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Synthesis of oligonucleotides with glucosamine at the 3′-position and evaluation of their biological activity ☆

Xiong Luo [a], Takahiro Sugiura [b], Remi Nakashima [a], Yoshiaki Kitamura [b], Yukio Kitade [a,b,*]

[a] United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[b] Department of Biomolecular Science, Faculty of Engineering, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan

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ABSTRACT

Short interfering RNA (siRNA) has been proven to be an utilizable tool for post-transcriptional gene silencing research. In this study, we designed and synthesized two glucosamine analogues and tried to modify the siRNA using these two glucosamine analogues at the 3′-overhang region of siRNAs to improve the nuclease resistance and to overcome some other weak points. The siRNAs modified with glucosamine analogues had almost no effect of the thermal stability and showed strong resistance to nuclease degradation. Some of them kept the same gene silencing activity level as unmodified siRNA.

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RNA interference (RNAi) has been a vital area of post-transcriptional gene silencing research because of its high efficiency and sequence specificity in plants[1] and mammalian cells.[2] Moreover, a chemically synthesized short interfering RNA (siRNA) duplex was reported to induce an RNAi effect.[3] RNAi technology, as a breakthrough of gene therapy, benefits many incurable diseases;[4–8] however, there are still a number of obstacles for *in vivo* experiments and further clinical testing, such as low nuclease resistance, poor membrane permeability, and off-target effects. In the wake of the elucidation of the RNAi mechanism, chemical modification[9–12] of siRNA is considered a potential approach to overcome these siRNA-related problems.

Until now, many chemical modifications of siRNAs at the 3′-overhang region or 5′-end have been designed and synthesized,[13–16] most of which showed better thermal stability, nuclease resistance, and other biological properties than the natural molecules. In our laboratory, aromatic compounds or abasic nucleosides were introduced at the 3′-overhang region in previous studies (Fig. 1).[14–16] However, none of these modifications were aimed at improving their affinity for the cell membrane or charge neutralization. Therefore, we searched for a residue with high membrane affinity and a positive charge that could be introduced into the 3′-overhang region of siRNA, which was expected to improve cell membrane penetration, nuclease resistance, and other properties.

The amino sugar glucosamine, which is a structural element of the polysaccharides chitosan and chitin, is considered to be a good candidate. It is known that glucosamine shows high membrane affinity.[17] As a positively charged molecule, it can also neutralize the negatively charged phosphates of siRNA. Furthermore, since chitosan exists widely in organisms, such as the exoskeleton of crustaceans and other arthropods and the cell wall of fungi,[18] glucosamine shows low toxicity to the human body.

Synthesis of modified glucosamine monomer units. To introduce glucosamine into the 3′-end of both sense and antisense siRNA strands, we prepared glucosamine monomer unit **12**, with a 1′-C_2 linker and 6′-controlled pore glass (CPG) resin, as the solid support for the DNA/RNA synthesizer from the starting material glucosamine hydrochloride **1** (Scheme 1). Since directly introducing a thiophenyl group into the 1′-position of nonprotected glucosamine would result in an α/β mixture, it was necessary to protect the amino and hydroxyl groups using phthalic anhydride and acetic anhydride, respectively, to generate fully protected **2** in yield of 68%. Treatment of thiophenol in the presence of a Lewis acid ($BF_3{\cdot}OEt_2$) produced only β type **3** in 93% yield. Because of the failure to remove the phthalyl group during the post-treatment step of oligonucleotide (ON) synthesis, we changed the protecting group to a trifluoroacetyl group, which could be removed easily by aqueous ammonia. Deprotection of the acetyl and phthalyl groups of **3** followed by silylation of 6′-OH, trifluoroacetylation of 2′-NH_2, and

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* Corresponding author. Tel./fax: +81 58 293 2640.
E-mail address: ykkitade@gifu-u.ac.jp (Y. Kitade).

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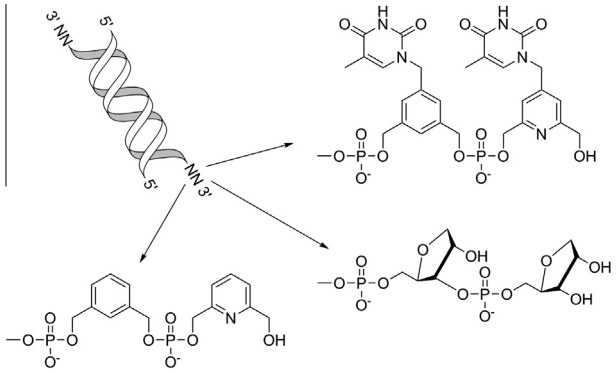


Figure 1. Structures of the modified siRNAs.

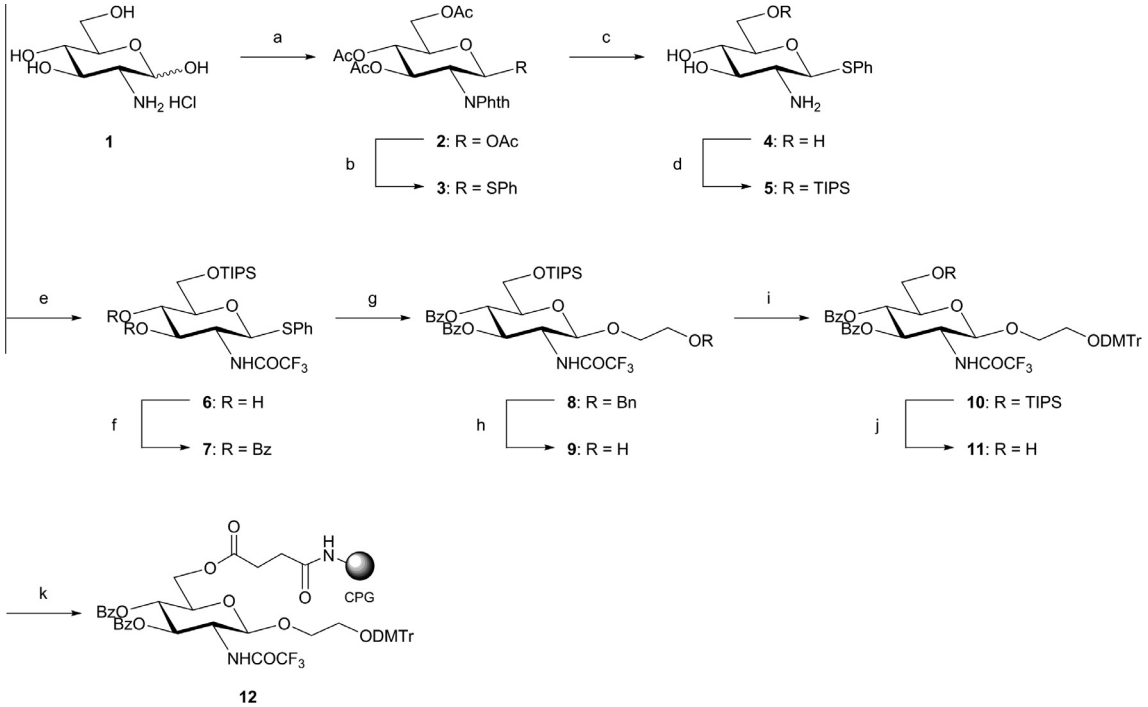
benzoylation of 3′,4′-OH afforded **4**–**7** in yields of 60, 86, 79, and 83%, respectively. In the presence of trifluoromethanesulfonic acid, treatment of 2-benzyloxyethanol, *N*-iodosuccinimide with molecular sieves 4 Å gave **8** in 78% yield. To remove the end of the 2C linker, $Pd(OH)_2$/C-catalyzed hydrogenating debenzylation and 4,4′-dimethoxytritylation were carried out to produce **9** and **10** in respective yields of 89% and 98%. Next, compound **10** was desilylated by treatment with tetrabutylammonium fluoride to afford **11** in yield of 68%. Subsequently, compound **11** was succinated to yield the corresponding succinate linked to the CPG resin to generate solid support **12** (19 μmol/g).

Furthermore, in order to examine the difference of glucosamine-modified siRNAs with/without a base moiety, we also synthesized another type of glucosamine monomer unit that included thymine at the 6′-position by an improved synthesis route (Scheme 2). In the presence of $BF_3{\cdot}OMe_2$, the treatment of 2-benzyloxyethanol to **2** could directly introduce a C_2 linker into the 1′-position of glucosamine to give **13** in 67% yield, thereby skipping the thiophenylation step. On account of the side reaction of the later Mitsunobu reaction, we deprotected the acetyl groups of **13** by treatment with MeONa in MeOH to give **14** in 88% yield. N^3-Benzoylthymine was introduced into the 6′-position of **14** by the Mitsunobu reaction to afford **15** in 84% yield. After removing the phthalyl group of the 2′-position and the benzoyl group of thymine, the synthesis strategy was the same as Scheme 1 to generate **16**–**19** in 87, 90%, quant., and 79% yields, respectively. Subsequently, the CPG resin-linked solid support **20** (37 μmol/g) was synthesized by the succination of **19**.

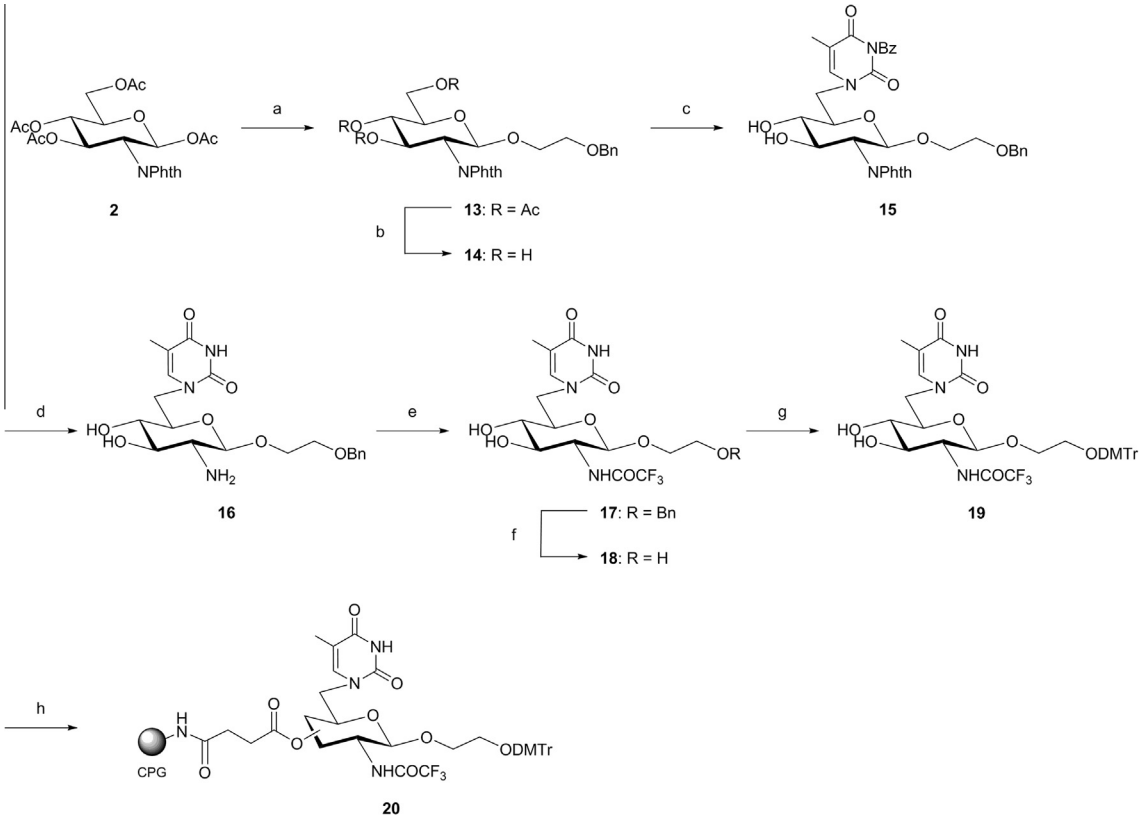
Synthesis of glucosamine analogue-modified siRNAs. We chose the *Renilla* luciferase gene as the target sequence. The basic siRNA sequences were as follows: *Renilla* sense, 5′-CUUCUUCGUCGAGACCAUG-3′; *Renilla* antisense 5′-CAUGGUCUCGACGAAGAAG-3′. By using **12** and **20**, ONs containing X or Y were synthesized using an automatic DNA/RNA synthesizer (Fig. 2). Each of these siRNA sequences were specified by their 3′-overhang region (0–2 molecules of thymidine and glucosamine analogue X or Y), and fluorescent amidites were also introduced at the 5′-end of ONs **42**–**44** for further assay tests.

The synthesized ONs were post-treated with the conventional process[11] and analyzed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The observed molecular weights of these ONs coincided with their calculated molecular weight.[19]

Thermal stability. The melting temperatures (T_m) of these siRNAs were recorded using a UV–visible spectrophotometer in a buffer of 10 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0) containing 100 mM NaCl. Absorbance at 260 nm was measured as a function of temperature from 20 to 95 °C with a 0.5 °C/min increase. The T_m values of these siRNAs are shown in Table 1. Neither of these 3′-end modified siRNAs showed a significant decrease of T_m compared with the unmodified molecule TT (T_m = 76.1 °C). Thus, we found that introduction of glucosamine analogues at the 3′-end of the ONs had



Scheme 1. Synthesis route of 6′-abasic glucosamine monomer unit. Reagents and conditions: (a) (i) MeONa, MeOH, rt; (ii) phthalic anhydride, MeOH, rt; (iii) Ac_2O, pyridine, rt, 68%; (b) PhSH, $BF_3{\cdot}OEt_2$, CH_2Cl_2, rt, 93%; (c) ethylenediamine, MeCN, reflux, 60%; (d) TIPSCl, pyridine, rt, 86%; (e) $(CF_3CO)_2O$, pyridine, rt, 79%; (f) BzCl, pyridine, rt, 83%; (g) 2-benzyloxyethanol, NIS, TfOH, CH_2Cl_2, MS4 Å, rt, 78%; (h) H_2, $Pd(OH)_2$/C, THF, rt, 89%; (i) DMTrCl, pyridine, rt, 98%; (j) TBAF, THF, rt, 68%; (k) (i) succinic anhydride, DMAP, pyridine, rt; (ii) CPG, EDC, DMF, rt, 19 μmol/g.



Scheme 2. Synthesis route of 6′-basic glucosamine monomer unit. Reagents and conditions: (a) 2-benzyloxyethanol, $BF_3{\cdot}OMe_2$, MeCN, reflux, 67%; (b) MeONa, MeOH, rt, 88%; (c) N^3-benzoylthymine, PPh_3, DEAD, THF, rt, 84%; (d) ethylenediamine, EtOH, reflux, 87%; (e) $(CF_3CO)_2O$, pyridine, rt, 90%; (f) H_2, $Pd(OH)_2/C$, THF, rt, quant; (g) DMTrCl, pyridine, rt, 79%; (h) (i) succinic anhydride, DMAP, pyridine, rt; (ii) CPG, EDC, DMF, rt, 37 μmol/g.

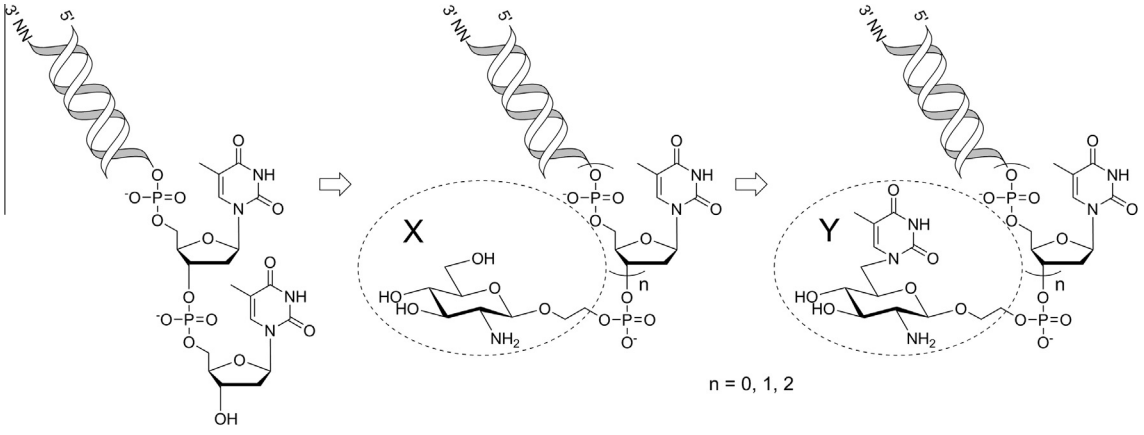


Figure 2. Structures of the siRNAs in this study.

almost no effect on the thermal stability of the siRNA duplexes. Moreover, the T_m values of the X- and Y-end siRNAs (**22**/**23**, **24**/**25**, and **26**/**27**) were slightly different, which could indicate that the nonthymine-containing glucosamine (X) siRNAs were more thermally stable than the thymine-containing glucosamine (Y) siRNAs.

Nuclease resistance. We tested the nuclease resistance of the 3′-end modified ONs **43** and **44** and the unmodified ON **42** against a 3′-exonuclease, snake venom phosphodiesterase (SVPD). After incubation in SVPD solution, degradation of the ONs was evaluated using polyacrylamide gel electrophoresis analysis under denaturing conditions (Fig. 3). The unmodified ON **42** was degraded by more than 95% after 3 min, in contrast, the modified ONs **43** and **44** were degraded by less than 40% after 3 min and approximately 30% still remained after 5 min. Therefore, the introduction of glucosamine analogues at the 3′-end of siRNAs significantly improved their resistance against SVPD nuclease.

Gene silencing of Renilla luciferase. Furthermore, we examined the gene silencing activity of each of these modified siRNAs using a dual-luciferase assay in HeLa cells targeted to the *Renilla* luciferase gene.[20] HeLa cells were co-transfected with the psiCHECK-2 vector containing *Renilla* luciferase and firefly luciferase and the indicated amount of siRNA. The results were normalized by firefly luciferase activity and the gene silencing efficacy of each siRNA

Table 1
Sequences of synthesized ONs used in this study

No. of siRNA	No. of ON	Sequence	T_m (°C)
siRNA **21**	ON **28**	5′-CUUCUUCGUCGAGACCAUGtt-3′	76.1
	ON **29**	5′-CAUGGUCUCGACGAAGAAGtt-3′	––
siRNA **22**	ON **30**	5′-CUUCUUCGUCGAGACCAUGX-3′	75.4
	ON **31**	5′-CAUGGUCUCGACGAAGAAGX-3′	––
siRNA **23**	ON **32**	5′-CUUCUUCGUCGAGACCAUGY-3′	75.2
	ON **33**	5′-CAUGGUCUCGACGAAGAAGY-3′	––
siRNA **24**	ON **34**	5′-CUUCUUCGUCGAGACCAUGtX-3′	75.5
	ON **35**	5′-CAUGGUCUCGACGAAGAAGtX-3′	––
siRNA **25**	ON **36**	5′-CUUCUUCGUCGAGACCAUGtY-3′	75.1
	ON **37**	5′-CAUGGUCUCGACGAAGAAGtY-3′	––
siRNA **26**	ON **38**	5′-CUUCUUCGUCGAGACCAUGttX-3′	74.5
	ON **39**	5′-CAUGGUCUCGACGAAGAAGttX-3′	––
siRNA **27**	ON **40**	5′-CUUCUUCGUCGAGACCAUGttY-3′	74.0
	ON **41**	5′-CAUGGUCUCGACGAAGAAGttY-3′	––
––	ON **42**	F-5′-CAUGGUCUCGACGAAGAAGtt-3′	––
––	ON **43**	F-5′-CAUGGUCUCGACGAAGAAGtX-3′	––
––	ON **44**	F-5′-CAUGGUCUCGACGAAGAAGtY-3′	––

[a]Upper case letters indicate ribonucleosides and lower-case letters show 2′-deoxyribonucleosides.
[b]F denotes fluorescein.

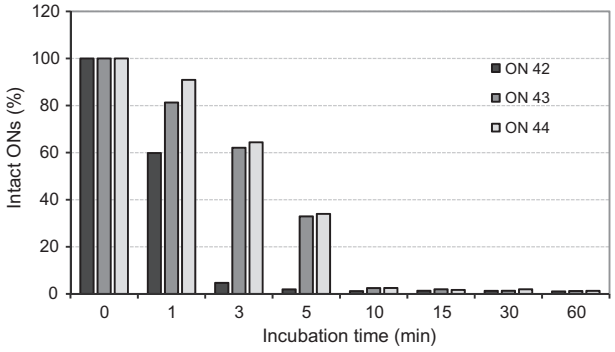


Figure 3. Nuclease resistance of ONs **42**–**44** against SVPD.

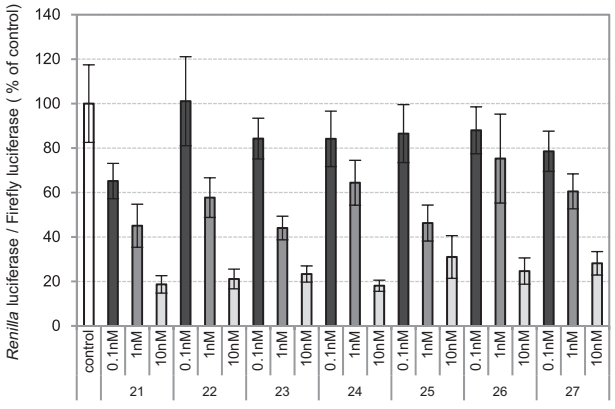


Figure 4. Dual-luciferase assay.

was assessed as a percentage of the control sample (Fig. 4). *Renilla* luciferase was suppressed by each of these siRNAs in a dose-dependent manner. Moreover, the Y-modified (thymine-containing) siRNAs showed better activity (siRNA **23** > siRNA **22**, siRNA **25** > siRNA **24**, siRNA **27** > siRNA **26**) than the X-modified (non-thymine-containing) siRNAs at 1 nM. We supposed that the thymine glucosamine could be a better choice for the 3′-overhang region modification of siRNAs.

In conclusion, we demonstrated a simple method for preparing glucosamine monomer units (with/without a base moiety at the 6′-position) that could be introduced to the 3′-overhang region of siRNAs. Compared with unmodified siRNA, glucosamine modification of siRNAs did not affect their thermal stability. It was also found that the X-modified siRNAs were more stable than the Y-modified molecules. These modified siRNAs showed an obvious improvement of resistance to nuclease degradation. All of these siRNAs suppressed *Renilla* luciferase in a dose-dependent manner and the Y-modified siRNAs showed better activity than the X-modified siRNAs at 1 nM.

Besides ribose and aromatic series modifications at the 3′-overhang region of siRNA, modification with a glucosamine analogue represents a new direction for siRNA chemical modification research. In this study, glucosamine monomer unit-modified siRNAs showed some improvement of nuclease resistance. It is expected that further study of glucosamine dimers or other base-containing analogues will overcome more of the problems associated with RNAi technology.

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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.bmcl.2013.05.036.

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19. *MALDI–TOF/MS analyses of ONs*. ON 28: calculated mass, 6579.0; observed mass, 6579.9. ON 29: calculated mass, 6727.5; observed mass, 6729.0. ON 30: calculated mass, 6256.7; observed mass, 6257.9. ON 31: calculated mass, 6405.2; observed mass, 6407.0. ON 32: calculated mass, 6365.7; observed mass, 6365.9. ON 33: calculated mass, 6514.9; observed mass, 6515.0. ON 34: calculated mass, 6561.7; observed mass, 6562.0. ON 35: calculated mass, 6712.0; observed mass, 6711.0. ON 36: calculated mass, 6669.5; observed mass, 6670.0. ON 37: calculated mass, 6819.0; observed mass, 6819.0. ON 38: calculated mass, 6865.7; observed mass, 6866.0. ON 39: calculated mass, 7010.1; observed mass, 7015.1. ON 40: calculated mass, 6973.9; observed mass, 6974.0. ON 41: calculated mass, 7122.4; observed mass, 7123.1. ON 42: calculated mass, 7265.4; observed mass, 7266.1. ON 43: calculated mass,

7248.3; observed mass, 7248.1. ON 44: calculated mass, 7355.7; observed mass, 7356.1.

20. *Dual-luciferase assay*. HeLa cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 in air in D-MEM (Wako) supplemented with 10% bovine serum (Sigma). 24 h before transfection, HeLa cells (4×104/mL) were transferred to 96-well plates (100 μL per well). They were transfected, using TransFast (Promega), according to instructions for transfection of adherent cell lines. Cells in each well were transfected with a solution (35 μL) of 20 ng of psi-CHECK-2 vector (Promega), the indicated amounts of siRNAs, and 0.3 μg of TransFast in Opti-MEM I reduced-serum medium (Invitrogen), and incubated at 37 °C. Transfection without siRNA was used as a control. After 1 h, D-MEM (100 μL) containing 10% bovine serum and antibiotics was added to each well, and the whole was further incubated at 37 °C. After 24 h, cell extracts were frozen at −80 °C. Activities of firefly and *Renilla* luciferases in cell lysates were determined with a dual-luciferase assay system (Promega). The results were confirmed by at least three independent transfection experiments with four cultures each and are expressed as the average from three experiments as mean ± SD.