherol **10** (0.2495 g, 0.58 mmol) was dissolved in dry *N*,*N*-dimethylformamide (6 mL). To the solution, sodium hydride (60% purity, 91.6 mg, 2.3 mmol, 4 equiv) and propargyl bromide (200 μL, 2.3 mmol, 4 equiv) were added. After 2 h, methanol was added and the mixture was concentrated to dryness. The mixture was dissolved in dichloromethane (50 mL) and washed with water (30 mL × 2). The crude product was purified by silica gel column chromatography (dichloromethane-hexane (1:9, v/v)) to give **11** as a pale yellow oil (0.2229 g, 0.474 mmol, 82%).

^{1}H NMR ($CDCl_3$) δ 4.36 (d, *s* = 2.7, 2H, HC≡C*CH*$_2$O), 2.57 (t, *J* = 6.6, 2H, Ar*CH*$_2$), 2.48 (d, *J* = 2.1, 1H, *HC*≡C), 2.20–2.08 (s × 3, 9H, Ar*CH*$_3$), 1.81–0.83 (m, 38H).

^{13}C NMR ($CDCl_3$) δ 127.9, 126.0, 122.9, 117.5, 74.9, 74.5, 60.5, 40.0, 39.3, 37.4, 37.3, 32.8, 32.7, 31.2, 28.0, 24.8, 24.4, 23.9, 22.7, 22.6, 21.0, 20.6, 19.7, 19.6, 13.0, 12.2, 11.8.

4.10. Compound 13

Compound **8** (10.5 mg, 7.5 μmol) was coevaporated with dry toluene (1 mL × 3). Sodium azide (5.0 mg, 75 μmol, 10 equiv) was added and the mixture was dissolved in dry *N*,*N*-dimethylformamide (2 mL). The suspending solution was heated to 80 °C, stirred for 19 h and then cooled to rt. The solution was concentrated, diluted with ethyl acetate (20 mL), and washed with water (20 mL × 3). The aqueous layer was back extracted with toluene (20 mL) and the combined organic layer was dried over $MgSO_4$, filtered, and concentrated. To the crude product including **12**, **11** (14.2 mg, 30 μmol, 4 equiv) and copper powder (9.1 mg) were added. The mixed solvent (*t*-butanol–water (2:1, v/v, 3.0 mL)) was added to the mixture and the solution was stirred and heated to 65 °C. After 14 h, the solution was cooled to rt, filtered and purified by silica gel column chromatography (dichloromethane (3% to 4% methanol)) to afford the pure **13** as a colorless oil (9.0 mg, 4.9 μmol, 66%).

^{1}H NMR ($CDCl_3$) δ 8.02–7.60 (24H, N*Phth*), 7.49 (s, 1H, triazole-H), 5.69 (d, *J* = 8.7, 1H, H-1), 5.43 (d, *J* = 7.8, 1H, H-1), 4.83 (d, *J* = 7.8, 1H, H-1), 4.72 (s, 2H), 4.60 (m, 1H), 4.35–3.40 (m, 21H), 3.14–3.10 (m, 3H), 2.95 (d, *J* = 11.1, 1H), 2.58 (t, *J* = 6.6, 2H), 2.18–2.09 (s × 3, 9H), 1.88–0.80 (m, 40H).

^{13}C NMR (75.5 MHz, $CDCl_3$) δ 168.5, 168.2, 168.0, 148.0, 147.9, 144.4, 134.4, 133.9, 133.8, 132.1, 131.9, 131.7, 131.6, 131.5, 127.9, 126.0, 123.8, 123.5, 123.1, 122.9, 117.6, 99.5, 99.2, 98.5, 75.6, 74.8, 72.6, 72.2, 70.8, 69.6, 69.0, 68.5, 66.3, 65.6, 54.5, 54.1, 53.5, 47.0, 40.1, 39.8, 39.3, 37.5, 37.4, 37.3, 32.8, 32.7, 31.2, 30.2, 29.7, 28.0, 24.8, 24.4, 23.8, 22.7, 22.6, 21.0, 20.6, 19.7, 19.6, 12.9, 12.0, 11.8.

MALDI-TOF MS: *m*/*z* calcd for $C_{101}H_{107}N_9NaO_{24}$ $[M+Na]^+$: 1853.74 Found: 1852.79.

4.11. Compound 14

Compound **13** (3.9 mg, 2.1 μmol) was dissolved in ethanol (2.0 mL) and hydrazine monohydrate (60 μL) were added and the mixture was stirred and heated to 80 °C. After 4.5 h, the mixture was cooled to rt and concentrated. Eighth part of the entire crude product was purified by C4 reversed-phase HPLC (0.05% TFA, water-acetonitrile) and lyophilized from water to give **14** as a colorless solid (78.5 nmol, 29%).

MALDI-TOF MS: *m*/*z* calcd for $C_{53}H_{95}N_9NaO_{12}$ $[M+Na]^+$: 1072.70 Found: 1072.52.

4.12. Compound 16

Compound **15** (38.5 mg, 83 μmol) was coevaporated with dry toluene (10 mL × 4) and dissolved in dry tetrahydrofurane (1.5 mL). To the solution, sodium hydride (60% purity, 15.1 mg, 377 μmol, 4.5 equiv) and propargyl bromide (15 μL, 188 μmol, 2.3 equiv) were added. The mixture was heated to 60 °C and stirred for 16 h. After the solution was cooled to rt, methanol (1 mL) was added and concentrated. The crude product was dissolved in dichloromethane (30 mL) and washed with saturated brine (10 mL × 3) and the organic layer was dried over Na_2SO_4, filtered and concentrated. The crude product was dissolved in dry tetrahydrofurane (1.5 mL) and tetrabutylammonium fluoride (37.2 mg, 150 μmol) was added. After the solution was stirred for 20 min, the reaction was quenched by addition of saturated aqueous solution of $NaHCO_3$ and the solution was concentrated. The crude product was purified by silica gel column chromatography (dichloromethane-hexane (6:4, v/v)) to afford the pure **16** as a pale yellow oil (26.2 mg, 62 μmol, 75%).

^{1}H NMR ($CDCl_3$) δ 4.25 (s, 1H, Ar*OH*), 4.13 (d, *J* = 2.7, 2H, HC≡*CCH*$_2$O), 3.50 (t, *J* = 6.6, 2H, O*CH*$_2$CH$_2$), 2.59 (t, *J* = 6.9, Ar*CH*$_2$), 2.41 (t, *J* = 2.4, *HC*≡C), 2.15–2.10 (s × 2, 9H, Ar*CH3*), 1.81–1.22 (m, 21H).

^{13}C NMR (75.5 MHz, $CDCl_3$) δ 145.5, 144.5, 122.5, 121.0, 118.5, 117.3, 80.0, 74.4, 74.0, 70.3, 58.0, 39.4, 31.5, 30.1, 29.5, 29.5, 29.4, 26.0, 23.8, 23.6, 20.7, 12.2, 11.7, 11.3.

MALDI-TOF MS: *m*/*z* calcd for $C_{25}H_{39}O_3$ $[M+H]^+$: 387.3 Found: 386.8.

4.13. Compound 17

Compound **8** (4.5 mg, 3.2 μmol) was coevaporated with dry toluene (1 mL × 4), sodium azide (8.6 mg, 132 μmol, 41 equiv) was added and the mixture was dissolved in dry *N*,*N*-dimethylformamide (2.0 mL). The suspending solution was heated to 80 °C, stirred for 15 h and then cooled to rt. The solution was concentrated, dissolved in ethyl acetate (20 mL), and washed with water (20 mL × 3). The aqueous layer was back extracted with toluene (10 mL) and the combined organic layer was dried over $MgSO_4$, filtered, and concentrated. To the crude product including **12**, **16** (2.7 mg, 7.0 μmol, 2.2 equiv) and copper powder were added. Mixed solvent (*t*-butanol–water (2:1, v/v, 1.5 mL)) was added to the mixture and the solution was stirred and heated to 80 °C. After 3.5 h, the solution was cooled to rt, filtered and purified by silica gel column chromatography (dichloromethane (2.5–3.5% methanol)) to afford the pure **13** as a colorless oil (5.1 mg, 2.9 μmol, 91%).

^{1}H NMR ($CDCl_3$) δ 8.01–7.61 (24H, N*Phth*), 7.31 (s, 1H, triazole-H), 5.69 (d, *J* = 8.7, 1H, H-1), 5.44 (d, *J* = 8.4, 1H, H-1), 4.82 (d, *J* = 7.8, 1H, H-1), 4.63–4.56 (m, 1H), 4.50 (s, 2H), 4.40–3.37 (m, 26H), 3.13–3.07 (m, 3H), 2.91 (d, *J* = 11.1, 1H), 2.58 (t, *J* = 6.6, 2H), 2.14–2.10 (s × 3, 9H), 1.83–1.22 (m, 21H).

^{13}C NMR (75.5 MHz, $CDCl_3$) δ 168.6, 168.2, 168.0, 145.4, 144.8, 144.5, 134.5, 133.8, 132.1, 131.9, 131.5, 123.8, 123.5, 123.2, 122.5, 121.1, 118.6, 117.3, 99.4, 99.2, 98.4, 75.6, 74.5 72.6, 72.2, 70.7, 69.6, 69.0, 68.4, 65.4, 64.0, 54.5, 54.1, 53.5, 46.9, 39.9, 39.2, 37.5, 31.5, 30.1, 29.6, 29.5, 29.4, 26.1, 23.9, 23.5, 20.7, 12.3, 11.8, 11.3.

MALDI-TOF MS: *m*/*z* calcd for $C_{94}H_{93}N_9NaO_{25}$ $[M+Na]^+$: 1771.62 Found: 1770.43.

4.14. Compound 18

Compound **17** (5.1 mg, 2.9 μmol) was dissolved in ethanol (2.0 mL) and hydrazine monohydrate (60 μL) were added and the mixture was stirred and heated to 90 °C. After 4 h, the mixture was cooled to rt and concentrated. Four-twenty ninth of the entire crude product was purified by C18 reversed-phase HPLC (0.05% TFA, water–acetonitrile) and lyophilized from water to give **18** as colorless solid (235 nmol, 59%).

MALDI-TOF MS: *m*/*z* calcd for $C_{46}H_{81}N_9NaO_{13}$ $[M+Na]^+$: 990.59 Found: 990.49.

4.15. Compound 19

4-Monomethoxytrityl chloride (0.6416 g, 2.0 mmol) was dissolved in dry pyridine (20 mL) and stirred. To the solution, propargyl alcohol (150 μL, 2.54 mmol, 1.27 equiv) was added and stirred for 3 d. The solvent was evaporated. And the mixture was dissolved in dichloromethane (30 mL) and washed with saturated aqueous solution of $NaHCO_3$ (20 mL × 2). The aqueous layer was back extracted with dichloromethane (10 mL × 4). The combined organic layer was dried over Na_2SO_4, filtered and concentrated. The crude product was purified by silica gel column chromatography

(dichloromethane–hexane (3:1, v/v)) to afford the pure **19** as a colorless solid (0.5989 g, 1.82 mmol, 91%).

^{1}H NMR ($CDCl_3$) δ 7.49–7.45 (m, 4H, Ar), 7.37–7.20 (m, 8H, Ar), 6.87–6.82 (m, 2H, Ar), 3.80 (s, 3H, CH_3OAr), 3.75 (d, J = 2.4, HC≡CH_2), 2.39 (d, J = 2.4, HC≡CH_2).

^{13}C NMR ($CDCl_3$) δ 158.7, 143.8, 135.0, 130.2, 128.2, 127.9, 127.0, 113.2, 87.2, 80.5, 55.2, 52.7.

4.16. Compound 20

Compound **8** (4.2 mg, 3.0 μmol) was coevaporated with dry toluene (1 mL × 3), sodium azide (6.4 mg, 100 μmol, 33 equiv) was added and the mixture was dissolved in dry *N*,*N*-dimethylformamide (1.0 mL). The suspending solution was heated to 80 °C, stirred for 18 h and then cooled to rt. The solution was concentrated, dissolved in ethyl acetate (20 mL), and washed with water (20 mL × 3). The aqueous layer was back extracted with toluene (10 mL) and the combined organic layer was dried over $MgSO_4$, filtered, and concentrated. To the crude product including **12**, **19** (6.4 mg, 20 μmol, 6.7 equiv) and copper powder (3.8 mg) were added. Mixed solvent (*t*-butanol–water (2:1, v/v, 1.0 mL)) was added to the mixture and the solution was stirred and heated to 65 °C. After 12 h, the solution was cooled to rt, filtered and purified by silica gel column chromatography (dichloromethane (3% methanol)) to afford the pure **20** as a colorless oil (5.1 mg, 3.0 μmol, quant).

^{1}H NMR ($CDCl_3$) δ 8.04–7.23 (m, 37H, Ar), 6.84 (d, J = 9.0, 2H, Ar), 5.72 (d, J = 8.1, 1H, H-1), 5.49 (d, J = 8.1, 1H, H-1), 4.86 (d, J = 8.1, 1H, H-1), 4.62–4.55 (m, 1H), 4.35–3.42 (m, 26H), 3.21–3.03 (m, 3H), 2.80 (d, J = 11.7, 1H), 1.83–1.78 (m, 2H).

^{13}C NMR (75.5 MHz, $CDCl_3$) δ 168.6, 168.2, 167.9, 158.6, 145.5, 144.2, 135.4, 134.5, 134.0, 133.8, 132.1, 131.9, 131.7, 131.5, 130.3, 128.4, 127.9, 126.9, 124.1, 123.8, 123.6, 123.2, 122.3, 113.2, 99.3, 99.1, 98.5, 86.9, 78.5, 75.3, 72.7, 72.6, 72.3, 71.1, 69.7, 69.2, 68.3, 65.7, 58.6, 55.2, 54.5, 54.3, 53.6, 46.9, 39.8, 37.3, 30.1.

MALDI-TOF MS: m/z calcd for $C_{92}H_{75}N_9NaO_{24}$ $[M+Na]^+$: 1712.48 Found: 1712.16.

4.17. Compound 21

Compound **20** (5.1 mg, 3.0 μmol) was dissolved in dry ethanol (3.0 mL) and hydrazine monohydrate (90 μL) were added and the mixture was stirred and heated to 80 °C. After 4 h, the mixture was cooled to rt and concentrated. Eighth part of the entire crude product was purified by C18 reversed-phase HPLC (0.05% TFA, water–acetonitrile). A half of the purified product was dissolved in 80% aqueous solution of acetic acid (5 mL) and then the solvent was evaporated after 1.5 h. The product was dissolved to water (30 mL) and washed with dichloromethane (10 mL × 3). Water was evaporated and 1% aqueous solution of trifluoroacetic acid to the product. After coevaporation with water (2 mL × 3), the product was lyophilized from water to give **21** as a colorless solid (27 nmol, 14%).

MALDI-TOF MS: calcd for $C_{24}H_{47}N_9NaO_{11}$ m/z $[M+Na]^+$: 660.33 Found: 660.05.

4.18. Biological experiments

4.18.1. Cell culture

Rat hepatocellular carcinoma (McA-RH7777) cells were maintained in Dulbecco's modified Eagle's medium (Sigma–Aldrich, St Louis, MO) only or supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 units/ml penicillin, and 100 μg of streptomycin at 37 °C in 5% CO_2.

4.18.2. In vitro siRNA activity assay

To determine in vitro activity of siRNAs, McA-RH7777 cells (kindly gifted from Hiroyuki Arai, PhD) were transfected with 10 nM siRNAs in absence and presence of equally amount of compound **18** respectively using Lipofectamine™ 2000 (Invitrogen). The cells were harvested 24 h after transfection. Total RNA was extracted and the amount of endogenous *apoB* mRNA was measured by quantitative real-time polymerase chain reaction (qRT-PCR).

4.18.3. Quantitative RT-PCR assay

DNase-treated 2 μg of RNAs were reverse transcribed with Super Script III and Random Hexamers (Life Technologies, Carlsbad, CA). The cDNAs were amplified by the quantitative TaqMan system using the Light Cycler 480 Real-Time PCR Instrument (Roche Diagnostics, Mannheim, Germany). The primers and probes for rat *apoB* (NM_019287) and rat *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*; NM_017008) were designed by Applied Biosystems (Foster City, CA).

4.18.4. Statistical analysis

Student's two-tailed *t*-test was used to determine the significance of differences between control and transfected groups in quantitative RT-PCR assay. Data are presented as means ± standard error of the means (SEMs); P <0.05 was considered significant.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.12.060.

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