Simple Solid-Phase Synthesis and Biological Properties of Carbohydrate−Oligonucleotide Conjugates Modified at the 3′-Terminus

Yutaka Ikeda,[†] Daisuke Kubota,[‡] and Yukio Nagasaki*

Grauate School of Pure and Applied Science, Tsukuba Research Center for Interdisciplinary Materials Science (TIMS), Center for Tsukuba Advanced Reserch Aliance (TARA), International Center for Materials Nanoarchitectonics Satellite (MANA), and National Institute for Materials Science (NIMS), University of Tsukuba, CREST, Japan Science and Technology Agency (JST), and Master's School of Medical Sciences, Graduate School of Comprehensive Human Science. Received April 28, 2010; Revised Manuscript Received June 30, 2010

A novel synthesis method for oligonucleotides possessing a functional moiety at the 3′-terminus was established based on solid-phase synthesis. In order to install the functional group at the 3′-terminus of the oligonucleotide, a solid support possessing the functional group was prepared. A carbohydrate was employed in this study for the functionalization of the oligonucleotide. To prepare a glycosylated solid support, a novel glycosyl acceptor (**2**) was synthesized using 4,4-dihydroxymethyl-cyclopenta-1-ene as the starting compound. The glycosylation reaction proceeded smoothly (yield = 95%) to yield the suitable glycosylated compound (**3**). After **8** was immobilized on the solid support, it was subjected to solid-phase oligonucleotide synthesis by the standard phosphoramidite coupling method. An oligonucleotide possessing a sugar moiety at the 3′-terminus was obtained after the products were deprotected and cleaved from the solid support. The stability of the carbohydrate-modified oligonucleotide was greatly increased even in the serum buffer, indicating that the sugar moiety at the 3′-position improved the resistance against enzymatic degradation. This technique was also applied to RNA synthesis. Galactose-ended siRNA was prepared and was confirmed to possess enough ability, at a concentration of 10 nM, to regulate the expression of the target gene.

INTRODUCTION

Oligonucleotide-based drugs such as antisense, aptamer, and siRNA drugs have attracted considerable attention as promising therapeutic agents in the treatment of human diseases (*1, 2*). Especially, siRNA is considered to be a new class of drug because it can suppress the expression of the target gene even at low concentrations (*3*). Several oligonucleotide drugs are now being evaluated in clinical trials; however, at present, only two drugs, Macugen and Vitramune, have been approved by the U.S. Food and Drug Administration. This reflects the present difficulty of exploiting therapeutic oligonucleotides. Several issues must be overcome in the development of drugs based on oligonucleotides, including the instability of oligonucleotides against enzymatic degradation and the requirement that the drug delivery system should be efficient and safe.

To increase the stability of oligonucleotide drugs, various chemical modifications of oligonucleotides have been attempted. Such chemical modifications must be designed so that they do not inhibit the activity of the oligonucleotide (*4*). For example, in the case of siRNA, the modifications should not interfere with the recognition of siRNA by the RNA-induced silencing complex (RISC), which mediates the destruction of the target RNA in RNAi, and should also not interfere with subsequent reactions, including the degradation of the target mRNA.

In general, the activity of oligonucleotide drugs strongly depends on their modification sites in the sequence. For example, when the 3′-terminus of siRNA is modified with a rigid nucleic acid, called a "locked nucleic acid (LNA)", in an overhang fashion, the gene-silencing efficiency is retained at almost the original level regardless of whether the modification is made in one or both of the strands. However, the function of siRNA is substantially impaired by modification with LNA at the 5′-terminus of the antisense strand (*5*). Terminal modification of the oligonucleotide with a biologically functional molecule is very useful, because this type of modification can be anticipated not only to increase the stability of the oligonucleotide, but also to incorporate the additional biological function into the oligonucleotide drug. For example, Soutschek et al. have reported that the modification of the 3′-terminus of the sense strand in siRNA with cholesterol improves its pharmacokinetic properties *in vivo*, probably due to the enhancement of the binding of the cholesterol-ended siRNA (chol-siRNA) to serum albumin (*6*). Even 24 h after the injection of the conjugate into mice, significant levels of chol-siRNA were detected in the liver, heart, kidney, adipose tissue, and lung (*6*).

Cationic cell-penetrating peptides (CPPs), such as the HIV-derived Tat peptides and the Antennapedia peptide, are known to be taken up by eukaryotic cells, and are therefore one important candidate as ligands to deliver a variety of molecules into cells by conjugation (*7*). Because CPPs possess a positive charge, the design of the conjugates must be carefully considered. When an anionic oligonucleotide is conjugated with a cationic CPP, for example, electrostatic aggregation cannot be avoided; this lowers the cell-penetrating ability and subsequently decreases the delivery efficiency of the conjugate (*8*). Uncharged antisense oligonucleotides, such as peptide nucleic acids (PNAs) (*9*) and phosphorodiamidate morpholino oligomers (PMOs) (*10*), must be employed instead.

Another candidate for conjugation with oligonucleoptides is carbohydrates. Carbohydrates are ubiquitous in living systems and are present either as free carbohydrates or as glycoconjugates, such as glycoproteins or glycolipids. They play roles in

* yukio@nagalabo.jp.

[†] Grauate School of Pure and Applied Science,TIMS, and TARA, University of Tsukuba, and CREST, JST.

[‡] Grauate School of Pure and Applied Science, and TIMS, University of Tsukuba.

10.1021/bc100205v
Published on Web 08/12/2010

bacterial and viral adhesion, tumor cell metastasis, the regulation of hormone and enzyme activities, and so forth (*11*). For example, various membrane lectins mediate the sugar-ligand-assisted endocytosis of complexes followed by complex dissociation in the acidic environment of the endosomes (*12*). Lectins also play an important role in delivering conjugates to the right cellular compartment, such as the cytosol or the nucleus. Since most carbohydrates are electrically neutral, the undesirable interactions mentioned above can be avoided through covalent conjugation with different types of biomolecules. Thus, these biological activities and the chemical properties of carbohydrates are useful in the development of functional conjugates for drug delivery systems.

To date, several groups have reported the development of methods to modify oligonucleotides with carbohydrates (*13*). Conjugation of a reactive carbohydrate with an oligonucleotide in solution is one of the major methods of modification (*14*). For mass production, however, it is obvious that conjugation on a solid phase during the synthesis of the oligonucleotide is much more advantageous than conjugation in solution. One of the most evident ways to incorporate a carbohydrate residue into an oligonucleotide on a solid phase is to synthesize a phosphoramidite derivative. If sugar-conjugated phosphoramidite derivatives are newly prepared, the carbohydrate can be introduced not only at the 5′-terminus, but at any site in the oligonucleotide sequence (*15, 16*).

Few studies have been carried out on the modification of the 3′-terminus of the oligonucleotide, due to the rather complicated modification reaction at this terminus. Adinolfi et al. have reported the installation of a carbohydrate at the 3′-terminus of an oligonucleotide (*16*). They used a protected carbohydrate on a solid phase and synthesized the oligonucleotide from the C6 position of the carbohydrate. To our knowledge, only Maier's group has developed the glycosylated solid support for the synthesis of sugar-conjugated oligonucleotides, in which the C1 position of the carbohydrate is linked to the 3′ terminus of the oligonucleotide. They prepared an oligonucleotide possessing a carbohydrate at the 3′-terminus by using ε-aminocapronic acid as a bifunctional linker. They prepared 1-β-aminocarbohydrate, followed by condensation with protected ε-aminocapronic acid (*17*). Recently, they developed another method (*18*) of conjugation based on the method previously reported by Reed et al. (*19*). In their method, however, the carbohydrate has to be converted to aminocarbohydrate before the condensation reaction with the bifunctional linker, resulting in a complicated reaction scheme for the preparation of the glycosylated solid support. Accordingly, we tried to establish an alternative method of modifying the oligonucleotide at the 3′-terminus with the C1 position of the carbohydrate.

In this report, we describe our novel, simple, solid-phase synthesis method of fabricating carbohydrate−oligonucleotide conjugates modified at the 3′-terminus of the oligonucleotide. We created a novel glycosylated solid support for efficient 3′-terminus modification. In our method, a protected carbohydrate is directly conjugated by the glycosylation reaction, without any transformation. The modified oligonucleotide is much more stable against enzymatic degradation. We also evaluated the effect of the modification on the activity of siRNA. The gene-silencing activities of the modified siRNAs were well retained after the conjugation.

EXPERIMENTAL PROCEDURES

Synthesis. *(1-(Benzyloxymethyl)cyclopent-3-enyl)methanol (***2***).* Compound **1** was synthesized according to a previously reported method (*20*). Compound **1** (300 mg, 2.3 mmol) was dissolved in 6 mL of *N,N*-dimethylformamide (DMF). To the solution, NaH (140 mg, 2.3 mmol) in DMF (2 mL) was added. After stirring at room temperature for 1 h, benzyl bromide (306 μL, 2.6 mmol) was added and stirred at room temperature for 12 h. The reaction mixture was extracted with ethyl acetate and saturated ammonium chloride. The organic solvent was washed with brine, dried over $MgSO_4$, and concentrated. Purification on a silica gel column with hexane−ethyl acetate gave compound **2** (440 mg, 86%). ^{1}H NMR ($CDCl_3$, 270 MHz) δ 7.42−7.27 (5H, m), 5.63 (2H, s), 4.56 (2H, s), 3.64 (2H, d, J = 5.8), 3.55 (2H, s), 2.34−2.17 (4H, m). ESI TOF/MS *m/z*: $[M+Na]^+$ 241.1769.

*(1-(Benzyloxymethyl)cyclopent-3-enyl)methyl 2,3,4,6-tetra-O-acetyl-β-*D*-galactopyranoside (***3***).* Compound **2** (100 mg, 0.46 mmol) and 1,2,3,4,6-penta-*O*-acetyl-D-galactose (179 mg,0.46 mmol) were co-evaporated 3 times with dry toluene and dissolved in 4 mL of CH_2Cl_2. To the solution, boron trifluoride diethyl etherate (84.9 μL, 0.69 mmol) was added. After stirring at room temperature for 12 h, the reaction was quenched with aqueous $NaHCO_3$. The reaction mixture was extracted with ethyl acetate. The organic solvent was washed with brine, dried over $MgSO_4$, and concentrated. Purification on a silica gel column with hexane−ethyl acetate gave compound **3** (240 mg, 95%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.38−7.27 (5H, m), 5.58 (2H, s), 5.38 (1H, dd, J = 2.4, 3.5), 5.21 (1H, dd, J = 8.0, 10.5), 5.00 (1H, dd, J = 10.5, 3.5), 4.53 (2H, d, J = 2.9), 4.44 (1H, d, J = 8.0), 4.15 (2H, m), 3.89 (1H, dd, J = 6.83, 2.4), 3.85 (1H, d, J = 9.3), 3.50 (1H, J = 9.3), 3.40 (1H, d, 8.7), 3.34 (1H, d, 8.7), 2.22 (2H, d, 11.7), 2.17 (3H, s), 2.14 (2H, d, 11.7), 2.06 (3H, s), 2.05 (3H, s), 2.00 (3H, s). ESI TOF/MS *m/z*: $[M+Na]^+$ 571.2296.

*(1-(Benzyloxymethyl)-3,4-dihydroxycyclopentyl)methyl 2,3,4,6-tetra-O-acetyl-β-*D*-galactopyranoside (***4***).* Compound **3** (240 mg, 0.44 mmol) was dissolved in acetone/H_2O/acetonitrile (1:1:1, v/v/v). To the solution, 4-methylmorpholine *N*-oxide (103 mg, 0.87 mmol) and osmium oxide-immobilized catalyst I (Wako) (3 mg) were added. After stirring at room temperature for 12 h, the reaction mixture was extracted with ethyl acetate and brine. The organic solvent was dried over $MgSO_4$ and concentrated. Purification on a silica gel column with hexane−ethyl acetate gave compound **4** in diastereomers (170 mg, 67%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.37−7.32 (5H, m), 5.38 (1H, d, J = 2.9), 5.21 and 5.17 (1H, dd, J = 7.4, 10.5 and J = 7.9, 10.5), 5.01 (1H, dd, J = 3.6, 10.5), 4.58 (1H, d, J = 6.05), 4.48 (1H, d, 6.05), 4.47 and 4.39 (1H, d, J = 7.4 and J = 7.9), 4.17 (1H, dd, J = 4.8, 11.2), 4.12 (1H, dd, J = 4.4, 11.2), 4.00 (1H, m), 3.94 (1H, m), 3.86 (1H, m), 3.71 and 3.24 (1H, d, J = 9.0 and 8.8), 3.44 and 3.22 (1H, d, J = 9.0 and 8.8), 3.36 and 3.22 (1H, d, J = 8.4 and 9.1), 3.30 and 3.20 (1H, d, J = 8.4, 9.1), 3.00 and 2.98 (1H, d, J = 9.1), 2.48 and 2.44 (1H, d, J = 7.7), 2.17 (3H, s), 2.04 (3H, s), 2.03 (3H, s), 1.99 (3H, s), 1.88 (1H, dd, J = 6.4, 13.8), 1.81 (1H, dd, J = 9.1, 13.8), 1.76 (1H, dd, J = 7.7, 13.5), 1.70 (1H, dd, J = 5.2, 13.5). ESI TOF/MS *m/z*: $[M+Na]^+$ 605.6320.

*(3-Acetoxy-1-(benzyloxymethyl)-4-hydroxycyclopentyl)methyl 2,3,4,6-tetra-O-acetyl-β-*D*-galactopyranoside (***5***).* Compound **4** (470 mg, 0.82 mmol) was dissolved in 8.2 mL of toluene. To the solution, acetic anhydride (114 μL, 1.2 mmol) was added. After stirring at room temperature for 12 h, the reaction mixture was extracted with aqueous 1 N HCl and ethyl acetate. The organic solvent was washed with aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated. Purification on a silica gel column with hexane−ethyl acetate gave compound **5** (292 mg, 57%) in diastereomers. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.37−7.22 (5H, m), 5.38 (d, J = 2.8), 5.19 (1H, dd, J = 7.8, 10.5), 4.99 (1H, dd, J = 2.8, 10.5), 4.55 (1H, d, J = 4.6), 4.5 (1H, d, J = 4.6), 4.42 (1H, d, J = 7.8), 4.22 (1H, m), 4.18 (1H, m), 3.88 (1H, m), 3.76 and 3.37 (1H, d, J = 9.0 and 8.4), 3.46 and 3.33 (1H, d, J = 9.0 and 8.4), 3.35 and 3.29 (1H, d, J =

10.3 and 8.8), 3.13 and 3.23 (10.3 and 8.8), 2.83 (1H, d, J = 7.7), 2.16 (3H, s), 2.10 (3H, s), 2.05 (3H, s), 2.01 (3H, s), 1.98 (3H, s), 1.93 (1H, m), 1.81 (1H, m), 1.72 (1H, m), 1.65 (1H, m). ESI TOF/MS *m/z*: $[M+Na]^+$ 647.7208.

*(3-Acetoxy-4-hydroxy-1-(hydroxymethyl)cyclopentyl)methyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside (***6***).* A stirred mixture of compound **5** (600 mg, 0.96 mmol) and palladium on carbon (Pd/C) (488 mg) in methanol (13 mL) was hydrogenated at ambient pressure (balloon) and temperature for 24 h. The mixture was filtered through Celite. The solvent was evaporated, and the residue was chromatographed on silica gel with hexane−ethyl acetate to give compound **6** in diastereomers. This material was used in the next step without further purification.

*(3-acetoxy-1-O-(4,4′-dimethoxytrityl)methyl-4-hydroxy)cyclopentyl)methyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside (***7***).* Compound **6** (200 mg, 0.37 mmol) was dissolved in 3 mL of pyridine. To the solution, 4,4′-dimethoxytrityl chloride (140 mg, 0.41 mmol) was added. After stirring at room temperature for 12 h, the reaction mixture was extracted with aqueous $NaHCO_3$ and ethyl acetate. The organic solvent was washed with brine, dried over MgSO4, and concentrated. Purification on a silica gel column with hexane−ethyl acetate gave compound **7** (250 mg, 70% from **5**) in diastereomers. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.42−7.22 (5H, m), 6.85−6.82 (4H, m), 5.39 (1H, d, J = 3.6), 5.19 (1H, dd, J = 7.9, 10.5), 4.99 (1H, dd, J = 3.6, 10.5), 4.46 (1H, d, J = 7.9), 4.19 (1H, m), 4.15 (1H, m), 4.13 (1H, m), 4.11 (1H, m), 4.00 and 3.90 (1H, d, J = 9.0 and 9.1), 3.47 and 3.37 (1H, d, J = 9.0 and 9.1), 3.13 and 3.05 (1H, d, J = 9.0 and 8.6), 3.00 and 2.84 (1H, d, J = 9.0 and 8.6), 2.16 (3H, s), 2.12 (3H, s), 2.01 (3H, s), 1.98 (3H, s), 1.93 (3H, s), 1.89 (1H, m), 1.82 (1H, m), 1.74 (1H, m), 1.65 (1H, m). ESI TOF/MS *m/z*: $[M+Na]^+$ 859.3449.

Synthesis of the Solid Support. Compound **7** (70 mg, 0.083 mmol) was dissolved in 2 mL of CH_2Cl_2. To the solution, 4-(dimethylamino)pyridine (26 mg, 0.21 mmol) and succinic anhydride (33 mg, 0.33 mmol) were added. After stirring at room temperature for 12 h, the reaction mixture was extracted with 10% citric acid and ethyl acetate. The organic solvent was washed with brine, dried over MgSO4, and concentrated. Purification on a silica gel column with hexane−ethyl acetate gave compound **8** (77 mg, 98%) in diastereomers.

An LCAA CPG resin (915 mg) was suspended in CH_2Cl_2 (5 mL). To the solution, compound **8** (30 mg, 32 μmol) and *N,N′*-dicyclohexylcarbodiimide (20 mg, 97 μmol) were added. After stirring at room temperature for 24 h, the resin was filtered and washed with CH_2Cl_2 and acetonitrile. A capping solution (pyridine−acetic anhydride, 9;1, v/v) was added to the resin and stirred at room temperature for 12 h. The resin was washed with CH_2Cl_2 and acetonitrile and dried *in vacuo*. The amount of compound **8** loaded onto the solid support was calculated to be 11 μmol/g, based on the calculation of the dimethoxytrityl cation released by the use of a solution of 60% $HClO_4$−EtOH (3:2, v/v).

Oligonucleotide Synthesis. Oligonucleotides were synthesized on a Nihon Techno Service NTS H-8 DNA/RNA synthesizer. Deprotection of the bases and phosphates was performed in concentrated NH_4OH/EtOH (3:1, v/v) at 55 °C for 5 h. In the case of RNA synthesis, the 2′-TBDMS groups were removed by triethylamine trihydrofluoride at room temperature for 24 h. The deprotected DNA and RNA were purified with a Sep-Pak C18 cartridge, followed by denaturing 20% polyacrylamide gel electrophoresis to afford DNAs and RNAs. These oligonucleotides were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS).

Serum Stability Assay. The serum stability of the oligonucleotides was evaluated by treatment with medium containing 10% serum at 37 °C. The modified oligonucleotides, which were labeled with fluorescence at the 5′-end of the strand and galactose at the 3′-end of the strand, and the control oligonucleotide, which was labeled with fluorescence at the 5′-end of the strand, were assayed for serum stability. Each oligonucleotide (400 pmol) was incubated with medium (100 μL) containing 10% of serum at 37 °C for the indicated time. After the incubation, the solution was diluted with formamide (100 μL) and heated at 100 °C for 2 min to quench the degradation. The samples were subjected to polyacrylamide gel electrophoresis and visualized by fluorescence scanning on a Typhoon 9400 (GE Healthcare).

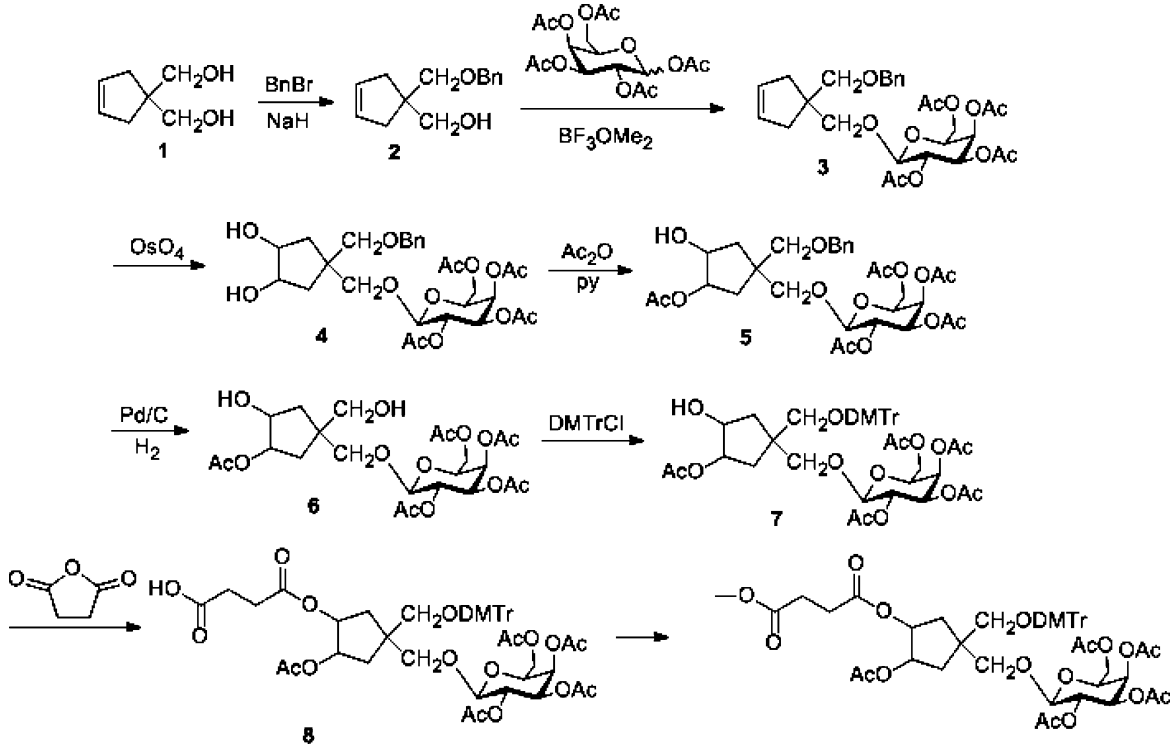
Transfection of siRNA for Surviving and Quantification of Survivin mRNA. HeLa cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, Mo) supplemented with 10% heat-inactivated fetal bovine serum and grown at 37 °C in a humidified atmosphere of 5% CO_2. 1×10^4 HeLa cells were cultured in 96-well culture plates in 100 μL of DMEM supplemented with 10% FBS, and incubated for 24 h. The indicated amounts of siRNA were transfected by Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. siRNA was mixed with 24 μL of OPTI-MEM. 0.5 μL of Lipofectamine 2000 was diluted in 24.5 μL of OPTI-MEM. After 5 min of incubation, they were combined and incubated for 20 min at room temperature. This solution was added to the cells, and the cells were incubated for an additional 48 h.

The amounts of survivin mRNA were determined by quantitative RT-PCR on a Thermal Cycler Dice Real Time System (TP-800) (TaKaRa). Isolation of the total RNA was performed using a CellAmp Direct RNA Prep Kit for One-Step RT-PCR (TaKaRa). Synthesis of cDNA and quantitative RT-PCR were performed using a One-Step SYBR PrimeScript RT-PCR Kit II (TaKaRa). The gene-specific primers used in the quantitative PCR were as follows: survivin forward primer, 5′-AGAACTGGCCCTTCTTGGAGG-3′; survivin reverse primer, 5′-CTTTTTATGTTCCTCTATGGGGTC-3′; GAPDH forward primer, GGTGGTCTCCTCTGACTTCAACA-3′; and GAPDH reverse primer, 5′-GTTGCTGTAGCCAAATTCGTTGT-3′. The relative quantification of gene expression was performed as described in the above-mentioned user's manual, using GAPDH as an internal standard.

RESULTS

In order to synthesize an oligonucleotide possessing a carbohydrate the at 3′-terminus, solid-phase synthesis was employed in this study. The synthesis procedure is summarized in Scheme 1. Compound **1** was synthesized as previously reported (*20*). Before the glycosylation reaction, we protected one of the hydroxyl groups of compound **1** with benzyl bromide, because the electron-donating benzyl group is known to increase the reactivity in the glycosylation reaction. Acetylated galactose was introduced by the glycosylation reaction via boron trifluoride methyl etherate as a Lewis acid. The yield of this glycosylation reaction was 95%. After the oxidation of compound **3** with osmium oxide, one of the hydroxyl groups on the cyclopentane was protected by acetyl chloride to afford compound **5**. The deprotection of the benzyl group of compound **5** was carried out with Pd/C, followed by protection with DMTr-Cl. Finally, compound **8** was immobilized onto the controlled pore glass (CPG). Carbohydrate−oligonucleotide conjugates were synthesized by a DNA/RNA synthesizer using the glycosylated CPG as the matrix. After the solid-phase synthesis, the obtained oligomer was deprotected and cleaved from the solid support with concentrated NH_4OH/EtOH (3:1, v/v) at 55 °C for 5 h to obtain carbohydrate-ended oligoDNA. In the case of glycosyl-ended oligoRNA, protected RNAs were incubated with triethylamine trihydrofluoride at room temperature for 24 h. The

Scheme 1[a]



[a] Reagents and conditions: (a) benzyl bromide, NaH, DMF, rt; (b) pentaacetyl-D-galactose, boron trifluoride methyl etherate, CH_2Cl_2, rt; (c) OsO_4, 4-methylmorpholine *N*-oxide, H_2O/acetonitrile/acetone (1:1:1, v/v), rt; (d) Ac_2O, pyridine, rt; (f) H_2, Pd/C, rt; (g) DMTrCl, pyridine, rt; (h) succinic anhydride, DMAP, CH_2Cl_2, rt; (i) LCAA-CPG, DCC, CH_2Cl_2, rt.

Table 1. Synthesized Galactose−Oligonucleotide Conjugates

name	sequence	calcd.	found
DNA1	Fluorescein-(dT)20-galactose	7011.4	7012.1
RNA1	GGACCACCGCAUCUCUACAdTdT-galactose	6992.3	6996
TNA2	UGUAGAGAUGCGGUGGUCCdTdT-galactose	7145.5	7151.8

deprotected DNA and RNA thus obtained were purified with a Sep-Pak C18 cartridge, followed by denaturing 20% polyacrylamide gel electrophoresis to afford sugar-ended oligomers. These oligomers were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS). Table 1 shows the sequences and the results of the MALDI-TOF-MS analysis of the synthesized oligonucleotide conjugates.

The serum stability of the oligonucleotide−carbohydrate conjugates was then investigated. The oligonucleotide which was modified with galactose at the 3′-terminus and the control oligonucleotide without galactose at the 3′-terminus were incubated with medium containing 10% of serum at 37 °C. Both oligonucleotides possessed a fluorescence probe at the 5′-terminus. After the incubation, the samples were subjected to polyacrylamide gel electrophoresis and visualized by fluorescence scanning. As shown in Figure 1, the oligonucleotide without 3′-galactose showed a degradation band, while that with 3′-galactose showed no change in molecular mass, indicating the serum stability of the oligonucleotide conjugated with galactose at the 3′-terminus. It was confirmed that ca. 80% of the incubated 3′-galactose-ended oligonucleotide remained after 180 min of incubation. It was also surprising that more than 40% of the oligonucleotide remained even after 24 h of incubation, as shown in Figure 1b.

In order to obtain further information on the modification of the oligonucleotide with the carbohydrate at the 3′-terminus, the activities of carbohydrate-modified siRNAs were investigated. For this objective, four samples were prepared, which are listed in Table 2. siRNA-targeting survivin, which is a member of the apoptosis inhibitor family (*21*), was transfected into HeLa cells with Lipofectamine 2000. After incubation for 48 h, the amount of survivin mRNA was analyzed by quantitative RT PCR. Figure 2 shows the silencing activities of the siRNAs conjugated with galactose. siRNA2, which was conjugated with galactose at the 3′-terminus of sense strand, did not show any significant change in activity at a concentration of 10 nM, as compared to the unmodified siRNA (siRNA1). Although the modification at the 3′-terminus of the antisense strand (siRNA3) reduced the silencing activity of siRNA to some extent, siRNA3 maintained its ability to reduce the amount of mRNA to 20% at 10 nM. It is worth noting that, even in the case of siRNA4, which was modified at the 3′-terminus of both strands, the amount of mRNA was reduced to 20%, i.e., almost the same level as that of siRNA3.

DISCUSSION

A safe and efficient system of delivering oligonucleotides is in great demand for the development of oligonucleotide-based therapy. However, research in this area suffers from the instability of oligonucleotides *in vivo* and the inefficient and nonselective cellular uptake of oligonucleotide drugs. To overcome these problems, we focused on glycoconjugates in our present study, because of the biologically important properties of carbohydrates. To date, various methods of delivering plasmid DNA or oligonucleotides into cells via molecules modified with a carbohydrate, such as glycosylated polyamine and glycosylated streptavidin, have been described (*22*). In these systems, the glycosylated molecules can form complexes with DNA via noncovalent interactions. In contrast to noncovalent formation, covalent oligonucleotide−carbohydrate conjugates have several advantages, e.g., the structural homogeneity of the product and the low toxicity of the components of the conjugate

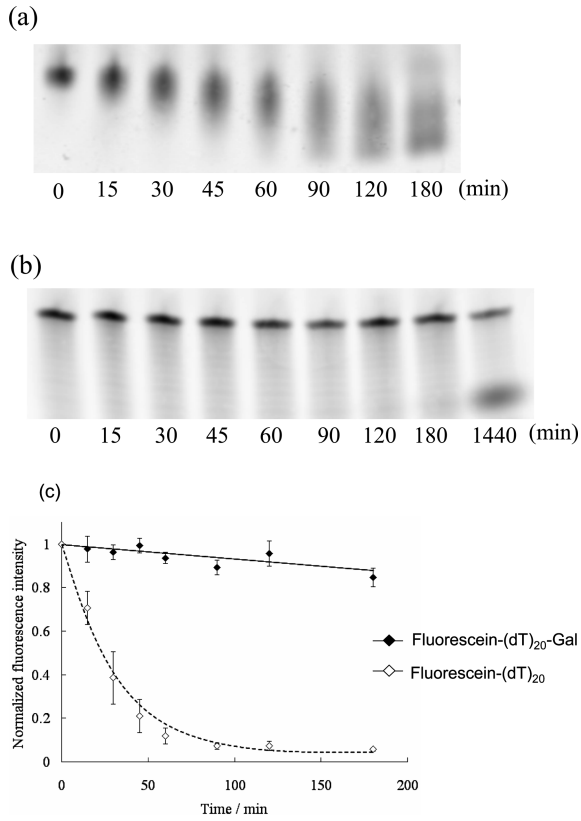


Figure 1. Comparison of the serum stabilities of the oligonucleotides. Each oligonucleotide, which was modified or not modified with galactose, was incubated in 10% serum at 37 °C for the durations indicated. All oligonucleotides were labeled with fluorescein at the 5′-end. The oligonucleotides, not conjugated with galactose (a) or conjugated with galactose (b), were subjected to polyacrylamide gel electrophoresis and visualized by fluorescence scanning. (c) Time-course of the degradation of the oligonucleotides. This result indicates that 3′-end modification with galactose improved the serum stability of the oligonucleotides.

Table 2. Sequences of the Oligonucleotides and siRNAs Used in This Study

siRNA	ON	sequence
siRNA1	ON1	5′-GGACCACCGCAUCUCUACAdTdT-3′
	ON3	3′-dTdTCCUGGUGGCGUAGAGAUGU
siRNA2	ON1	5′-GGACCACCGCAUCUCUACAdTdT-3′
	ON4	3′-**Gal**-dTdTCCUGGUGGCGUAGAGAUGU
siRNA3	ON2	5′-GGACCACCGCAUCUCUACAdTdT-**Gal**3′
	ON3	3′-dTdTCCUGGUGGCGUAGAGAUGU
siRNA4	ON2	5′-GGACCACCGCAUCUCUACAdTdT-**Gal**3′
	ON4	3′-**Gal**-dTdTCCUGGUGGCGUAGAGAUGU

compared to those of polycationic compounds such as poly(L-lysine), which is toxic at concentrations above 100 μM.

In this study, we developed an easy solid-phase synthesis method of producing carbohydrate−oligonucleotide conjugate modified at the 3′ terminus. In the synthesis scheme, the glycosylation reaction is the key reaction. Especially, when an expensive carbohydrate such as a rare sugar is used for the conjugation, the high yield of glycosylation reaction is desirable. In general, the yield of the glycosylation reaction is dependent on the protecting groups on the glycosyl donor and the glycosyl acceptor. Electron-withdrawing groups, such as the acetyl or the benzoyl group, have been found to decrease the reactivity of the donor/acceptor, and electron-donating groups, such as the benzyl group, have been found to increase the reactivity of the donor/acceptor. In our case, compound **2**, which is a glycosyl acceptor, contained a benzyl group, resulting in a good yield of the glycosylation reaction. In contrast to a previous study in

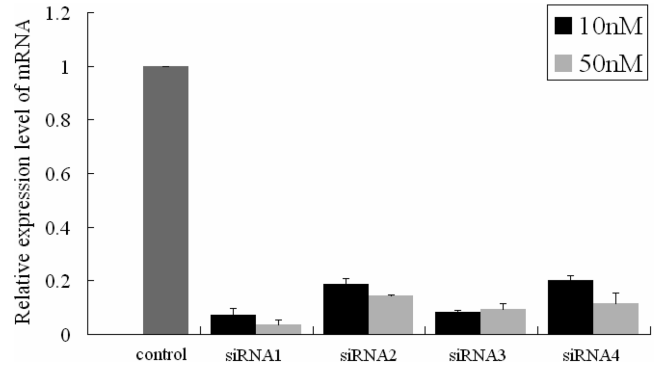


Figure 2. Survivin mRNA level in HeLa cells. HeLa cells were transfected with siRNA1, siRNA2, siRNA3, and siRNA4 at the indicated concentrations. After incubation for 48 h, the amount of survivin mRNA was measured by quantitative RT-PCR. The relative amount of survivin mRNA from the HeLa cells without any treatment was employed as a control.

which a carbohydrate was conjugated by condensation via a bifunctional linker, our approach is based on the general method of carbohydrate synthesis. Accordingly, compound **2**, which is a good glycosyl acceptor, can be easily conjugated with other glycosyl donors that have been well studied and synthesized in the field of oligosaccharide synthesis (*23*).

In an effort to increase the stability of oligonucleotides against enzymatic degradation, several chemical modifications resulting in the formation of compounds, such as phosphorothioate oligonucleotides and 2′-*O*-methyl oligonucleotide, have been reported. These modifications stabilize the oligonucleotides; however, in some cases, the modified oligonucleotides have been found to be toxic. For example, the application of phosphorothioate oligonucleotide to the central nervous system produces irritation and toxicity (*24*). In our system, oligonucleotides can be stabilized to a remarkable degree by modifying them only at the 3′-terminus. In the case of oligodeoxynucleotides, degradation usually starts to occur at the 3′-terminus of the strand. Accordingly, protection at the 3′-terminus of the strand is very important for the stabilization of the oligonucleotides (*25*). This method can be applied to oligodeoxynucleotide-based drugs, such as antisense, decoy DNA, and aptamer drugs.

We also evaluated the activities of RNA interference of carbohydrate−oligonucleotide conjugates. In some cases, the position of the modification affects the activity of the oligonucleotide drugs. For example, Kitade et al. have conjugated several lipophilic molecules at the 3′-terminus of either the sense or the antisense stand of siRNA and evaluated the activities of siRNA (*26*). In the case of modification at the 3′-terminus of the antisense strand, the activity of siRNA was slightly reduced, especially in the case of modification with cholesterol, and the silencing activity was substantially impaired (*26*). These results indicate that the properties of the compounds conjugated with oligonucleotides and the site of conjugation are important factors for efficient gene silencing. The modified siRNA in this study maintained its ability to reduce the amount of target mRNA to 20% at 10 nM, even in the case of modification at the 3′-terminus of both the sense and the antisense strands of siRNA. This indicates that two ligands can be introduced at the 3′-termini of the sense and antisense strands. Although only galactose was employed for conjugation with the end of siRNA in this work, it is anticipated from the obtained data that two kinds of ligands can be introduced, one at each end of siRNA. For example, cholesterol and a carbohydrate might be introduced at the 3′-terminus of the sense and antisense strands of siRNA, respectively, without great impairment of the siRNA activity.

In conclusion, a novel method of modifying oligonucleotides at the 3′-terminus was developed based on solid-phase synthesis.

In this study, a carbohydrate was conjugated at the 3′-terminus of an oligonucleotide on a novel glycosylated solid support. The stability of the oligonucleotide increased greatly upon carbohydrate modification. The activity of the modified siRNA, in which galactose was conjugated at the 3′-terminus, was found to possess sufficient ability to regulate the gene expression of the target gene. The novel modification method described in this report can be applied not just to carbohydrate modification, but to any modification of oligonucleotides. Studies thereon are currently being undertaken.

LITERATURE CITED

(1) Moschos, S. A., Spinks, K., Williams, A. E., and Lindsay, M. A. (2008) Targeting the lung using siRNA and antisense based oligonucleotides. *Curr. Pharm. 14*, 3620–3627.

(2) Que-Gewirth, N. S., and Sullenger, B. A. (2007) Gene therapy progress and prospects: RNA aptamers. *Gene Ther. 14*, 283–291.

(3) Castanotto, D., and Rossi, J. J. (2009) The promises and pitfalls of RNA-interference-based therapeutics. *Nature 457*, 426–433.

(4) Juliano, R., Bauman, J., Kang, H., and Ming, X. (2009) Biological barriers to therapy with antisense and siRNA oligonucleotides. *Mol. Pharm. 6*, 686–695.

(5) Elmén, J., Thonberg, H., Ljungberg, K., Frieden, M., Westergaard, M., Xu, Y., Wahren, B., Liang, Z., Ørum, H., Koch, T., and Wahlestedt, C. (2005) Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. *Nucleic Acids Res. 33*, 439–447.

(6) Soutschek, J., Akinc, A., Bramlage, B., Charisse, K., Constien, R., Donoghue, M., Elbashir, S., Geick, A., Hadwiger, P., Harborth, J., John, M., Kesavan, V., Lavine, G., Pandey, R. K., Racie, T., Rajeev, K. G., Röhl, I., Toudjarska, I., Wang, G., Wuschko, S., Bumcrot, D., Koteliansky, V., Limmer, S., Manoharan, M., and Vornlocher, H. P. (2004) Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature 432*, 173–178.

(7) Fonseca, S. B., Pereria, M. P., and Kelley, S. O. (2009) Recent advances in the use of cell-penetrating peptides for medical and biological applications. *Adv. Drug Delivery Rev. 61*, 953–964.

(8) Meade, B. R., and Dowdy, S. F. (2007) Exogenous siRNA delivery using peptide transduction domains/cell penetrating peptides. *Adv. Drug Delivery Rev. 59*, −40.

(9) Koppelhus, U., Shiraishi, T., Zachar, V., Pankratova, S., and Nielsen, P. E. (2008) Improved cellular activity of antisense peptide nucleic acids by conjugation to a cationic peptide-lipid (CatLip) domain. *Bioconjugate Chem. 19*, 1526–1534.

(10) Amantana, A., Moulton, H. M., Cate, M. L., Reddy, M. T., Whitehead, T., Hassinger, J. N., Youngblood, D. S., and Iversen, P. L. (2007) Pharmacokinetics, biodistribution, stability and toxicity of a cell-penetrating peptide-morpholino oligomer conjugate. *Bioconjugate Chem. 18*, 1325–1331.

(11) Taylor, M. E., and Drickamer, K. (2003) *Introduction to glycobiology*, Oxford University Press, United Kingdom.

(12) Vasta, G. R. (2009) Roles of galectins in infection. *Nat. Rev. Microbiol. 7*, 424–438.

(13) Zatsepin, T. S., and Oretskaya, T. S. (2004) Synthesis and applications of oligonucleotide-carbohydrate conjugates. *Chem. Biodivers. 1*, 1401–1417.

(14) Oishi, M., Nagasaki, Y., Itaka, K., Nishiyama, N., and Kataoka, K. (2005) Lactosylated poly(ethylene glycol)-siRNA conjugate through acid-labile beta-thiopropionate linkage to construct pH-sensitive polyion complex micelles achieving enhanced gene silencing in hepatoma cells. *J. Am. Chem. Soc. 127*, 1624–1625.

(15) Sheppard, T. L., Wong, C. H., and Joyce, G. F. (2000) Nucleoglycoconjugates: design and synthesis of a new class of DNA-carbohydrate conjugates. *Angew. Chem., Int. Ed. Engl. 39*, 3660–3663.

(16) Adinolfi, M., Napoli, L. D., Fabio, G. D., Iadonisi, A., Montesarchio, D., and Piccialli, G. (2002) Solid phase synthesis of oligonucleotides tethered to oligo-glucose phosphate tails. *Tetrahedron 58*, 6697–6704.

(17) Maier, M. A., Yannopoulos, C. G., Mohamed, N., Roland, A., Fritz, H., Mohan, V., Just, G., and Manoharan, M. (2003) Synthesis of antisense oligonucleotides conjugated to a multivalent carbohydrate cluster for cellular targeting. *Bioconjugate Chem. 14*, 18–29.

(18) Manoharan, M., Rajeev, K. G., Narayanannair, J. K., and Maier, M. U. S. Patent Appl. US 2009/0239814 A1.

(19) Reed, M. W., Adams, A. D., Nelson, J. S., and Meyer, R. B., Jr. (1991) Acridine- and cholesterol-derivatized solid supports for improved synthesis of 3′-modified oligonucleotides. *Bioconjugate Chem. 2*, 217–225.

(20) Kang, J. H., Chung, H. E., Kim, S. Y., Kim, Y., Lee, J., Lewin, N. E., Pearce, L., Blumberg, P. M., and Marquez, V. E. (2003) Conformationally constrained analogues of diacylglycerol (DAG). Effect on protein kinase C (PK-C) binding by the isosteric replacement of Sn-1 and Sn-2 esters in DAG-lactones. *Bioorg. Med. Chem. 11*, 2529–2539.

(21) Ryan, B. M., O'Donovan, N., and Duffy, M. J. (2009) Survivin: a new target for anti-cancer therapy. *Cancer Treat. Rev. 35*, 553–562.

(22) Lemarchand, C., Gref, R., and Couvreur, P. (2004) Polysaccharide-decorated nanoparticles. *Eur. J. Pharm. Biopharm. 58*, 327–341.

(23) Khan, S. H., and O'Neill, R. A. (1996) *Modern methods in carbohydrate synthesis*, Harwood Academic Publishers, United States.

(24) Peng Ho, S., Livanov, V., Zhang, W., Li, J., and Lesher, T. (1998) Mdificatin of phosphorothiate oligonucleotides yields potent analogs with mininmal toxicity for antisensene experiments in the CNS. *Brain Re. Ml. Brain Res. 62*, 1–11.

(25) Gamper, H. B., Reed, M. W., Cox, T., Virosco, J. S., Adams, A. D., Gall, A. A., Scholler, J. K., and Meyer, R. B., Jr. (1993) Facile preparation of nuclease resistant 3′ modified oligodeoxynucleotides. *Nucleic Acids Res. 21*, 145–150.

(26) Ueno, Y., Kawada, K., Naito, T., Shibata, A., Yoshikawa, K., Kim, H. S., Wataya, Y., and Kitade, Y. (2008) Synthesis and silencing properties of siRNAs possessing lipophilic groups at their 3′-termini. *Bioorg. Med. Chem. 16*, 7698–7704.

BC100205V