Bioconjugate Chemistry

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Diels–Alder Cycloadditions on Synthetic RNA in Mammalian Cells

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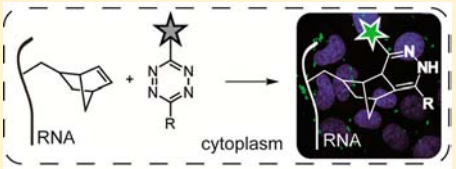
Supporting Information

ABSTRACT: Inverse electron demand Diels–Alder cycloadditions are extremely useful tools for orthogonal labeling of biomolecules such as proteins or small molecules in a cellular context. In-cell labeling of dienophile-modified RNA oligonucleotides using Diels–Alder cycloaddition reactions has not been demonstrated before. In this study we report site-specific labeling of RNA oligonucleotides modified with norbornene derivatives at a predefined sequence position within an RNA sequence *in vitro* and in mammalian cells using various tetrazine–fluorophore conjugates. The approach could in future be used as a chemical tool for the detection and investigation of RNA functions in cells minimizing the presumed distortion of RNA functions by a large chemical reporter group such as a fluorophore.



INTRODUCTION

Recently, copper-free 'click' reactions on biomolecules have gained high interest due to their mild and high-yielding labeling reactions *in vitro* and in a cellular context.[1] So far, reactions on genetically encoded alkenes in proteins and on alkene-modified small molecules in mammalian cells have been reported.[2−7]

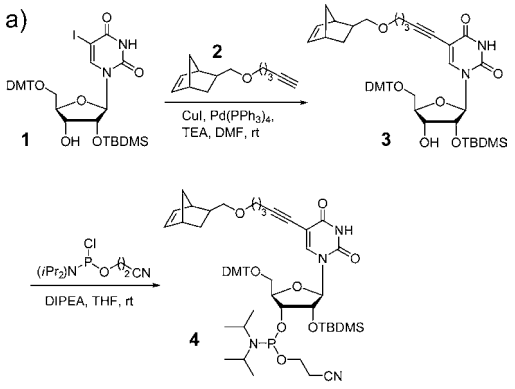
Norbornenes and other strained alkenes react readily with tetrazines in an additive-free inverse electron demand Diels–Alder cycloaddition (iEDDA) with high specificity and minimal apparent cell toxicity.[5,8,9] Tetrazine–fluorophore conjugates have been developed which exhibit a strong turn-on fluorescence after reacting with an appropriate dienophile.[10,11] Taken together, the mild reaction conditions of this cycloaddition, its high yields in aqueous solutions, and the notable quenching properties of tetrazines on adjacent fluorophores emitting between 510 and 530 nm[10] make these compounds extremely useful for labeling procedures in a cellular environment.

Labeling of oligonucleotides via click reactions has extensively been performed using copper-catalyzed alkyne–azide click reactions. Site-specific[12] as well as metabolic labeling[13] of DNA and RNA has been demonstrated. Norbornene derivatives have been incorporated into DNA oligonucleotides[14−16] and so far have been successfully used in tetrazine Diels–Alder cycloadditions.[15,16] Additionally, one example of RNA labeling via a norbornene attached to the 5′-end of RNA oligonucleotides *in vitro* is known.[17] Intrastrand labeling of modified RNA by inverse electron demand Diels–Alder cycloadditions and, in general, a nontemplate-directed, direct labeling of modified RNA (and DNA) by iEDDA in a cellular context has not been achieved so far.

RESULTS AND DISCUSSION

We aimed to utilize the Diels–Alder cycloaddition between norbornenes and tetrazines for the attachment of small molecules (e.g., fluorophores) at specific positions within RNA oligonucleotide sequences *in vitro* and in mammalian cells. For this, a norbornene modification was introduced into three different RNA strands at specific positions within the sequence during solid-phase RNA synthesis via the norbornene-modified uridine phosphoramidite **4** (Scheme 1). Synthesis of compound **4** started from the TBDMS- and DMT-protected 5-iodo-uridine **1**. Sonogashira coupling with the alkyne-bearing (*exo*-) norbornenyl derivative **2**[14] yielded

Scheme 1. (a) Synthesis of the Norbornene-Modified Phosphoramidite 4; (b) List of the Synthesized RNA Oligonucleotides Using 4 (see Table S1, Supporting Information for mass spectrometry analysis of the purified oligonucleotides)



b) RNA1: 5′-CGAUG**U***CAGUGG-3′
RNA2: 5′-G**U***GCAUACCUGGUGCUCUGUGCAC-3′
RNA3: 5′-GG**U***GAAGUUCAAGGGCGUGdTdT-3′
U* = modified unit using **4**

Received: April 17, 2014
Revised: July 28, 2014
Published: July 28, 2014

dx.doi.org/10.1021/bc500302y | *Bioconjugate Chem.* 2014, 25, 1438−1443

nucleoside **3** (with 60% yield). Subsequent phosphitylation of **3** using 2-cyanoethyl-*N*,*N*-diisopropyl-chlorophosphoramidite gave nucleoside **4** (with 75% yield). Incorporation of **4** into oligonucleotides RNA1−3 was performed under standard RNA synthesis conditions.

As a reaction partner for the inverse electron demand Diels−Alder cycloaddition we compared various tetrazine derivatives **5**-**8** containing the fluorophore Oregon Green 488 (Figure 1a).

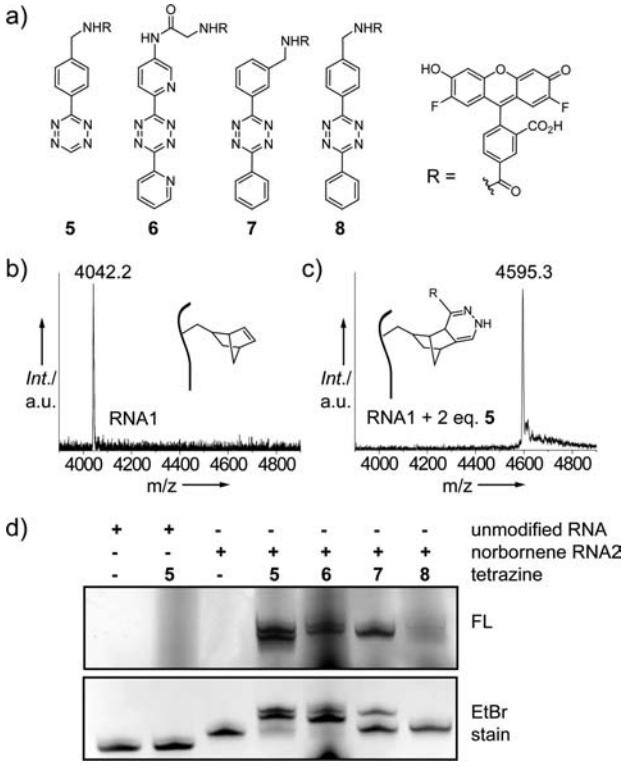


Figure 1. Inverse electron-demand Diels−Alder cycloaddition of tetrazine−Oregon Green 488 conjugates **5**−**8** with norbornene-modified RNA oligonucleotides. (a) Synthesized tetrazine−Oregon Green 488 conjugates **5**−**8**. (b) MALDI-TOF mass spectrum of purified RNA1 (*m*/*z* calc: 4038.7), (c) MALDI-TOF mass spectrum of RNA1 after reaction with 2 equiv **5** (*m*/*z* calc: 4593.8). (d) Denaturing 20% PAGE analysis of the reaction of 3 equiv of **5**−**8** with norbornene-modified oligonucleotide RNA2. The upper image shows the fluorescence scan of the gel (excitation 473 nm, emission 520 nm). The fluorescent click product is clearly visible for tetrazines **5**, **6**, and **7**. The lower image shows the same gel after staining with ethidium bromide. Here, unmodified control oligonucleotides and the norbornene-modified RNA2 are additionally detected. For an overlay of both images see Figure S4 in the SI.

The compounds were synthesized starting from the corresponding aromatic nitriles, the fluorophore was introduced as an activated carboxyl ester in the final step. Compound **5** has already been reported,[9] **6** is known with other fluorophores.[9] The detailed synthesis[9,18] and purification of the tetrazine-fluorophore conjugates **5**-**8** is described in the Supporting Information (SI). All conjugates show significant turn-on fluorescence and reactivity in the Diels−Alder reaction with norbornene derivatives (Figure S2, SI). Oregon Green 488 was chosen due to its high photostability and high quantum yield which is required for fluorescence cell microscopy applications.[19]

Reaction of the norbornene-modified oligonucleotide RNA1 with a 3-fold excess of the tetrazine−fluorophore conjugate **5** in aqueous solution results in the cycloaddition product which was confirmed with MALDI-TOF mass spectrometry (Figure 1c). Here, no unreacted educt is detected after incubation of both reaction partners at room temperature for 1 h. Denaturing PAGE analysis of the reaction of **5** with RNA1 proved to be difficult, as the excess of the unreacted tetrazine−fluorophore conjugate shows a similar migration behavior on the denaturing gel compared to the short, 12mer RNA oligonucleotide RNA1 (Figure S3 in the SI). Therefore, we performed PAGE analysis of the click reaction of tetrazines **5**−**8** with the longer RNA oligonucleotide RNA2 which is designed to fold into a hairpin structure bearing the norbornene modification within its stem region. This allows us to demonstrate that the click reaction also works if the norbornene modification is positioned in a double-stranded region of a structured RNA. Denaturing PAGE now shows a nearly quantitative reaction of the tetrazines **5** and **6** with the norbornene-modified oligonucleotide RNA2 (Figure 1d, yields 96% (**5**) and 97% (**6**) calculated from the band intensities).[20] Moreover, for both tetrazines, **5** and **6**, two bands of the cycloaddition product are detected via PAGE analysis (Figure 1d). This observation is also made for RNA1 (Figure S3 in the SI), where only one molecular mass is detected for the mixture (Figure 1c). Likewise, RNA3 reacts with tetrazine **5** showing two cycloaddition products (Figure S3 in the SI). We conclude that PAGE analysis is sensitive enough to separate two different product isomers of the click reaction, most likely the two isomers arising from exoaddition of the tetrazine.[15,21] To verify that isomers are present, we analyzed the crude reaction mixture via LC−MS as well as excised the bands for MS analysis (Figure S9 and S10 in the SI). Only one molecular mass is detected for both bands and several HPLC product peaks. Interestingly, tetrazine **6** partly degrades with time. An additional band besides the two cycloaddition products is detected (Figure 1d/S4 and S9 in the SI). Mass spectrometry analysis proves that tetrazine **6** has reacted; however loss of the fluorophore due to cleavage of the aliphatic amide bond has occurred (see Figure S9 in the SI).

Surprisingly, tetrazine **8** shows hardly any reaction with the RNA2 oligonucleotide. In contrast, the meta-substituted tetrazine **7** does react, however, with low yield (∼40%). Moreover, only one product band is detected—possibly one isomer of the cycloaddition is favored due to the attachment of the fluorophore in the meta position. We assume that steric effects of the disubstituted tetrazines with the RNA reduce the overall yield of the click reaction. This is possibly compensated by the more electron-deficient and therefore more reactive tetrazine ring in **6**.

In mammalian cells, tetrazine cycloadditions have only been used so far for the labeling of small molecules and proteins,[3,4,6,7,9,10] a recent exception being the template-directed cycloaddition between two DNA strands.[16] Therefore, we investigated whether these reactions proceed also in mammalian cells with alkene-modified RNA. As a proof of concept, we transfected a modified 21mer RNA duplex into NCI-H460 and HEK 293 cells (Figure S7 in the SI) cells using Lipofectamine 2000 as transfection reagent. The duplex with 3′-dTdT overhanging ends consists of the norbornene-modified sense strand (RNA3) and the corresponding antisense strand, either unmodified or ATTO647 labeled. This siRNA-like duplex was chosen due to the well-established transfection protocols for siRNAs and its application potential.[22] Cells were

dx.doi.org/10.1021/bc500302y | *Bioconjugate Chem.* 2014, 25, 1438−1443

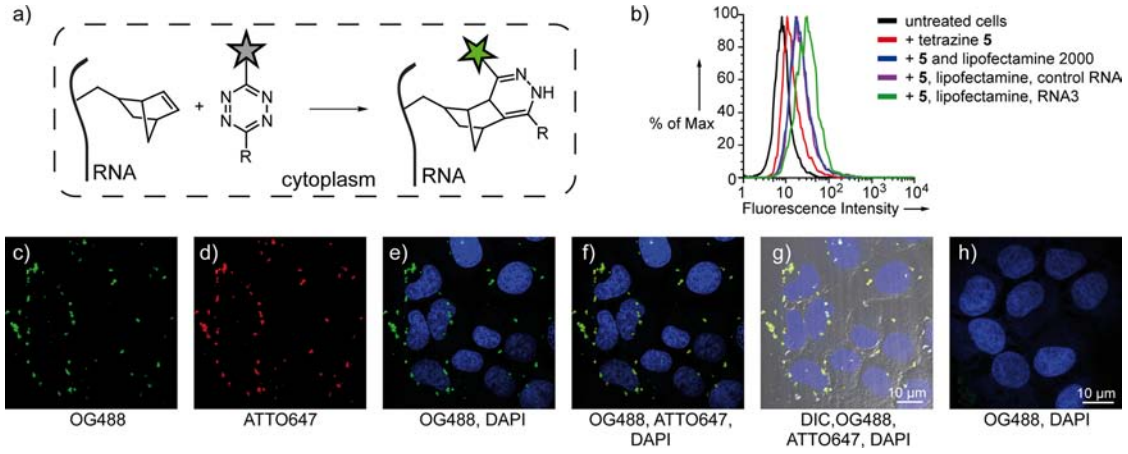


Figure 2. Click reaction of norbornene-modified RNA and tetrazine–fluorophore conjugates performed in mammalian cells. NCI-H460 cells were transfected with an RNA duplex consisting of the norbornene-modified RNA3 and the corresponding antisense oligonucleotide either unlabeled or labeled with ATTO647 prior to transfection. (a) Subsequent to transfection of the norbornene-modified RNA, 4 h incubation and several washing steps, the cells were incubated with tetrazine–fluorophore conjugate **5** for 1 h, and the fluorescence was analyzed via flow cytometry (b) and fluorescence microscopy (c–h). (b) Flow cytometry analysis of the click reaction in H460 cells (green curve). Various controls using unlabeled RNA of the same sequence as RNA3 (purple) or tetrazine **5** with and without transfection reagent (blue and red curve) are performed to determine the background fluorescence. (c–g) Confocal fluorescence microscopy images of fixed H460 cells after in-cell click reaction with tetrazine **5** on double-labeled siRNA (norbornene modification on sense strand and ATTO647 on antisense strand). Images were obtained using an *Olympus FV1000* confocal laser scanning microscope. (c) RNA2 labeled using **5** is visible as green (OG488) fluorescence; (d) ATTO647 labeled antisense strand of the siRNA duplex is shown as red fluorescence (633 nm excitation signal); (e) merged green fluorescence image (488 nm excitation signal) and nuclei stain (DAPI, 405 nm excitation signal); (f) merged image of green, red, and DAPI fluorescence; (g) merged image of differential interference contrast (DIC), green, red, and DAPI fluorescence; (h) H460 cells after transfection with unlabeled control RNA and incubation with **5**. The images of green and DAPI fluorescence are overlaid. Images of further control experiments are found in Figure S8 in the SI.

transfected with the RNA duplex (4 h), washed several times to ensure that unincorporated RNA was removed, and subsequently incubated with a 10-fold excess of the correspondent tetrazine–fluorophore conjugate (Figure 2a). The increase in fluorescence intensity upon click reaction in transfected cells was detected using flow cytometry and fluorescence microscopy.

Results of the flow cytometry analysis for the cycloaddition reaction in H460 cells using tetrazine **5**, the RNA duplex containing RNA3, and an unlabeled antisense strand are depicted in Figure 2b. An increase in fluorescence is observed for controls such as the tetrazine **5** itself, tetrazine **5** in combination with lipofectamine 2000 or in combination with unlabeled control siRNA due to the background fluorescence of the tetrazine–fluorophore conjugate. Nevertheless, for the reaction of **5** with norbornene-modified RNA in live mammalian cells we do observe a strong increase in fluorescence (Figure 2b, green curve) which is comparable to the fluorescence of cells containing RNA labeled with Oregon Green 488 prior to transfection, either via tetrazine click in media or directly at its 5′-end via an amino-modified RNA and the NHS-activated fluorophore (Figure S5 in the SI). Comparing the fluorescence intensities resulting from the click reaction with **5** to the fluorescence intensities resulting from the background, the population comparison gives a value of 63.6% (Figure S6 in the SI). We also performed the cycloaddition reaction with tetrazines **6** and **7** in H460 cells (see SI, Figure S6). For tetrazine **7**, a similar increase in fluorescence intensity after cycloaddition compared to tetrazine **5** is measured (62.4%). However, for tetrazine **6** the increase in fluorescence intensity is remarkably weaker (20.9%). Tetrazine **6** shows a high reactivity on RNA *in vitro* (Figure 1d), though it seems not to react efficiently in cells (17.4%). We cannot rule out the possibility that the uptake of **6** into cells is not as efficient compared to that of tetrazine **5**. Moreover, loss of the fluorophore in tetrazine conjugate **6** due to cleavage of the aliphatic amide bond is observed with time (Figure 1d and Figure S9 in the SI) which makes this tetrazine–fluorophore conjugate less suitable for the application.

We subsequently attempted to localize the Diels–Alder reaction product of tetrazine–fluorophore conjugate **5** with modified RNA in mammalian cells using fluorescence microscopy. For cell microscopy, we used a double-labeled siRNA, the sense strand bearing the norbornene modification (RNA3) and the antisense strand an ATTO647 fluorophore label. After 4 h transfection of the siRNA, followed by incubation with tetrazine **5**, cells were fixed with paraformaldehyde, and the nuclei were stained with DAPI and examined using a confocal laser scanning microscope within 12 h past fixation. A strong turn-on of the fluorescence after reaction of **5** with RNA3 in H460 cells can clearly be detected (Figure 2c, e, and g). The tetrazine–Oregon Green 488-labeled RNA is localized to discrete foci in the cytoplasm. Fixation of the cells occurred 5 h after transfection of the siRNA; thus, the RNA is still concentrated in lipid vesicles from the transfection carrier.[23−25] This does not prevent efficient labeling by the tetrazine–fluorophore conjugate. The ATTO647-labeled antisense strand of the siRNA duplex colocalizes exactly with the green fluorescence of the tetrazine **5**–RNA cycloaddition product (Figure 2f and g). Five hours post transfection, separation of sense and antisense strand of a siRNA duplex has not yet occurred.[24] This further strengthens our conclusion that successful transfection of the norbornene-modified siRNA duplex and subsequent cycloaddition with tetrazine–fluorophore conjugate **5** was achieved.

dx.doi.org/10.1021/bc500302y | *Bioconjugate Chem.* 2014, 25, 1438−1443

CONCLUSION

In summary, we incorporated a norbornene−uridine derivative as phosphoramidite into RNA via solid-phase RNA synthesis and performed successful copper-free click reactions with various tetrazine−fluorophore conjugates *in vitro* and demonstrate that the click reaction can be effectively performed on RNA in mammalian cells. We further showed that the reactivity of different prepared tetrazine−fluorophore conjugates on RNA differs remarkably both *in vitro* and in cells presumably due to steric effects, diffusion, and/or cell permeability. Moreover, for one construct (**6**) instability of the fluorophore linkage is observed. Thus, for broader applications an individual optimization of the employed tetrazine derivatives for cycloaddition on dienophile-modified RNA is required. This high-yielding and mild-labeling reaction on RNA in mammalian cells can be useful for future work toward time-dependent localization of RNA probes bound to specific targets in cells, especially when the attachment of a large label to the RNA probe needs to be separated from the initial target recognition process. The presented labeling strategy could in future be employed as a chemical tool for the detection and investigation of RNA functions in cells by complementary RNA probes. In contrast to already known techniques such as molecular beacon, transfection with fluorescently labeled RNA, DNA, or even PNA[21], our approach attempts to minimize the presumed distortion of RNA transport within the cell and functions by the chemical reporter (e.g., fluorogenic dye) which can be attached to the RNA probe subsequently to target binding.

EXPERIMENTAL PROCEDURES

Synthesis and Characterization of the Norbornene-Modified Phosphoramidite 4. *Synthesis of 3.* Triethylamine (0.4 g, 3.1 mmol) was added to a degassed solution of 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-*tert*-butyldimethylsilyl-5-iodoridine **1**[26] (0.10 g; 0.13 mmol), CuI (0.02 g, 0.11 mmol), and $Pd(PPh_3)_4$ (0.05 g, 0.04 mmol) in dry DMF (10 mL). The solution was stirred for 10 min at room temperature. Alkyne **2**[14,27] (0.4 g, 2.1 mmol) was added, and the reaction was stirred at room temperature overnight. The solvents were removed *in vacuo*, and the crude product was purified via flash chromatography (SiO_2,CH_2Cl_2/MeOH 95/5) to yield compound **3** (66 mg, 0.08 mmol, 60%) as yellow foam. R_f = 0.18 (cyclohexane/ethyl acetate: 3/1); ^{1}H NMR (500 MHz, DMSO-d_6): δ = 11.68 (s, 1H; N*H*), 7.93 (s, 1H, H-6), 7.44−7.23 (m, 9H, C*H* aryl DMT), 6.92−6.88 (m, 4H, C*H* aryl DMT), 6.09−6.04 (m, 2H; C*H* norbornene olefin), 5.81 (d, *J*(H,H) = 5.1 Hz, 1H; C1′-H), 5.18 (d, *J*(H,H) = 5.9 Hz, 1H; 3′−OH), 4.34 (t, *J*(H,H) = 5.1 Hz, 1H; C2′-H), 4.10−4.07 (m, 1H, C3′-H), 4.05−4.03 (m, 1H; C4′-H), 3.75 (s, 6H; CH_3 DMT), 3.33−3.16 (m, 6H; C5′-H, ≡CCH_2CH_2CH_2O-, ≡CCH_2CH_2CH_2OCH_2-), 2.76 (s, 1H; C*H* bridgehead norbornene), 2.63 (s, 1H; C*H* bridgehead norbornene), 2.15−2.10 (m, 2H; ≡CCH_2), 1.56−1.50 (m, 1H; C*H* norbornene), 1.45−1.40 (m, 2H; ≡CCH$_2$CH_2-), 1.28−1.24 (m, 1H; CH_2 bridge), 1.20−1.18 (m, 1H; CH_2 bridge), 1.15−1.03 (m, 2H; CH_2 norbornene), 0.86 (s, 9H; TBDMS *t*-Bu methyls), 0.08 (s, 3H; TBDMS methyl), 0.06 ppm (s, 3H; TBDMS methyl); ^{13}C NMR (126 MHz, DMSO) δ = 161.55, 158.09, 149.52, 144.67, 141.45, 136.34, 136.30, 135.34, 135.09, 133.53, 129.65, 129.62, 127.87, 127.46, 126.66, 113.23, 113.18, 99.58, 93.08, 88.03, 86.04, 83.51, 75.74, 74.41, 71.97, 69.82, 68.60, 63.00, 55.01, 44.61, 43.20, 40.97, 38.45, 29.15, 28.02, 25.58, 17.88, 15.63, −4.84, −5.20 ppm.; ESI-HRMS: *m*/*z* [M + Na]$^+$ calcd for $C_{49}H_{60}N_2O_9SiNa$: 871.3960, found: 871.3937.

Synthesis of 4. Compound **3** (66 mg, 0.08 mmol) and *N,N*-diisopropylethylamine (0.05 g, 0.39 mmol) were dissolved in dry THF (1.0 mL). 2-Cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (0.04 g, 0.17 mmol) was added, and the reaction was stirred for 3 h at room temperature. The solvents were removed *in vacuo*, and the crude product was purified via flash chromatography (SiO_2,CH_2Cl_2/MeOH 20/1) to yield **4** (61 mg, 0.06 mmol, 75%) as white foam. Compound **4** is stable over 6 months stored under argon at −20 °C. R_f = 0.15 (CH_2Cl_2/MeOH: 20/1); ^{1}H NMR (600 MHz, $CDCl_3$): Slow inversion at the phosphorus results in two diastereoisomers a and b of approximately equal intensities. The specific assignment is given whenever possible. δ = 8.15 (b), 8.09 (a) (s, 1H, H-6), 7.46−7.28 (m, 9H, C*H* aryl DMT), 6.86−6.82 (m, 4H, C*H* aryl DMT), 6.08 (a), 5.99 (b) (d, *J*(H,H) = 7.0(a), 6.3(b) Hz, 1H; C1′-H), 6.08−6.03 (m, 2H; C*H* norbornene olefin), 4.57(a), 4.47(b) (dd, *J*(H,H)= 4.5, 7.0 Hz (a), *J*(H,H) = 5.0, 6.2 Hz (b), 1H; C2′-H), 4.31−4.30 (b), 4.18−4.17 (a) (m, 1H; C4′-H), 4.24−4.20 (a), 4.17−4.15 (b) (m, 1H, C3′-H), 3.99−3.86 (a/b), 3.65−3.56 (b/a) (m, 2H; OCH_2CH_2CN), 3.79 (a/b), 3.78 (b/a) (s, 6H; CH_3 DMT), 3.58−3.13 (m, 7H; C5′-H, ≡CCH_2CH_2CH_2O-, ≡CCH_2CH_2CH_2OCH_2-, C*H* *i*Pr), 2.77 (s, 1H; C*H* bridgehead norbornene), 2.66 (s, 1H; C*H* bridgehead norbornene), 2.68−2.60 (a/b), 2.35−2.26 (b/a) (m, 2H; OCH_2CH_2CN), 2.13−2.03 (m, 2H; ≡CCH_2), 1.62−1.56 (m, 1H; C*H* norbornene), 1.43−1.31 (m, 2H; ≡CCH$_2$CH_2-), 1.28−1.00 (m, 4H; CH_2 bridge, CH_2 norbornene, CH_3 *i*Pr), 0.90 (a/b), 0.87 (b/a) (s, 9H; TBDMS *t*-Bu methyls), 0.07 ppm (br s, 6H; TBDMS methyl); ^{31}P NMR (122 MHz, $CDCl_3$) δ = 149.7; 151.7 ppm.; ESI-HRMS: *m*/*z* [M + Na_2]$^{2+}$ calcd for $C_{58}H_{77}N_4O_{10}PSiNa_2$: 547.2465, found 547.2465.

The chemical synthesis and characterization of the tetrazines in conjugates **6**, **7**, and **8** and the preparation of all conjugates with Oregon Green 488 are described in the SI (For spectra see figure S12). RNA synthesis and incorporation of **4** into RNA oligonucleotides was performed on an ABI3400 DNA Synthesizer (*Applied Bioscience*) using standard conditions and 2′-TBDMS-protected phosphoramidites. The antisense strand of the siRNA has the following sequence: 5′-CACGCCCU-UGAACUUCACCdTdT-3′ (either unlabeled or 5′-ATTO647-labeled). Oligonucleotide molecular weights were confirmed via ESI-MS (see SI).

Click Reactions *in Vitro* and in Mammalian Cells. In general, the *in vitro* click reactions were performed with a 3-fold excess of the respective tetrazine−fluorophore conjugate; for the click reaction in mammalian cells, a 10-fold excess of the conjugate was used. The *in vitro* click reaction conditions of norbornene-modified RNA1 and **3** with tetrazine−fluorophore conjugate **5** are described in the SI (Figure S3).

Cell Culture. The human lung cancer cell line NCI-H460 was used for all experiments. The cell line was maintained in RPMI 1640 media supplemented with 10% fetal calf serum, suspended using 0.05% trypsin/0.53 mM EDTA, washed with 1× PBS and 1× HBSS and transfected in Opti-MEM reduced serum media (*Gibco*). Twenty-four hours before the transfection, 80,000 cells were seeded in 1 mL media per well of 12-well plates. They were transfected in subconfluent wells using 3 μL lipofectamine 2000 (*Life Technologies*) in 100 μL Opti-MEM and 20 pmol norbornene-modified siRNA in 100 μL Opti-MEM for 4 h at 37 °C in a total volume of 1 mL per well.

dx.doi.org/10.1021/bc500302y | *Bioconjugate Chem.* 2014, 25, 1438−1443

After transfection, the cells were washed three times with PBS to remove all remaining lipofectamine and norbornene-modified siRNA. A mixture of the correspondent tetrazine−fluorophore conjugate in Opti-MEM reduced serum medium was added to a final concentration of 200 nmol/L and incubated for 1 h at 37 °C. After incubation, the cells were washed three times with PBS to remove all remaining tetrazine−fluorophore conjugate.

Flow Cytometry. Into each well, 400 μL PBS was added. A cell scraper was used to suspended cells from the wells, and the samples were transferred into FACS tubes. Fluorescence intensities were determined by a *FACSCanto II* flow cytometer (*BD Biosciences*) counting 10,000 events and evaluated using the *FlowJo* software package.

Fluorescence Microscopy. Cells were grown on glass coverslips and washed three times with HBSS after all steps. The cells were fixed using 250 μL 4% paraformaldehyde in PBS for 20 min at RT, and the nucleus was stained using 200 μL DAPI in PBS (1:1000). The coverslips were mounted on microscopy slides using *Fluoro-Gel* (*EMS*). An *Olympus FV1000* laser-scanning confocal microscope (*Olympus*) was used to image fixed cells with a 60× oil immersion objective lens.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures for the chemical synthesis of tetrazine−fluorophore conjugates **5**−**8**, for detailed RNA synthesis conditions, and for the click reactions *in vitro* and in mammalian cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank M. Hoch and W. Kolanus (LIMES Institute, University of Bonn, Germany) for access to the microscope facilities. We further thank A. Heckel (Goethe University, Frankfurt, Germany) for performing high-resolution ESI mass spectrometry of RNA oligonucleotides, and M. Famulok (LIMES Institute, University of Bonn, Germany) for laboratory facilities. S.K.-S. thanks the Fonds der Chemischen Industrie for financial support (Liebig-Fellowship).

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