

LNA-DNA gapmer 型の 13 塩基からなる一本鎖 ASO (ssASO) に相補的な 13 塩基長の cRNA に対して、上記化学修飾を施した。合成は、一般的な核酸合成法に従い実施し、得られた各 cRNA

(cRNA0~cRNA4) は逆相 HPLC にて精製した後、MALDI-TOF-MS によってその構造を決定している。以下に用いた ASO 並びに各 cRNA の配列とその化学修飾様式を示す。

ASO:

5'-G(L)\*A(L)\*A\*G\*G\*T\*C\*A\*T\*G\*G(L)\*C(L)\*A(L)

cRNA0: 5'-u\*g\*c\*CAUGACCU\*u\*c-3'

cRNA1: 5'- u\*g\*c\*C\*AU\*GAC\*C\*U\*u\*c-3'

cRNA2: 5'- u\*g\*c\*C\*A\*U\*G\*A\*C\*C\*U\*u\*c-3'

cRNA3: 5'- u\*g\*c\*CAUGACCU\*u\*c-3'

cRNA4: 5'- u\*g\*c\*cAuGAccu\*u\*c-3'

なお、上記配列中の化学修飾様式は以下の通りである。

X(L): LNA, X: DNA, X: RNA, x: 2'-OMe, X: 2'-F,

\*: PS 結合

### 3-2) 腸管内をミミックした評価系における酵素耐性能の評価

ICR 雄性マウス (8~10 週齢) を麻酔下、腸管ループを作製し、生理食塩水を注入して 2 時間インキュベートした。残存する腸内インキュベーション液及び粘膜上皮組織を採取し、生理食塩水を加えてホモジナイズした後、4°C、3,000×g にて遠心分離して沈殿を除去した。RNase A 活性を  $1 \times 10^{-5}$  unit/ $\mu$ l に調整した粘膜ホモジネート液又は腸内インキュベーション液に、cRNA 又は ASO(2.5 $\mu$ M) を添加し、37°C でインキュベーションした後、一定時間後に酵素反応を停止したものをサンプルとした。各サンプルをポリアクリルアミド (15%) 電気泳動後、核酸染色し、分解性の評価を行った。

(倫理面への配慮)

動物実験は、動物愛護及び実験動物の適正管理、動物実験の適正化の観点から、大阪大学、大阪大谷大学及び東京医科歯科大学の各動物実験委員会規定

と動物実験指針に基づいて行った。

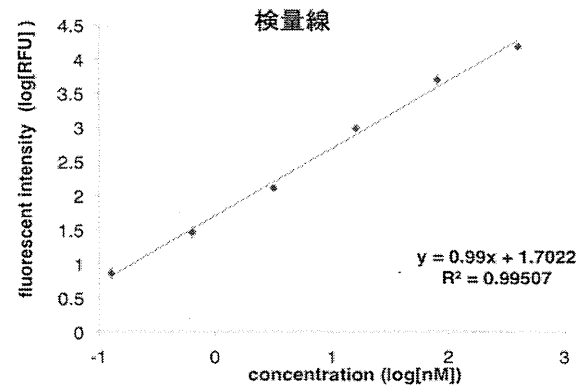
## C. 研究結果

### 1) 核酸医薬の薬物動態評価に関する研究

#### 1-1) ELISA 法における検量線の作成

ASO の濃度と測定した蛍光強度をそれぞれ対数化し、得られた値から検量線を作成した (図 2)。広い濃度範囲において高い直線性を有する値を得た。

図 2 検量線



#### 1-2) ELISA 法の同時再現性の検証

上で得られた検量線より、各サンプルの実濃度を算出した。各サンプルの理論値と実測濃度の平均値及び標準偏差値より、真度及び精度を算出した (表 2)。定量範囲 0.128~400 nM において、真度: 80.2~128%、精度: 16.8% 以下という非常に高い正確性を有する評価系であることが見いだされた。

表 2 真度及び精度

理論値(nM)	400	80	16	3.2	0.64	0.128
実測濃度 (nM)	317.9	121.3	24.9	2.84	0.55	0.158
	298.1	109.2	26.6	3.05	0.64	0.092
	296.0	109.5	18.5	2.67	0.56	0.174
	310.9	90.8	23.6	2.22	0.61	0.181
	351.6	109.3	20.8	1.97	0.77	0.145
	318.2	95.0	18.6	1.55	0.40	0.148
	304.8	98.8	26.2	2.85	0.53	0.130
395.3	110.6	15.4	2.88	0.69	0.093	
平均値 (nM)	320.9	102.4	20.1	2.63	0.59	0.147
標準偏差 (nM)	22.2	9.1	2.8	0.44	0.09	0.016
真度 (%)	80.2	128.1	125.9	82.1	92.5	114.9
精度 (%)	6.9	8.9	14.1	16.8	14.8	11.1

### 1-3) 薬物投与量と組織蓄積量の比較

マウスに対して ASO を静脈内より単回投与 (5-70mg/kg/回) した場合の肝臓における蓄積量を評価した。ELISA 測定より得られた濃度データを肝臓の総タンパク質量で標準化し、肝臓タンパク質 1  $\mu\text{g}$  中の ASO 量 (ng) の形で表現した。検討の結果、投与量に正比例して肝臓内に蓄積することが見いだされた (図 3)。この時、肝臓における蓄積量の飽和現象は認められなかった。

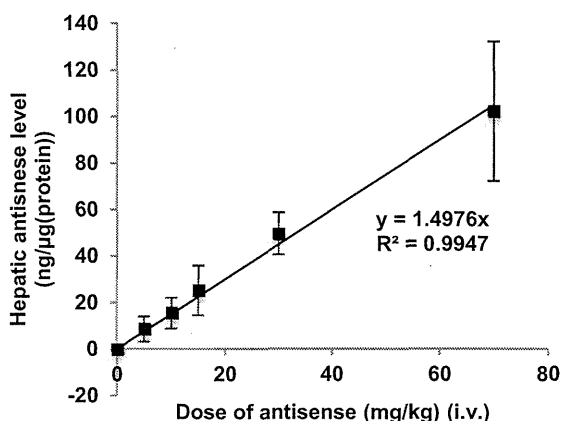


図 3 アンチセンス核酸の肝蓄積量

## 2) dsASO の開発研究

### 2-1) VE-dsASO の標的遺伝子発現抑制効果

まず、VE-ssASO をマウスに静注した際の肝における標的遺伝子発現抑制効果を確認した (図 4)。その結果、ビタミン E を直接 ASO に結合することで、ASO の標的遺伝子発現抑制効果が著明に減弱することが示された。

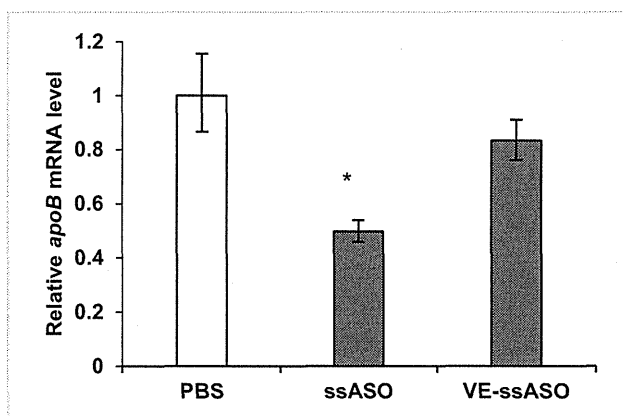


図 4 マウスに対して ssASO と VE-ssASO を 0.75 mg/kg で静脈注射後、3 日後における肝での標的遺伝子の発現抑制効果 ( $n = 3$ , \*:  $P < 0.05$ )

次に、VE-dsASO を 0.02~0.75 mg/kg の投与量で同様にマウスに静注した際の標的遺伝子発現抑制効果を確認した (図 5)。その結果、Toc-dsASO が投与した用量依存性に標的遺伝子発現抑制効果を示すこと、標的遺伝子の発現を 50% 抑制するのに必要な用量 ( $\text{ED}_{50}$ ) が 0.036 mg/kg であることが分かった。ssASO の  $\text{ED}_{50}$  は 0.75 mg/kg であることから、VE-dsASO では ssASO のおよそ 5% であり、ssASO に直接ビタミン E を結合させた場合と異なり、二本鎖として間接的にビタミン E を結合させることにより大幅な有効性の上昇が認められた。

今回用いた ASO は apoB に対するものであり、そのまま高脂血症の治療薬となり得る。そこで、高脂肪食を 2 週間摂取させて作成した高脂血症モデルマウスに対して、高脂肪食を継続したまま週 1 回 ssASO または VE-dsASO をそれぞれ 0.09 若しくは 0.75 mg/kg 静注した。計 4 回投与し、最終投与の 3 日後の血清を用いて LDL コレステロール (LDLC) 値を測定した (図 6)。その結果、特に VE-dsASO 投与群で LDLC 値の低下を認め、実際に VE-dsASO によって高脂血症に対する治療が可能になるものと考えられた。

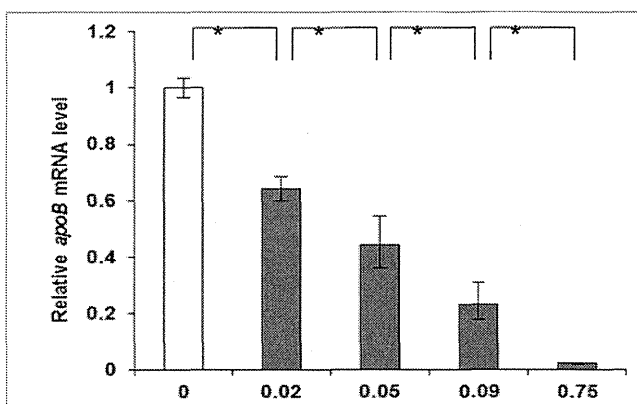


図 5 マウスに対して VE-dsASO を 0.02~0.75 mg/kg で静脈注射後、3 日後における肝での標的遺

伝子の発現抑制効果( $n=3$ , \*:  $P<0.05$ )

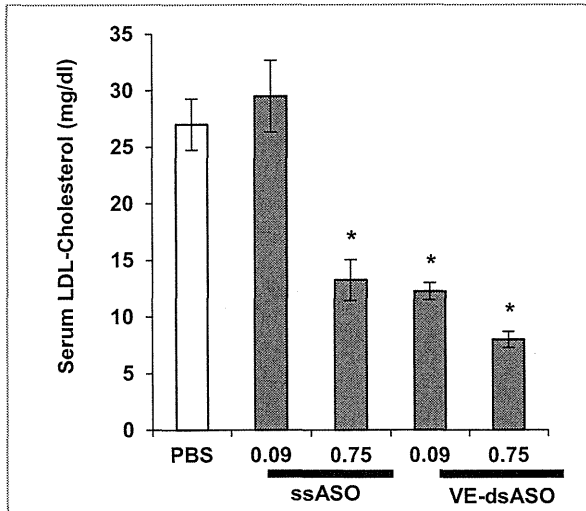


図 6 高脂血症モデルマウスに対して ssASO と VE-dsASO を 0.09 若しくは 0.75 mg/kg で毎週静脈注射後、最終投与 3 日後における血中 LDL コレステロール値( $n=4$ , \*:  $P<0.05$ )

### 2-2) 生体内での VE-dsASO の分布

蛍光標識した VE-dsASO を 0.75 mg/kg で静注し、投与 5 分、6 時間、7 日後の体内における分布を IVIS imaging system を用いてそれぞれ撮像した (図 7)。その結果、ssASO において 7 日後でも生体内に広汎に分布しているのに対し、VE-dsASO では投与 6 時間後にはほぼ肝に集積しており、ビタミン E を結合することにより大幅な生体内分布の改善を確認できた。

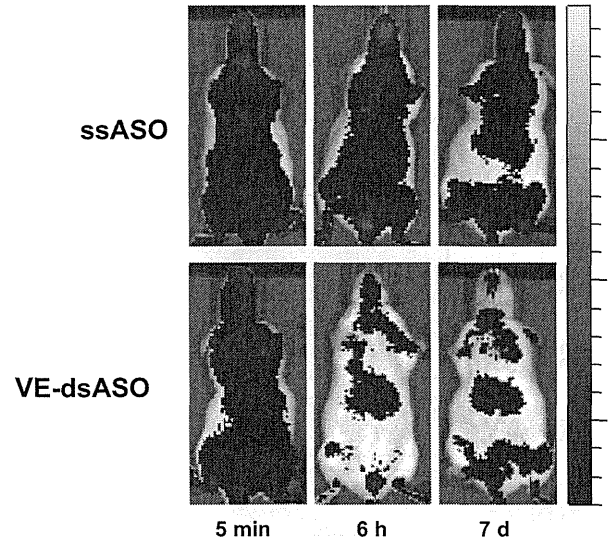


図 7 マウスに対して蛍光標識 ssASO と VE-dsASO を 0.75 mg/kg で静脈注射後、5 分、6 時間、7 日後における ASO の体内分布 (IVIS imaging system を用いて撮影)

### 2-3) VE-dsASO の安全性評価

VE-dsASO を 0.75 mg/kg で静注したマウスの血液サンプルの生化学的検査にて、VE-dsASO を静注投与した際の明らかな副作用は検出されなかった (表 3)。

	PBS only	VE-dsASO
BUN (mg/dl)	28.9 ± 1.6	26.5 ± 1.2
Cre (mg/dl)	0.16 ± 0.02	0.14 ± 0.01
AST (U/l)	73 ± 14	63 ± 3
ALT (U/l)	29 ± 2	35 ± 4

表 3 Toc-dsASO 静注時の血清生化学

### 3) 生体内安定性を高めた cRNA の創成研究

#### 3-1) cRNA 鎖の生体内安定性評価

化学合成した各種 cRNA のマウス結腸粘膜ホモジネート中での安定性を調べた (図 8)。

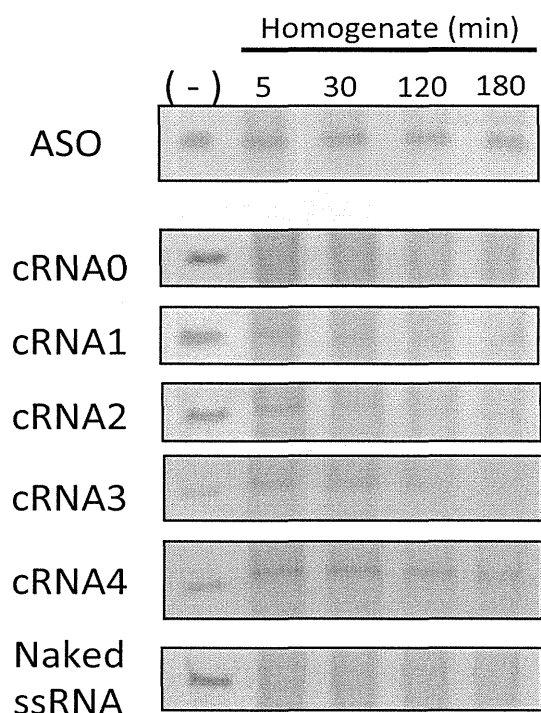


図 8 マウス結腸粘膜ホモジネート中における各 cRNA の安定性

その結果、コントロールとして用いた天然型 RNA(naked ssRNA)が5分以内に分解されるのに対し、cRNA0~cRNA4については、その化学修飾の種類や程度に応じて分解耐性特性が異なっていた。すなわち、配列の5'側3残基と3'側2残基のみ 2'-OMe 体の導入に加えリン酸ジエステル結合をホスホロチオアート化 (PS 結合化) した cRNA0 は、天然型 RNA と同じく5分以内に分解されたが、一方で、配列中央部のピリミジン残基部位を PS 化した cRNA1 においては、安定性向上が認められた。また、配列中央部を全て PS 化した cRNA2 については、cRNA1 と顕著な違いは認められず、プリン残基部分の PS 化による安定性向上は限定的である事が確認された。cRNA3 と cRNA4 については、配列中央部のピリミジン残基をそれぞれ 2'-F 体及び 2'-OMe 体に変換したものであり、配列の両末端側以外にリン酸ジエステル結合部分の PS 化修飾は行っていない。しかし、特に cRNA4 において優れた安定化効果が認められている。なお、本条件化において ASO は極めて高い酵素耐性能を示す事が確

認された。

#### D. 考察

##### 1) 核酸医薬の薬物動態評価に関する研究

アンチセンス分子の濃度 (濃度域 0.128~400 nM) と測定した蛍光強度をそれぞれ対数化し、得られた検量線では、決定係数  $R^2 \geq 0.99$  という高い相関性が認められた。本手法は、0.128~400 nM の非常に広い濃度範囲で LNA 搭載型アンチセンス核酸を定量することが可能である。

Viswanathan らの報告 (C. T. Viswanathan *et al.*, *AAPS Journal*, 2007, 9, 30) によれば、分析測定法において検量線サンプルの真度が 80-120% (但し、測定可能濃度域の定量下限、定量上限の検量線サンプルの真度は 75-125%)、検量線サンプルの精度が 20%以下 (但し、測定可能濃度域の定量下限、定量上限の検量線サンプルの精度が 25%以下) であることが望ましいとしている。今回の結果では精度は上記基準を満たしている。真度についても、一部の濃度では基準を若干程度上回っているが、67% (6 個中 4 個) 以上のサンプルが条件を満たしており、十分に正確性の高い測定系であることが言える。

静脈内投与に伴い、肝臓にアンチセンス分子が投与量に正比例して増加していることを見いだした。本結果から、70 mg/kg の高用量でも肝臓への蓄積に飽和が無く、また 1 mg/kg あたりの投与量に対して、0.65  $\mu$ g が肝臓タンパク質 1 g あたりに蓄積することを見出した。これは 1mg/kg あたりの投与量に対して 0.65 $\mu$ g が肝臓 1g あたりに蓄積する計算になり、投与量の約 6%が 72 時間後にも肝臓に未変化体として残存していることになる。これほどまでに肝臓が効率よくアンチセンス分子を取り込み、長い半減期を示したのは LNA の生体内での高い安定性はもとより、肝臓の豊富な血流量と類洞内の遅い血流速度、さらにその内部に広がる Disse 腔などの広い内腔にタンパク質結合型及び非結合型アンチセンスが取り込まれ、細胞内への取り込みや代謝の機会を増加させるからであると考えられる。

##### 2) dsASO の開発研究

ASO のデリバリーは、主に核酸間のホスホロチオエート結合が担っていると考えられており、ナノパーティクルや脂質結合といったデリバリー因子を要する siRNA 等と異なりデリバリー用の素子が必要とされていない。しかし、臓器への集積性の点で ASO にも何らかのデリバリー因子を用いることが、ASO による効果向上・副作用軽減の両面で必要と思われる。

ビタミン E 結合機能核酸は、siRNA で報告があるように集積の点で肝へのデリバリーに際して有用であることが示されていた。そこでビタミン E を直接 ASO に結合してみたものの、逆に有効性を損ねる結果となった。そのため間接的にビタミン E を ASO に結合させるために、ASO に対する相補鎖を設計し、それにビタミン E を結合させることで、肝特異的なデリバリー及び効果の著明な向上という二点を成し遂げることができた。

### 3) 生体内安定性を高めた cRNA の創成研究

今回、新たに構築した核酸の生体内安定性評価系、すなわち腸管内をミミックした酵素耐性能評価系を用いる事により、経口投与に資する人工核酸創成の基盤となるデータが得られた。

まず第一に、両末端を LNA 修飾し全てのリン酸ジエステル結合を PS 化した ASO については、腸管内をミミックした環境下においても極めて安定であり、核酸医薬としての高いポテンシャルを示す結果となった。

一方、cRNA2 は ASO と同じく配列中の全てのリン酸ジエステル結合が PS 化されているにも関わらず、安定化は限定的であった。この結果は、PS 化修飾が生体内安定性を大きく向上させるとされてきたこれまでの知見と必ずしも合致するものではなく、ある意味予想外の結果であった。ASO と cRNA2 は化学構造上大きな違いが何点かあるが、その最たる違いは ASO 鎖が DNA をベースにしたものであり、cRNA2 は RNA ベースの構造であるという点であろう。すなわち、RNA 鎖に対して PS 化を行なうことにより、一定程度の酵素耐性能は付与できるものの、腸管内をミミックした条件下においては、ASO、つ

まり DNA 鎖をベースにした配列ほどの安定性は得られない可能性が示唆された。これは今後 RNA 鎖を含む核酸医薬を開発していく際の重要な知見の一つであろう。

今回の検討に用いた cRNA の中で cRNA4 が最も優れた安定性を示した。配列中央部分を PS 化していないにも関わらず、cRNA4 が良好な成績を示した事は本研究の中で特筆すべき点であり、経口投与を視野に入れた RNA 鎖の化学修飾における 2'-OMe 体の有用性を端的に示した結果である。今回 cRNA4 では配列中央部分のピリミジン塩基のみを 2'-OMe 化しており、プリン塩基については天然の RNA を利用している。cRNA の配列を詳しく見てみると、配列中央部に存在するプリン鎖は 3 残基あり、そのうち 2 残基は連続している。すなわち、腸管内をミミックした系においても、2 残基程度の天然型プリン RNA の連続は十分に許容されるということが明らかとなった。

今後、様々な配列を用いて本結果の検証を進め、その一般性を明らかにしていく事により、経口投与可能な核酸医薬素材の開発研究が大きく進むと考えられる。

## E. 結論

本研究では、経口投与可能な核酸医薬の創成を目指し、1) 核酸医薬の薬物動態を評価するための技術構築を進め、ELISA 法を応用した *in vivo* 系でのアンチセンス核酸 (ASO) 定量法を構築した。また、2) ASO に脂質を結合することによる有効な核酸デリバリー技術の開発を検討し、通常一本鎖である ASO に相補鎖核酸 (cRNA) をハイブリダイズさせた新規の二本鎖 ASO (dsASO) の開発に成功した。さらに、3) 腸管環境をミミックした核酸のヌクレアーゼ耐性評価系を利用し、化学修飾型 cRNA 及び ASO の安定性を評価した。これらの成果は、従来の核酸医薬の有効性を大きく上回る新たな核酸創薬手法につながるばかりか、経口投与に資する核酸医薬の化学修飾のあり方を示唆するものであり、我が国の革新的医薬品創出に大きく貢献するものであると

言える。

## F.健康危険情報

該当なし

## G.研究発表

### 1. 論文発表

- Morihiro K, Kodama T, Mori S, Obika S. Photoinduced Changes in hydrogen bonding patterns of 8-thiopurine nucleobase analogues in a DNA strand. *Org Biomol Chem* 2014; 12: 2468-2473.
- Shrestha AR, Kotobuki Y, Hari Y, Obika S. Guanidine bridged nucleic acid (GuNA): an effect of cationic bridged nucleic acid on DNA binding affinity. *Chem Commun* 2014; 50: 575-577.
- Morihiro K, Kodama T, Waki R, Obika S. Light-triggered strand exchange reaction using the change in the hydrogen bonding pattern of the nucleobase. *Analog Chem Sci* 2014; 5: 744-750.
- Hari Y, Nakahara M, Ijitsu S, Obika S. The ability of 1-aryltriazole-containing nucleobases to recognize a TA base pair in triplex DNA. *Heterocycles* 2014; 88: 377-386.
- Yamamoto T, Obika S, Nakatani M, Yasuhara H, Wada F, Shibata E, Shibata M, Shiba M. Locked nucleic acid antisense inhibitor targeting apolipoprotein C-III efficiently and preferentially removes triglyceride from large very low-density lipoprotein particles in murine plasma. *Eur J Pharmacol* 2013; 723: 353-359.
- Hari Y, Morikawa T, Osawa T, Obika S. Synthesis and properties of 2'-O,4'-C-ethyleneoxy bridged 5-methyluridine. *Org Lett* 2013; 15: 3702-3705.
- Hari Y, Akabane M, Obika S. 2',4'-BNA bearing a chiral guanidinopyrrolidine-containing nucleobase with potent ability to recognize the CG base pair in parallel-motif DNA triplex. *Chem Commun* 2013; 49: 7421-7423.
- Akiyama H, Miyashita K, Hari Y, Obika S, Imanishi T. Synthesis of novel polyesteramine dendrimers by divergent and convergent methods. *Tetrahedron* 2013; 33: 6810-6820.
- Hari Y, Nakahara M, Obika S. Triplex-forming ability of oligonucleotides containing 1-aryl-1,2,3-triazole nucleobases linked via a two atom-length spacer. *Bioorg Med Chem* 2013; 21: 5583-5588.
- Hari Y, Kashima S, Inohara H, Ijitsu S, Imanishi T, Obika S. Base-pair recognition ability of hydroxyphenyl nucleobases in parallel triplex DNA. *Tetrahedron* 2013; 69: 6381-6391.
- Hari Y, Osawa T, Kotobuki Y, Yahara A, Shrestha AR, Obika S. Synthesis and properties of thymidines with six-membered amide bridge. *Bioorg Med Chem* 2013; 21: 4405-4412.
- Morihiro K, Kodama T, Kentefu, Moai Y, Veedu RN, Obika S. Selenomethylene-locked nucleic acid enables reversible hybridization in response to redox changes. *Angew Chem Int Ed* 2013; 52: 5074-5078.
- Hari Y, Osawa T, Obika S. Synthesis and duplex-forming ability of oligonucleotides containing 4'-carboxythymidine analogs. *Org Biomol Chem* 2012; 10: 9639-9649.

14. Kang JH, Tachibana Y, Obika S, Harada-Shiba M, Yamaoka T. Efficient reduction of serum cholesterol by combining a liver-targeted gene delivery system with chemically modified apolipoprotein B siRNA. *J Control Release* 2012; 163: 119-124.
  15. Yahara A, Shrestha AR, Yamamoto T, Hari Y, Osawa T, Yamaguchi M, Nishida M, Kodama T, Obika S. Amido-bridged nucleic acids (AmNAs): synthesis, duplex stability, nuclease resistance, and in vitro antisense potency. *ChemBioChem* 2012; 13: 2513-2516.
  16. Yamamoto T, Yasuhara H, Wada F, Harada-Shiba M, Imanishi T, Obika S. Superior silencing by 2',4'-BNANC-based short antisense oligonucleotides compared to 2',4'-BNA/LNA-based apolipoprotein B antisense inhibitors. *J Nucleic Acids Res* 2012; 707323.
  17. Wada S, Obika S, Shibata M, Yamamoto T, Nakatani M, Yamaoka T, Torigoe H, Shiba M. Development of a 2',4'-BNA/LNA based siRNA for dyslipidemia and assessment of the effects of its chemical modifications in vivo. *Mol Ther-Nucleic Acids* 2012; 1: e45.
  18. Rahman SMA, Baba T, Kodama T, Islam MA, Obika S. Hybridizing ability and nuclease resistance profile of backbone modified cationic phosphorothioate oligonucleotides. *Bioorg Med Chem* 2012; 20: 4098-4102.
  19. Yamamoto T, Harada-Shiba M, Nakatani M, Wada S, Yasuhara H, Narukawa K, Sasaki K, Shibata M, Torigoe H, Yamaoka T, Imanishi T, Obika S. Cholesterol-lowering action of BNA-based antisense oligonucleotides targeting PCSK9 in atherogenic diet-induced hypercholesterolemic mice. *Mol Ther-Nucleic Acids* 2012; 1: e22.
  20. Yamamoto T, Harada-Shiba M, Nakatani M, Wada S, Yasuhara H, Narukawa K, Sasaki K, Shibata M, Torigoe H, Yamaoka T, Imanishi T, Obika S. Cholesterol-lowering action of BNA-based antisense oligonucleotides targeting PCSK9 in atherogenic diet-induced hypercholesterolemic mice. *molecular therapy-nucleic acids*, 2012; 1: e22.
  21. Mori K, Kodama T, Obika S. Design, Synthesis and properties of boat-shaped glucopyranosyl nucleic acid. *Org Lett* 2011; 13: 6050-6053.
  22. Shrestha A R, Hari Y, Yahara A, Osawa T, Obika S. Synthesis and properties of a bridged nucleic acid with a perhydro-1,2-oxazin-3-one ring. *J Org Chem* 2011; 17: 7918-7926.
- 2.学会発表**  
(国内学会)
1. 小比賀聡. 核酸創薬の新展開. 大阪大学未来戦略機構創薬シンポジウム アカデミア基盤研究から未来創薬へ, 東京, 2014.3.27.
  2. 小比賀聡. 核酸創薬研究における新たな取り組み. 第7回レドックス・ライフイノベーションシンポジウム, 東京, 2014.3.6.
  3. 小比賀聡. 生命科学・医薬研究を拓くマイクロ RNA の研究最前線核酸創薬を目指した人工核酸の開発研究. 千里ライフサイエンスセミナー, 大阪, 2014.2.21.
  4. 小比賀聡. 核酸医薬開発の現状と今後の展開 -高機能化技術/核酸修飾技術-. JST 研究開発戦略センター 次世代バイオ医薬品の俯瞰に関するワークショップ, 東京, 2013.12.19.

5. 小比賀聡. 核酸医薬の有効性・安全性評価における課題. 第10回医薬品レギュラトリーサイエンスフォーラム, 東京, 2013.12.12.
6. 小比賀聡. 核酸創薬に向けた我々の取り組み～基礎から最前線まで～. 新科学技術推進協会ライフサイエンス技術部会・分科会講演会, 東京, 2013.11.14.
7. 小比賀聡. 新規架橋型人工核酸の開発研究. ヒューマンサイエンス振興財団規制基準委員会規制動向調査WG勉強会, 大阪, 2013.7.10.
8. 原孝志, 兒玉哲也, 竹垣裕美, 森廣邦彦, 伊藤浩介, 小比賀聡. 2',4'-BNA/LNA-プリン塩基類縁体の二重鎖形成能に及ぼす二面角  $\chi$  の影響. 日本薬学会第133年会, 横浜, 2013.3.28.
9. 森廣邦彦, 兒玉哲也, 肯特夫, 百合義大, Rakesh N. Veedu, 小比賀聡. セレノメチレン架橋型人工核酸の開発と酸化還元スイッチへの応用. 日本薬学会第133年会, 横浜, 2013.3.28.
10. 山本剛史, 斯波真理子, 安原秀典, 中谷萌夏, 小比賀聡. 高力価抗PCSK9アンチセンスの探索研究. 日本薬学会第133年会, 横浜, 2013.3.28.
11. Yasuhara H, Yamamoto T, Obika S, Harada-Shiba M. Quantitative measurement of hepatic accumulation of phosphorothioated antisense oligonucleotides modified with bridged nucleic acids by elisa-based oligonucleotide detection method. 日本薬物動態学会 第27回年会, 東京, 2012.11.22.
12. 藤坂朱紀, 張功幸, 田熊寛子, 逢娟娟, 今西武, 小比賀聡. 核酸塩基部にアデニン、グアニン、チミン、5-メチルシトシンを持つN-メチル-2',4'-BNA<sup>NC</sup>の合成. 第38回 反応と合成の進歩シンポジウム, 東京, 2012.11.6.
13. 安原秀典, 山本剛史, 中谷萌夏, 柴田雅朗, 小比賀聡, 斯波真理子. 抗ApoC-IIIアンチセンス核酸による高トリグリセリド血症治療薬の開発. アンチセンス・遺伝子・デリバリーシンポジウム2012, 仙台, 2012.9.25.
14. 安原秀典, 山本剛史, 和田俊輔, 中谷萌夏, 和田郁人, 鳥越秀峰, 山岡哲二, 柴田雅朗, 今西武, 斯波真理子, 小比賀聡. PCSK9阻害による家族性高コレステロール血症治療のための高機能性核酸医薬品の開発. アンチセンス・遺伝子・デリバリーシンポジウム2012, 仙台, 2012.9.24.
15. 壽悠太郎, 張功幸, Shrestha Ajaya Ram, 小比賀聡. 新規架橋型人工核酸の合成と機能評価;グアニジノ基による架橋部へのカチオン導入の効果. アンチセンス・遺伝子・デリバリーシンポジウム2012, 仙台, 2012.9.24.
16. 安原秀典, 山本剛史, 和田俊輔, 小比賀聡, 斯波真理子. PCSK9を標的とした人工核酸BNA搭載型アンチセンス医薬の開発. 第12回遺伝子・デリバリー研究会 夏季セミナー, 北九州, 2012.7.31.
17. Yamamoto T, Harada-Shiba M, Nakatani M, Wada S, Yasuhara H, Narukawa K, Sasaki K, Shibata MA, Torigoe H, Yamaoka T, Imanishi T, Obika S. Screening and Selection of Efficient BNA-Modified anti-PCSK9 Antisenses. 第44回日本動脈硬化学会総会・学術集会, 福岡, 2012.7.20.
18. Yamamoto T, Yasuhara H, Nakatani M, Shibata MA, Obika S, Harada-Shiba M. Hypertriglyceridemic Mice Were Responsive to Apolipoprotein C-III-lowering Therapy by BNA-modified Antisense Oligonucleotides. 第44回日本動脈硬化学会総会・学術集会, 福岡, 2012.7.19.



19. 森廣邦彦, 兒玉哲也, 小比賀聡. 光刺激により認識塩基がグアニンからアデニンに変化するシン配向型人工核酸の開発. 日本ケミカルバイオロジー学会第7回年会, 京都, 2012.6.9.
- (国際学会)
1. Mori K, Kodama T, Obika S. Synthesis and properties of a nucleic acid bearing boat-shaped pyranose sugar. 13th Tetrahedron Symposium - Asia Edition, Taipei, 2012.11.29, 30.
  2. Fujisaka A, Hari Y, Takuma H, Pang J, Imanishi T, Obika S. Synthesis and Property of Oligonucleotides Modified with 2',4'-BNANC Bearing Adenine, Guanine, Thymine and 5-Methylcytosine. 13th Tetrahedron Symposium - Asia Edition, Taipei, 2012.11.27, 28.
  3. Kotobuki Y, Hari Y, Shrestha AR, Obika S. Synthesis and properties of guanidine-bridged nucleic acid: The effect of cationic bridge on duplex stability. The 39th Internatinal Symposium on Nucleic Acids Chemistry, Nagoya, 2012.11.16.
  4. Morihiro K, Kodama T, Obika S. Light-triggered Strand-displacement Reaction Using Recognition-base-changeable Nucleic Acids . The 39th Internatinal Symposium on Nucleic Acids Chemistry, Nagoya, 2012.11.15.
  5. Yasuhara H, Yamamoto T, Nakatani M, Wada S, Torigoe H, Yamaoka T, Shibata MA, Imanishi T, Harada-Shiba M, Obika S. Design, Screening of Efficient BNA Antisense Okigonucleotides for PCSK9. 8th Annual Meeting of the Oligonucleotide Therapeutics Society, Boston, 2012.10.31.
  6. Wada S, Obika S, Shibata MA, Yamamoto T, Yamaoka T, Harada-Shiba M. In Vivo Assessment of the Chemical Modification of siRNA. 8th Annual Meeting of the Oligonucleotide Therapeutics Society, Boston, 2012.10.31.
  7. Yahara A, Shrestha AR, Yamamoto T, Hari Y, Osawa T, Yamaguchi M, Nishida M, Kodama T, Obika S. Amido-bridged Nucleic Acid: Synthesis, Duplex Stability, Nuclease Resistance, and In Vitro Antisense Potency. 8th Annual Meeting of the Oligonucleotide Therapeutics Society, Boston, 2012.10.30.
  8. Yamamoto T, Yasuhara H, Nakatani M, Shibata MA, Obika S, Harada-Shiba M. Correction of Murine Hypertriglyceridemia by BNA Antisense Oligonucleotides Targeting Apolipoprotein C-III. 8th Annual Meeting of the Oligonucleotide Therapeutics Society, Boston, 2012.10.29.
  9. Osawa T, Hari Y, Obika S. Development of a Novel Bridged Nucleic Acid Based on Intramolecular Hydrogen Bond Formation. The 7th Seoul-Kyoto-Osaka Joint Symposium on Pharmaceutical Sciences for Young Scientists, Kyoto, 2012.5.25.
  10. Mori K, Kodama T, Obika S. A Boat-Shaped Glucopyranosyl Nucleic Acid: Design, Synthesis and Hybridization Properties. The 7th Seoul-Kyoto-Osaka Joint Symposium on Pharmaceutical Sciences for Young Scientists, Kyoto, 2012.5.24.
  11. Yamamoto T, Harada-Shiba M, Nakatani M, Yasuhara H, Wada S, Narukawa K, Sasaki K, Torigoe H, Yamaoka T, Imanishi T, Obika S. Bridged nucleic acid-based

antisense therapeutics targeting PCSK9 reduced serum LDL-cholesterol levels of mice fed a diet in high cholesterol. The XVI International Symposium on Atherosclerosis (ISA2012), Sydney Australia, 2012.3.26-29.

出 願 人：東京医科歯科大学, 大阪大学  
発 明 人：横田隆徳, 仁科一隆, 水澤英洋,  
小比賀聡.

国際出願番号：PCT/JP2012/083180

出 願 日：2012.12.17

12. Yamamoto T, Harada-Shiba M, Nakatani M, Wada S, Sasaki K, Yasuhara H, Torigoe H, Yamaoka T, Imanishi T, Obika S. Evaluation of Efficacies of BNA-modified Anti-PCSK9 Antisenses. The 38th International Symposium on Nucleic Acids Chemistry, Sapporo, 2011.11.9-11 (Outstanding Poster Presentation Award)
13. Yamamoto T, Harada-Shiba M, Nakatani M, Yasuhara H, Wada S, Narukawa K, Sasaki K, Torigoe H, Yamaoka T, Imanishi T, Obika S. Potency Comparison of Series of BNA-Based Antisenses in Dyslipidemic Animals, 7th Annual Meeting of Oligonucleotide Therapeutics Society, Copenhargen. Denmark, 2011.9.8.
14. Hara T, Kodama T, Takegaki Y, Morihiro K, Ito KR, Obika S. Synthesis And Hybridization Property of Oligonucleotides Containing 2',4'-BNA-7-Deazaguanine Analog. XX IRT - 20th International Roundtable on Nucleosides, Montreal, 2012.8.6, 7.
15. Mori K, Kodama T, Obika S. Investigation of the Factor Affecting the Duplex-Forming Ability of Boat-Shaped Glucopyranosyl Nucleic Acid. XX IRT - 20th International Roundtable on Nucleosides, Montreal, 2012.8.6, 7.

2.実用新案登録

特になし

3.その他

特になし

## H.知的所有権の取得状況（予定を含む）

### 1.特許取得（出願）

発 明 の 名 称：キメラ二重鎖核酸

## Ⅱ. 研究成果の刊行に関する一覧表

## 研究成果の刊行に関する一覧表

### 英文原著

著者名	論文題名	雑誌名	巻	頁	出版 西暦年	GRANT への 謝辞の 有無
Iwata R, Nishina K, Yokota T, Wada T.	Synthesis and properties of double-stranded RNA-bindable oligodiaminogalactose derivatives conjugated with vitamin E.	Bioorg Med Chem	22	1394- 1404	2014	有
Pittella F, Cabral H, Maeda Y, Mi P, Watanabe S, Takemoto H, Kim HJ, Nishiyama N, Miyata K, Kataoka K.	Systemic siRNA delivery to a spontaneous pancreatic tumor model in transgenic mice by PEGylated calcium phosphate hybrid micelles.	J Control Release	178	18-25	2014	有
Takemoto H, Miyata K, Hattori S, Ishii T, Suma T, Uchida S, Nishiyama N, Kataoka K.	Acidic pH-responsive siRNA conjugate for reversible carrier stability and accelerated endosomal escape with reduced IFN $\alpha$ -associated immune response.	Angew Chem Int Ed	52	6218- 6222	2013	有
Maeda Y, Iwata R, Wada T.	Synthesis and properties of cationic oligopeptides with different side chain lengths that bind to RNA duplexes.	Bioorg Med Chem	21	1717- 1723	2013	有
Hirai T, Enomoto M, Machida A, Yamamoto M, Kuwahara H, Tajiri M, Hirai Y, Sotome S, Mizusawa H, Shinomiya K, Okawa A, Yokota T.	Intrathecal shRNA-AAV9 inhibits target protein expression in the spinal cord and dorsal root ganglia of adult mice.	Hum Gene Ther Methods	23	119- 127	2012	無
Kuwahara H, Yokota T, Mizusawa H.	Delivery of siRNA into the blood-brain barrier: recent advances and future perspective.	Ther Deliv	3	417- 420	2012	無
Suma T, Miyata K, Ishii T, Uchida S, Uchida H, Itaka K, Nishiyama N, Kataoka K.	Enhanced stability and gene silencing ability of siRNA-loaded polyion complexes formulated from polyaspartamide derivatives with a repetitive array of amino groups in the side chain.	Biomaterials	33	2770- 2779	2012	有
Kuwahara H, Nishina K, Yoshida K, Nishina T, Yamamoto M, Saito Y, Piao W, Yoshida M, Mizusawa H, Yokota T.	Efficient in vivo delivery of siRNA into brain capillary endothelial cells along with endogenous lipoprotein.	Mol Ther	19	2213- 2221	2011	無
Christie RJ, Miyata K, Matsumoto Y, Nomoto T, Menasco D, Lai TC, Pennisi M, Osada K, Fukushima S, Nishiyama N, Yamasaki Y, Kataoka K.	Effect of polymer structure on micelles formed between siRNA and cationic block copolymer Comprising thiols and amidines.	Biomacromole cules	12	3174- 3185	2011	有
Pittella F, Zhang M, Lee Y, Kim HJ, Tockary T, Osada K, Ishii T, Miyata K, Nishiyama N, Kataoka K.	Enhanced endosomal escape of siRNA-incorporating hybrid nanoparticles from calcium phosphate and PEG-block charge-conversional polymer for efficient gene knockdown with negligible cytotoxicity.	Biomaterials	32	3106- 3114	2011	有

## 英文総説

著者名	論文題名	雑誌名	巻	頁	出版西暦年
Nishina K, Mizusawa H, Yokota T.	Short interfering RNA and the central nervous system: development of nonviral delivery systems.	Expert Opin Drug Deliv	10	289-292	2013
Oka N, Sato K, Wada T.	Recent progress in the synthesis of glycosyl phosphate derivatives.	Trends in Glycosci. Glycotechnol	24	152-168	2012
Oka N, Wada T	Stereocontrolled synthesis of oligonucleotide analogs containing chiral internucleotidic phosphorus atoms	Chem Soc Rev	40	5829-5843	2011

### Ⅲ. 研究成果の刊行物、別刷



Contents lists available at ScienceDirect

# Bioorganic & Medicinal Chemistry

journal homepage: [www.elsevier.com/locate/bmc](http://www.elsevier.com/locate/bmc)

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

Rintaro Iwata<sup>a,b</sup>, Kazutaka Nishina<sup>c</sup>, Takanori Yokota<sup>c</sup>, Takeshi Wada<sup>a,b,\*</sup><sup>a</sup> Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda, Chiba 278-8510, Japan<sup>b</sup> Department of Medical Genome Sciences, Graduate School of Frontier Sciences, The University of Tokyo, Bioscience Building 702, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8562, Japan<sup>c</sup> Department of Neurology and Neurological Science, Graduate School, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

### ARTICLE INFO

#### Article history:

Received 10 October 2013

Revised 24 December 2013

Accepted 27 December 2013

Available online 4 January 2014

#### Keywords:

Oligodiaminogalactose

Vitamin E

RNA interference

RNA duplex

Drug delivery system

### 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)- $\beta$ -D-galactopyranose oligomers (oligodiaminogalactoses; ODAGals) conjugated with  $\alpha$ -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.

© 2014 Elsevier Ltd. All rights reserved.

### 1. Introduction

Since the discovery of RNA interference (RNAi),<sup>1</sup> short interfering RNAs (siRNAs) have been receiving a lot of attention as candidates for next-generation drugs.<sup>2–4</sup> 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.<sup>5</sup> Non-viral delivery strategies have included the use of a variety of RNA-conjugates and carriers, such as cationic lipids, polymers, and other molecules.<sup>6–8</sup> 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.<sup>9–11</sup>

We previously reported that 'oligodiaminosaccharides,' which have amino groups at the 2- and 6-positions of  $\alpha$ -1-4 linked oligo-D-glucose, exhibit A-type nucleic acid duplex-binding properties.<sup>12</sup> 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  $\alpha$ -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  $\alpha$ -tocopherol transfer protein ( $\alpha$ -TTP).<sup>13</sup>

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

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

\* Corresponding author. Tel./fax: +81 4 7121 3671.

E-mail address: [twada@rs.tus.ac.jp](mailto:twada@rs.tus.ac.jp) (T. Wada).

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- $\beta$ -D-oligogalactopyranoside are expected to have similar structures and functions as those of ODAGs. We thus propose these 'oligodiaminogalactoses (ODAGs)' as novel RNA duplex-bindable molecules.

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

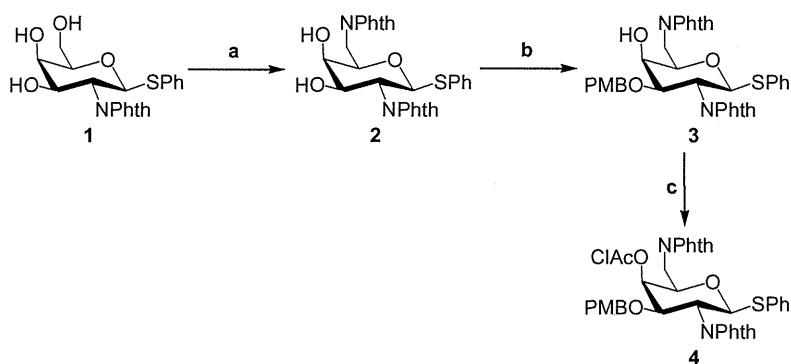
## 2. Results and discussion

### 2.1. Preparation of the glycosyl donor

To construct the  $\beta$ -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.<sup>18</sup> Next, the 6-OH group of **1** was converted to a phthalimide group via the Mitsunobu reaction to afford **2**.<sup>19</sup> The 3-OH group of **2** was then selectively protected with a *p*-methoxybenzyl (PMB) group using dibutyltin oxide to obtain **3**.<sup>20</sup> 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 ODAG **7** was obtained (Scheme 2).



**Scheme 1.** Synthesis of the glycosyl donor **4**. Reagents and conditions: (a) DIAD, phthalimide, PPh<sub>3</sub>, THF, rt, 1 h, 71%; (b) (i) Bu<sub>2</sub>SnO, 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%.

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 nearly all of the glycoside bonds were cleaved.

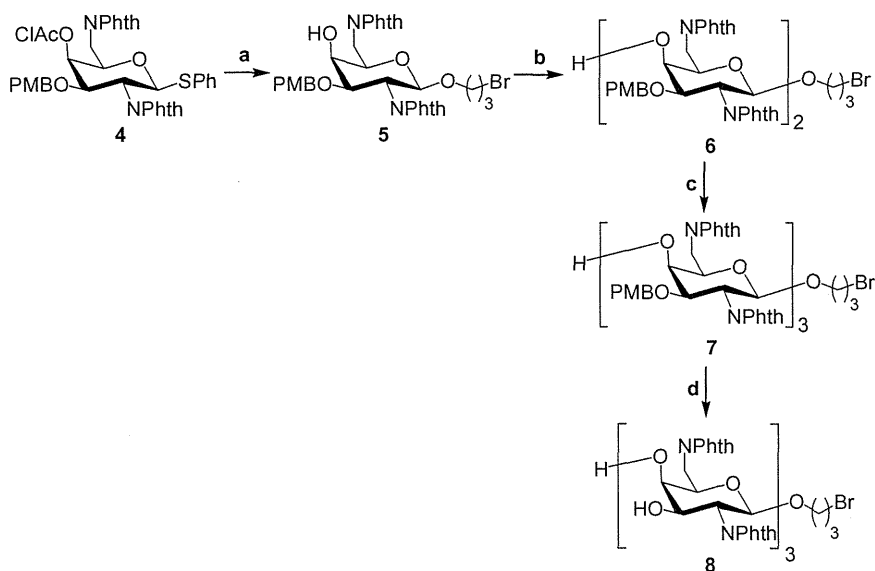
As shown in Schemes 4–6, the trisaccharide bromide **8** was azidated and conjugated with a propargylated vitamin E (**11**), vitamin E analog (**16**<sup>17</sup>), and the 4-methoxytriphenylmethyl (MMTr) group (**19**) via the Huisgen reaction<sup>21</sup> to obtain triazole-linked trisaccharides **13**, **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 ODAG derivatives **14** and **18**. Separately, the MMTr group was removed following dephthaloylation of **20** to obtain the non-conjugated ODAG **21**.

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

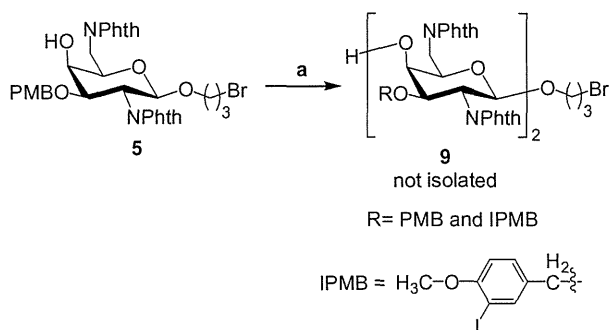
To evaluate whether ODAGs can interact with and induce structural changes or thermodynamic stabilization of RNA duplexes, UV melting, CD spectrometry, and fluorescence anisotropy measurements were carried out. All of the experiments were performed under near to physiological conditions with a 10 mM phosphate 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 ODAG **21** complexed with the RNA duplexes (5'-rCGCGAAUUCGCG-3')<sub>2</sub> (RNA-I) and (5'-rAAAAAUUUUUU-3')<sub>2</sub> (RNA-II) (1 equiv of **21** was added to the RNA duplex solution) respectively. The melting temperatures (*T*<sub>m</sub>) for the two systems increased by 2.0 °C and 2.2 °C, respectively (Table 1). These results suggest that the ODAG 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*<sub>m</sub> values were slightly decreased. These results suggest that an excess amount of ODAG thermodynamically destabilizes the duplexes, or aggregation of **21** occurs at such a high concentration.

The UV melting curves for the complexes of VE-bound ODAG **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*<sub>m</sub> values was not observed in either case. In addition, when 4 equiv of **14** were added, the *T*<sub>m</sub> values for RNA-I decreased and the UV melting curve showed an





**Scheme 2.** Synthesis of the protected tri-diaminogalactose **8**. Reagents and conditions: (a) (i) NIS, TFOH, 3-bromo-1-propanol,  $\text{CH}_2\text{Cl}_2$ ,  $0^\circ\text{C}$ , 20 min, (ii) NaOMe,  $\text{CH}_2\text{Cl}_2$ -MeOH,  $0^\circ\text{C}$ , 12.5 h, 85% over 2 steps; (b) (i) **4**, NIS, TFOH,  $\text{CH}_2\text{Cl}_2$ -Et<sub>2</sub>O,  $0^\circ\text{C}$ , 1.5 h, (ii) NaOMe,  $\text{CH}_2\text{Cl}_2$ -MeOH,  $0^\circ\text{C}$ , 13 h, 79% over two steps; (c) (i) **4**, NIS, TFOH,  $\text{CH}_2\text{Cl}_2$ -Et<sub>2</sub>O,  $0^\circ\text{C}$ , 1.5 h, (ii) NaOMe,  $\text{CH}_2\text{Cl}_2$ -MeOH,  $0^\circ\text{C}$ , 14 h, 77% over two steps; (d) 10%TFA in  $\text{CH}_2\text{Cl}_2$ ,  $0^\circ\text{C}$ , 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  $2.7^\circ\text{C}$  for RNA-I and  $9.4^\circ\text{C}$  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  $50$ – $60^\circ\text{C}$ . 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.

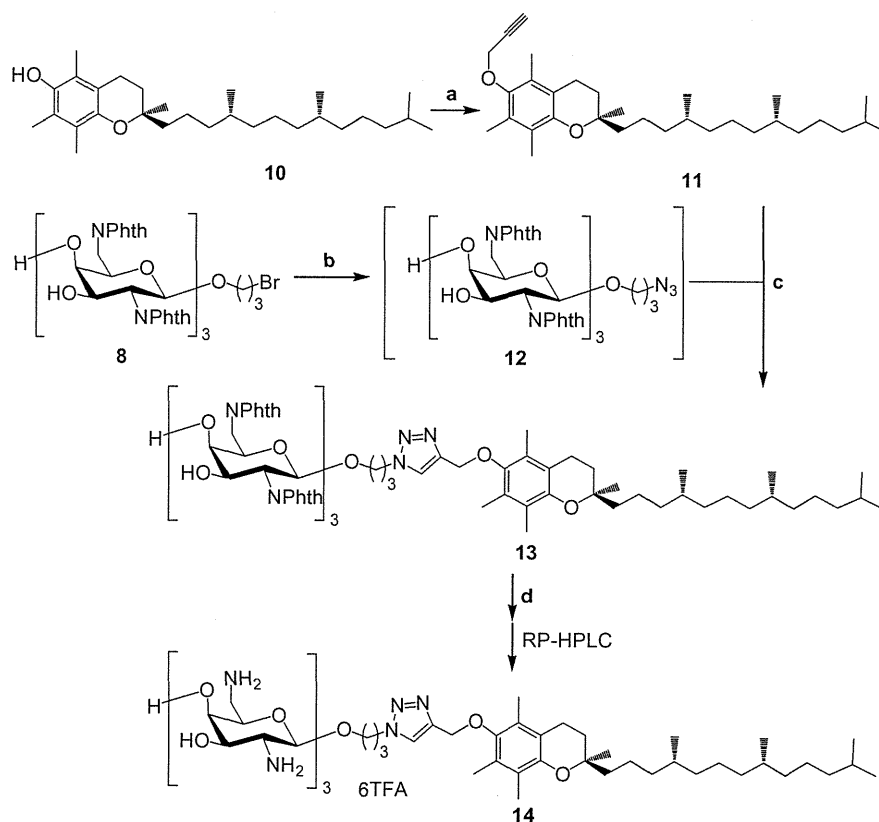
Next, to detect the structural changes in the RNA duplexes, CD spectra were measured for RNA-I and VE analog-bound ODAGal **18**, which possibly binds to the duplex. Figure 7 shows the CD spectra

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  $265\text{ nm}$  shifted  $1$ – $2\text{ nm}$  to a longer wavelength, its peak intensity increased, and the molar ellipticity near  $230\text{ nm}$  continuously changed. These changes in the peak at  $265\text{ nm}$  are very similar to those observed in our previous study upon addition of ODAGlcs to RNA duplexes.<sup>12</sup> 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.

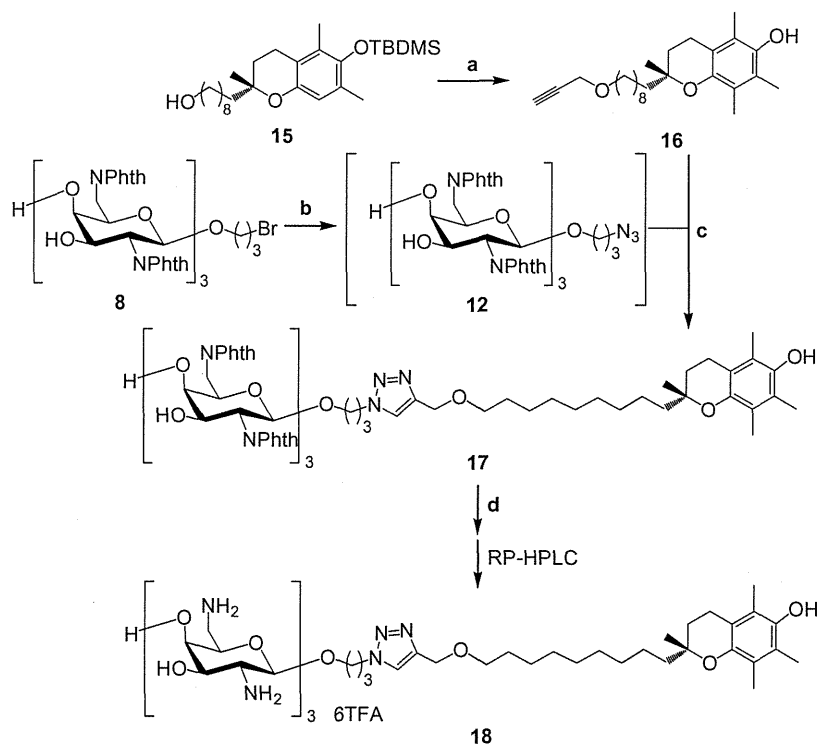
Finally, we measured the binding affinity of the VE derivative-bound ODAGals for RNA duplexes using direct fluorescence anisotropy titration. A fluorophore-labeled RNA duplex (5'-FAM-CGCGAAUUCGCG)<sub>2</sub> was used in this experiment. Figure 8 clearly shows that the VE analog-bound ODAGal **18** binds to the RNA duplex with  $K_d$   $3.8 \pm 1.2 (\times 10^{-8}\text{ M})$ . 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  $0.5\ \mu\text{M}$  **14**.

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

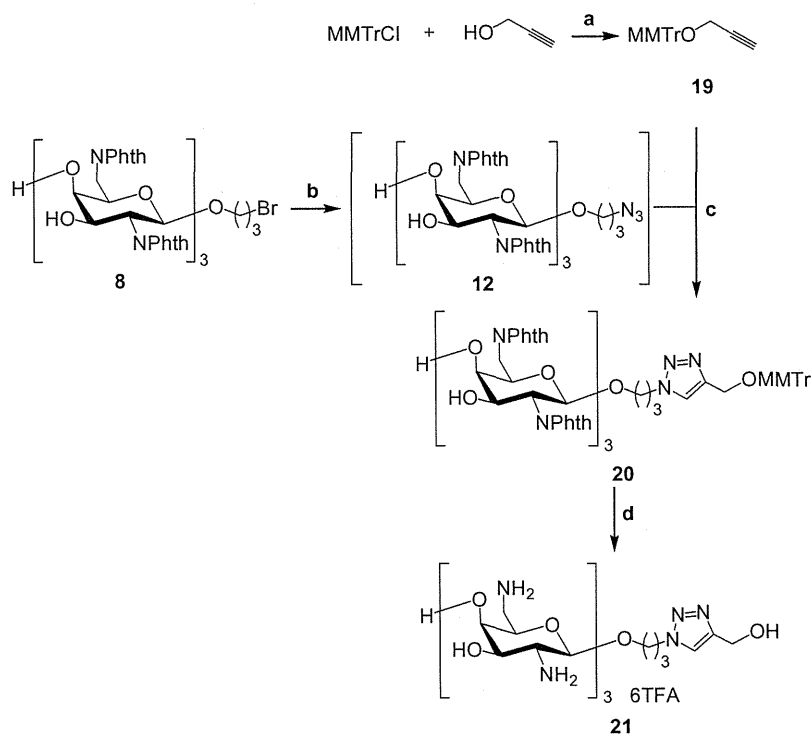
On the basis of the above results, it can be concluded that at least a portion of the ODAGal derivatives interact and form complexes with RNA duplexes. Therefore, experiments designed to evaluate RNA interference in the presence of the ODAGal derivatives were performed using an siRNA that targets apoB1 mRNA.<sup>22</sup> 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 ( $71^\circ\text{C}$ ). It has been previously shown that the oligodiaminosaccharides



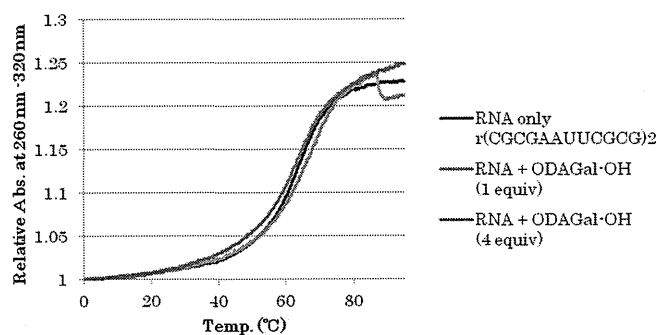
**Scheme 4.** Synthesis of the VE bound ODAGal 14. Reagents and conditions: (a) 3-bromopropyne, NaH, DMF, rt, 20 min, 82%; (b)  $\text{NaN}_3$ , DMF, 80 °C, 15 h; (c) Cu powder, *t*-BuOH–water, 80 °C, 19 h, 66% over two steps from 8; (d) 3% $\text{NH}_2\text{NH}_2\cdot\text{H}_2\text{O}$ , EtOH, 80 °C, 4.5 h, 29%.



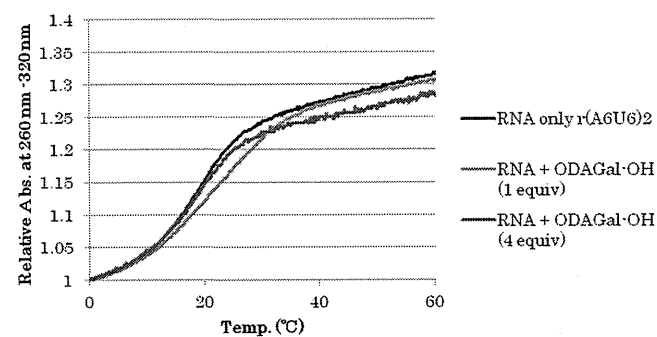
**Scheme 5.** Synthesis of the VE analog bound ODAGal 18. Reagents and conditions: (a) (i) 3-bromopropyne, NaH, THF, 60 °C, 6 h, (ii) 1 M TBAF, THF, rt, 30 min, 75% over two steps; (b)  $\text{NaN}_3$ , DMF, 80 °C, 12 h; (c) Cu powder, *t*-BuOH–water, 80 °C, 3.5 h, 91% over two steps from 8; (d) 3% $\text{NH}_2\text{NH}_2\cdot\text{H}_2\text{O}$ , 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)  $\text{NaN}_3$ , 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% $\text{NH}_2\text{NH}_2\cdot\text{H}_2\text{O}$ , EtOH, 90 °C, 4 h, (ii) 80%AcOH aq, 11% over two steps.



**Figure 1.** UV melting curves of (5'-rCGCGAAUUCGCG-3')<sub>2</sub> in the presence of VE unbound ODAGal **21**.

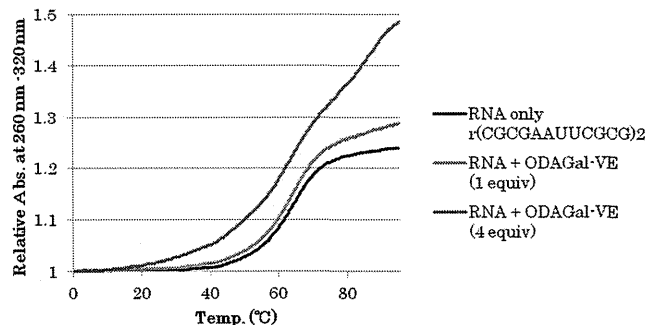


**Figure 2.** UV melting curves of (5'-rAAAAUUUUUU-3')<sub>2</sub> in the presence of VE unbound ODAGal **21**.

increase the  $T_m$  values of RNA duplexes that have low  $T_m$  values, and not high  $T_m$  values.<sup>12</sup> 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)	$\Delta T_m$ (°C)	$T_m$ (°C)	$\Delta T_m$ (°C)
<b>21</b>	RNA only	19.7	–	63.2	–
	1 equiv	21.9	2.2	65.2	2.0
	4 equiv	18.8	–0.9	62.4	–0.8
<b>14</b>	1 equiv	19.8	0.1	62.5	–0.7
	4 equiv	17.6	–2.1	n.d.	–
<b>18</b>	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')<sub>2</sub> 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**

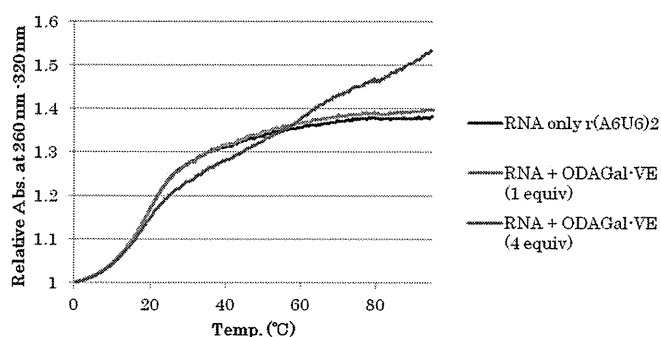


Figure 4. UV melting curves of (5'-rAAAAUUUUUU-3')<sub>2</sub> in the presence of VE unbound ODAGal 14.

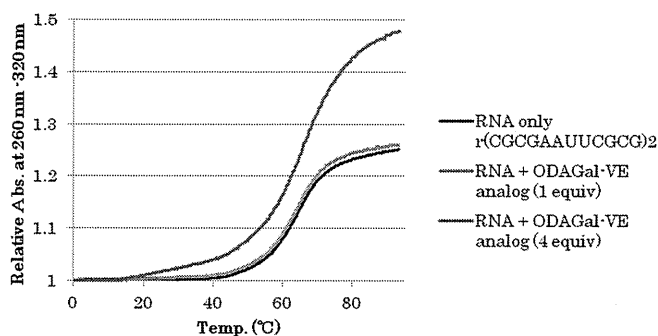


Figure 5. UV melting curves of (5'-rCGCGAAUUCGCG-3')<sub>2</sub> in the presence of VE analog bound ODAGal 18.

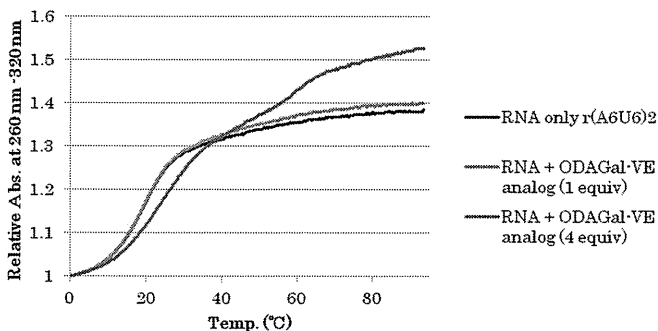


Figure 6. UV melting curves of (5'-rAAAAUUUUUU-3')<sub>2</sub> in the presence of VE analog bound ODAGal 18.

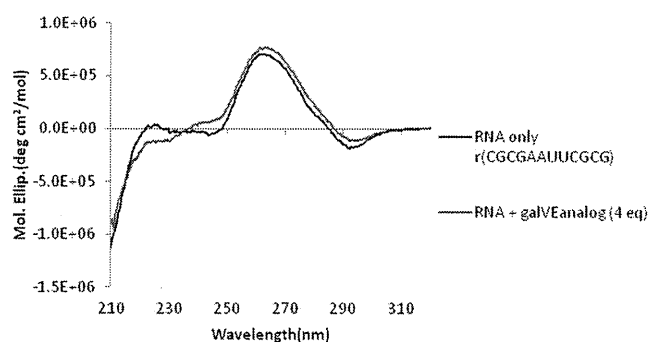


Figure 7. UV melting curves of (5'-rCGCGAAUUCGCG-3')<sub>2</sub> in the presence of VE analog bound ODAGal 18.

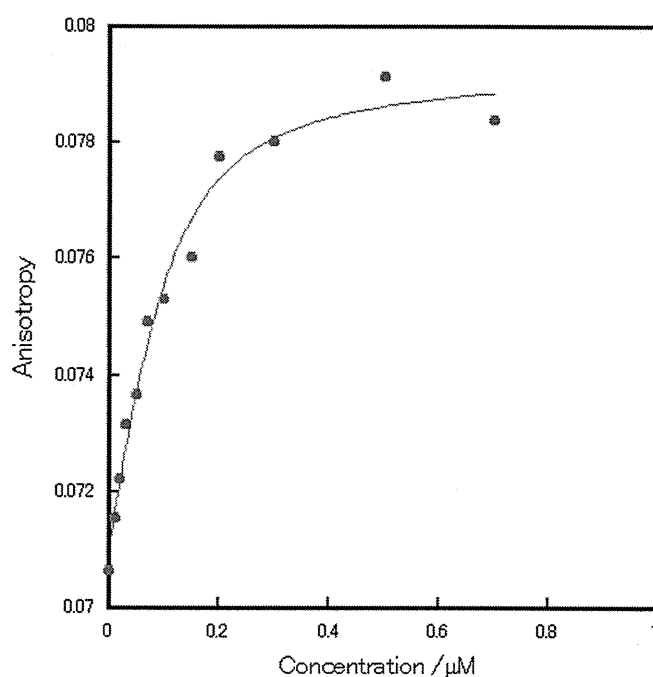


Figure 8. Fluorescence anisotropy of 100 nM of 5'-FAMlabeled RNA duplex was titrated by increasing concentration of VE analog-bound ODAGal 18 at 20 °C. The formation of the 18-RNA complex is reflected an increase in the observed anisotropy values.

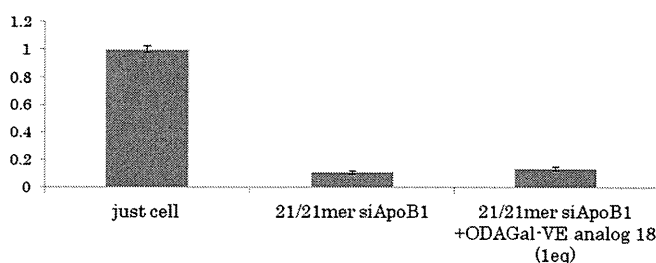


Figure 9. Evaluation of siRNA activity in the presence of ODAGal derivatives; Cell line name: McA-RH7777; Transfection reagent: Lipofectamine 2000 in culture cells of McA-RH7777.  $m \pm$  SEM Sense Sequence: 5'-GUCAUCACACUGAAUACCAAU-3' Antisense Sequence: 3'-CACAGUAGUGACUUAUGGU-5'.

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- $\beta$ -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  $\alpha$ -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.