Fluorescent Labeling of (Oligo)Nucleotides by a New Fluoride Cleavable Linker Capable of Versatile Attachment Modes

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Postsynthetic on Column RNA Labeling via Stille Coupling

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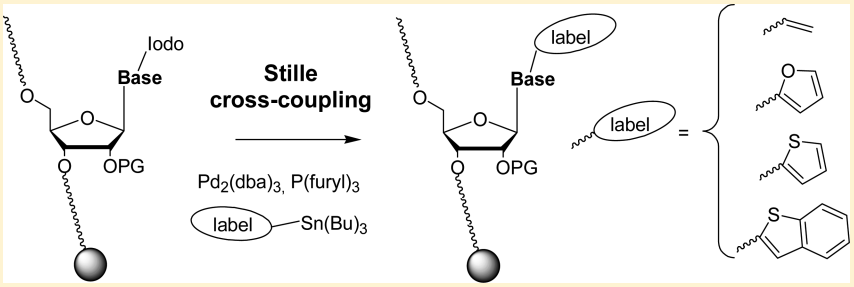

ABSTRACT: Stille Coupling is a versatile C−C bond forming reaction with high functional group tolerance under mild conditions. Our on column synthesis concept for RNA modification is based on the incorporation of iodo substituted nucleotide precursors to RNA during automated standard solid phase synthesis via TBDMS-, TC-, and ACE- protecting group strategies. Subsequently, the RNA, still bound on solid support, is ready for orthogonal postsynthetic functionalization via Stille cross-couplings utilizing the advantages of solid phase synthesis. Several monomer test reactions were employed with 2-iodo adenosine and 5-iodo uridine and organostannanes as coupling partners under different conditions, changing the catalyst/ligand system, temperature, and reaction time as well as conventional heating and microwave irradiation. Finally, Stille cross-couplings under optimized conditions were transferred to fully protected 5-mer and 12-mer RNA oligonucleotides on-column. Deprotection and cleavage from solid support resulted in site-specifically labeled oligonucleotides. Derivatizations via Stille cross-couplings were performed initially with vinyltributylstannane as well as later with 2-furanyl-, 2-thiophene-, and benzothiophene-2-tributylstannanes yielding fluorescently functionalized RNA.

INTRODUCTION

Over the past decades, synthetic oligonucleotides gained increased importance in the interdisciplinary field of natural sciences and medicine. Particularly RNA, which plays various key roles in cellular processes where it functions in catalysis and regulation, has become of great interest.[1−4] In addition to applications of chemically modified RNA in therapy,[5] such as the antisense strategy[6] and RNA-interference,[7] labeled oligonucleotides are crucial tools for analysis of structure and dynamics,[8] particularly, to investigate its function, folding and mechanisms by spectroscopic techniques such as fluorescence spectroscopy (e.g., FRET),[9] EPR, or NMR spectroscopy.[10,11]

Several chemical modifications are described for DNA as well as for RNA. For an improved cellular uptake and increased nuclease resistance, they are primarily found at the sugar phosphate backbone (e.g., thiophosphates etc.), on the termini (e.g., cholesterol or bile acid), and in the 2′-position of ribose (e.g., cationic or lipophilic groups or as locked nucleic acids).[12,13] Dyes or radicals (e.g., fluorescent or spin labels) for spectroscopic purposes and photolabile groups in caged oligonucleotides can be conjugated to the sugar−phosphate backbone or in the 2′-position but are mainly attached to the base moiety.[8,14−16]

Generally, in addition to the possibility of synthesizing functionalized oligonucleotides via enzymatic incorporation, chemical derivatization mainly consists of the synthesis of modified phosphoramidites. They can be directly incorporated to oligonucleotides or by postsynthetic approaches in which stable precursor nucleotides are inserted and subsequently modified with a range of functional groups.[17] The former mentioned strategy is straightforward in assembling during solid-phase synthesis and subsequent purification but is not convergent and suffers from the time-consuming and sometimes complicated preparation of differently modified phosphoramidites. They are potentially sensitive to the conditions of automated oligonucleotide synthesis and also might limit the overall yield after insertion. The second procedure turns out to follow a more sophisticated strategy providing greater flexibility and synthetic ease, but the challenge is to find suitable and selective methods qualified for postsynthetic applications.[18−21]

One of the first postsynthetic approaches reported in literature for oligonucleotides is the substitution reaction of convertible nucleotides[18,22−34] bearing leaving groups such as fluorine, triazole, or substituted phenyls at the base with, e.g., amine-, thio-, or alkoxy nucleophiles. It has been recently applied to introduce rigid nitroxide radicals to RNA for EPR spectroscopy.[35] Other substitution reactions are related to electrophilic alkylbromides[36,37] conjugated to the sugar, and

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also, postsynthetic reactions on thio-substituted nucleosides involved in transfer-RNA or playing a role in cross-linking reactions of DNA[29,34,38,39] are reported. Furthermore, thiols or amines and lately seleno nucleophiles[40] were converted postsynthetically with reactive electrophiles such as activated esters, thiocyanates, or acid chlorides.[17] Labeling biomolecules by new bio-orthogonal methods[17] comprises, e.g., the Diels–Alder reaction, Staudinger ligation, and the so-called Click reaction (Huisgen-Sharpless [3 + 2] cycloaddition). The latter one is intensely used for postsynthetic modification primarily for DNA[41−44] but also for RNA.[45] Further prominent alternatives for postsynthetic conjugation of labels to oligonucleotides are palladium or copper catalyzed coupling reactions. Most notable is the Sonogashira cross-coupling, first reported by Khan and Grinstaff et al.[46] for modifying DNA on solid support, followed by Wagenknecht et al.[47] who attached 1-ethinylpyrene to DNA and our work applying a slightly modified procedure to generate EPR probes by introducing spin labels to DNA[48−50] and also to RNA[50−52] (using the TBDMS as well as the ACE protecting group strategy) as well as performing the synthesis of fluorescent RNA oligonucleotides.[53] While all early publications describe the cross-coupling during oligonucleotide synthesis in a semiautomated manner at the 5′-end and subsequent continuation of synthesis to fully assembled oligonucleotides, the postsynthetic Sonogashira in recent years could be employed by us[54] and other groups[55] after the complete synthesis without interruption. This "total post-synthetic" strategy avoids lower coupling yields after functionalization during automated synthesis and also permits one to introduce labels that are sensitive to synthesizer reagents such as acid or oxidants. A further postsynthetic, metal-mediated reaction has been presented by Matsuda et al.[20] who attached functional molecules to DNA via copper-catalyzed oxidative acetylenic coupling on column. Recently, palladium cross-couplings were extended to the Suzuki–Miyaura cross-coupling, used in arylation of guanine.[56] Contrary to the afore cited Sonogashira cross-couplings and the oxidative acetylenic homocoupling, this method is performed on deprotected DNA, already cleaved from solid support.

These studies demonstrate the progress achieved in convenient synthesis of modified oligonucleotides, and further elaboration is encouraged; nevertheless, there is a growing demand[40] in developing advanced approaches, in particular for RNA on a postsynthetic level that is described less extensively than for DNA.

We are interested in the synthetic aspect concerning the conjugation of labels to RNA at the nucleobases with the objective to obtain site-specifically functionalized oligonucleotides. For this aim, we would like to profit from a combination of both concepts, the efficiency of the postsynthetic approach and the advantage of on column synthesis.[20,46] This provides a fast and economical strategy in which only very small absolute amounts of reagents (coupling partner and catalyst/ligand) are necessary. Furthermore, it benefits from the high-yield coupling as well as ease of separation and purification of the desired product on solid support.

The success of postsynthetic derivatization depends on the chemoselectivity and yield of the reaction.[21] Thus, a convenient, orthogonal, and mild chemical method that lacks side reactions and is compatible with RNA in regard to stability and solid support conditions is required. For our purposes, most promising candidates are palladium-catalyzed cross-couplings starting from halogenated base residues as phosphoramidite precursors that are commercially available or can be prepared without large synthetic effort. For reasons of versatility, we chose the Stille cross-coupling[57] that fulfils the qualifications for the postsynthetic reaction of RNA on solid-phase. This method tolerates a wide range of functionalities, proceeds under mild conditions without the need of a base, and utilizes reagents stable against air and moisture.[58,59] The Stille cross-coupling uses vinylic or aromatic alkylstannanes as coupling partners, which might be the only drawback in terms of the toxicity of organotin compounds. This handicap is diminished to some extent in our approach on solid-phase as only very small amounts of tin reagents are used and can be easily separated from the product on bead by simple washing.

In this article, we present the palladium mediated Stille cross-coupling on monomeric model compounds and demonstrate, for the first time, the feasibility of the Stille cross-coupling for the convenient synthesis of postsynthetically modified RNAs on column. To show the generality and applicability of our new approach, we synthesized a set of 5-mer and 12-mer RNAs via TBDMS, TC (thiomorpholine-carbothioate)- and ACE [bis-(acetoxyethyloxy)-methyl] protecting group strategies. There we attached different coupling partners to iodinated uridine and adenosine resulting in modified RNA that is ready for further modification or can be used for fluorescence spectroscopy.

EXPERIMENTAL PROCEDURES

General. All reagents were of the highest commercially available quality and were used as received. It must be noted that the organo(tributyl)tin reagents are toxic and can harm health via inhalation, skin contact, and ingestion.

NMR spectra were recorded on Bruker AM, DPX, and AV instruments at 250, 300, and 400 MHz and 300 K. Chemical shifts (δ) are reported in ppm relative to the solvent signal. The fine structure of proton signals was specified with s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublet), dt (doublet of triplet), and bs (broad singlet). Assignments were made by DEPT, COSY, HSQC, and HMBC experiments.

Thin layer chromatograms (TLC) were recorded on 60 F_{254} plates from Merck (thickness of layer 0.2 mm). Column chromatography was carried out on silica gel 60 (40–63 μm, Merck).

Reversed phase (RP) HPLC was performed on a Jasco LC-2000Plus HPLC system equipped with a Jasco UV 2075Plus detector (detection at 254 nm) and a preparative Phenomenex Jupiter Proteo C12 column (250 × 15 mm) or an analytical Jupiter Proteo C12 column (250 × 4.6 mm). Anion-exchange (AE) HPLC was carried out on a Jasco LC-900 HPLC system equipped with a Jasco UV-970 detector (detection at 254 nm) and a Dionex BioLC DNAPac PA-100 (250 × 9 mm) column.

ESI-Mass spectrometry for monomers was performed on a Fisons instrument equipped with a VG platform II with a quadrupol analyzer or on a Nano-ESI Mariner Biospectrometry Workstation from PerSeptive Biosystems.

High resolution mass spectrometry (HRMS) was provided by a MALDI Orbitrap LTQ XL instrument (Thermo Fisher) equipped with a 337 nm nitrogen laser. 4-Hydroxy alpha cyanocinnamic acid was applied as the matrix, and the matrix peaks were used as lock masses for internal calibration.

Oligonucleotides were analyzed via ESI mass spectrometry using a LCMS instrument with microTOF-Q II analyzer (Bruker). An Agilent 1200 Series HPLC using the eluent

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methanol/0.005 M TEAA buffer (gradient 0−60%) was applied as the LC system.

UV spectroscopy was carried out on a Jasco V650 spectrophotometer using 1.0 cm cuvettes typically in 10 μM concentration. Fluorescence spectroscopy was performed on a Hitachi F4500 fluorescence spectrometer using 0.3 cm cuvettes in 10 μM concentration.

The numbering of atoms in the nucleoside parts is according to IUPAC (the common numbering convention of nucleosides).

Stille Cross-Coupling on Monomers. Synthesis of modified nucleosides via Stille cross-coupling is based on procedures already reported. While we used the Stille cross-coupling on monomers as a model reaction to establish a setting qualified for transfer to RNA, we modified the conditions with respect to solvent, temperature, catalyst/ligand loading, and reaction time. In general, we applied two main procedures (method A following Nair et al[60−62] and method B following Farina et al[63]). Particular changes in the mentioned parameters are presented in the Results and Discussion section. To screen suitable conditions, optimized experiments were mostly controlled by RP-HPLC [analytical Phenomenex Jupiter Proteo C12 column (250 × 4.6 mm) using the gradient 8−17% C within 20 min, 10% A constantly (eluent A, 1 M TEAA buffer, pH 6.5; eluent B, Millipore water; eluent C, acetonitrile; 1 mL/min flow)] to monitor conversion rates. Experiments under defined setups were also accomplished to isolate, purify, and characterize the modified nucleosides after synthesis. For that purpose, we followed the procedures described below. The analytical data found for the known compounds **2**, **4**, **5**, **6**, **8**, **9**, and **10** are fully consistent with the data already reported. Compound **7** has not been described yet and is completely characterized here.

General Procedures. Method A 1 (Procedure According to Nair et al.[60−62]). In a dry 2-neck flask under argon, typically 100 mg of nucleoside was dissolved in 2 mL of DMF, and consecutively 4.4 mol % $Pd(MeCN)_2Cl_2$ and 1.1 equiv tributyl organostannane were added. The yellow reaction mixture was heated at 90 °C for 3 h and changed color over orange and brown to black. After cooling to ambient temperature, the catalyst was filtered off and washed with ethyl acetate. The yellow filtrate was concentrated under reduced pressure and the crude product purified by silica gel column chromatography using the gradient 9:1 DCM/MeOH. The product as an off-white solid was usually obtained in 90−99%.

Method A 2 (Procedure According to Nair et al.[60−62]). This procedure is identical to method A 1 with the difference that an increased amount of 5.4 mol % $Pd(MeCN)_2Cl_2$ and additionally 12 mol % ligand P(*o*-tol)$_3$ was applied.

Method B 1 (Based on the Procedure of Farina et al.[63]). In a dry 2-neck flask under argon, typically 100 mg of nucleoside was dissolved in 2 mL of DMF, 5 mol % $Pd_2(dba)_3$ was added, and the black-colored reaction mixture was stirred for 5 min. Subsequently, 1.1 equiv tributyl organostannane was dropped on the suspension over 5 min resulting in brightening up, and the green−yellow reaction mixture was stirred for typically 3 h at 60 °C. After filtration through Celite, the solvent was removed under reduced pressure, and the crude product was purified by column chromatography using the gradient 9:1 DCM/MeOH. The product as an off-white solid was usually obtained in a yield of 90−99%.

Method B 2 (Based on the Procedure of Farina et al.[63]). This procedure is identical to method B 1 with the difference that 5.4 mol % $Pd_2(dba)_3$ was used, and 12 mol % ligand P(fur)$_3$ was added at the same time.

2-Vinyladenosine[64,65] (2). Compound **2** was prepared, isolated, and purified following method A 1 (99% yield), method A 2 (99% yield), and method B 1 (95% yield).

R_F = 0.17 (CH_2Cl_2/MeOH = 9:1). ^{1}H NMR (400 MHz, DMSO-d_6,): δ = 3.53−3.59 (1H, m, 5′-C*H*), 3.65−3.70 (1H, m, 5′-C*H*), 3.94−3.98 (1H, m, 4′-C*H*), 4.16 (1H, bs, 3′-C*H*), 4.65 (1H, q, J = 5.2 Hz, 2′-C*H*), 5.20 (1H, d, J = 3.0 Hz, 3′-O*H*), 5.39 (1H, dd, J = 6.8 and 4.6 Hz, 5′-O*H*), 5.44 (1H, d, J = 5.6 Hz, 2′-O*H*), 5.55 (1H, dd, J_{cis} = 10.4 and J_{gem} = 2.2 Hz, vinyl-C*H2*$_a$″), 5.88 (1H, d, J = 6.4 Hz, 1′-C*H*), 6.37 (1H, dd, J_{trans} = 17.3 and J_{gem} = 2.2 Hz, vinyl-C*H2*$_b$″), 6.60 (1H, dd, J_{trans} = 17.3 and J_{cis} = 10.4 Hz, vinyl-C*H1*″), 7.29 (2H, bs, N*H*$_2$), 8.32 (1H, s, 8-C*H*) ppm.

^{13}C NMR (100 MHz, DMSO-d_6, 300 K): δ = 62.75 (5′-CH_2), 70.74 (3′-CH_2), 73.22 (2′-CH), 85.88 (4′-CH), 87.64 (1′-CH), 118.28 (5-*C*), 121.10 (HC=CH_2), 136.88 (HC=CH_2), 140.30 (8-*C*H), 149.58 (4-*C*), 155.65 (6-*C*NH$_2$), 157.69 (2-*C*-vinyl) ppm.

ESI(+)-MS (m/z) calcd, 293.11 [$C_{12}H_{15}N_5O_4$]; found, 294.1 [M + H]$^+$. HRMS (M + H): calcd, 294.11968 and 295.12304; found, 294.11969 and 295.12219.

5-Vinyluridine[63,66] (4). Compound **4** was prepared, isolated, and purified following method B 2 with a reaction time of only 2 h (control via RP-HPLC) in a yield of 90%.

R_F = 0.14 (CH_2Cl_2/MeOH = 9:1). ^{1}H NMR (250 MHz, DMSO-d_6): δ = 3.54−3.62 (1H, m, 5′-C*H*), 3.66−3.74 (1H, m, 5′-C*H*), 3.84−3.88 (1H, m, 4′-C*H*), 3.98−4.09 (2H, m, 2′-C*H* and 3′-C*H*), 5.06 (1H, d, J = 5.2 Hz, 3′-O*H*), 5.12 (1H, dd, J_{cis} = 11.5 and J_{gem} = 2.0 Hz, vinyl-C*H2*$_a$″), 5.23 (1H, t, J = 4.8 Hz, 5′-O*H*), 5.40 (1H, d, J = 5.3 Hz, 2′-O*H*), 5.78 (1H, d, J = 4.5 Hz, 1′-C*H*), 5.92 (1H, dd, J_{trans} = 17.7 and J_{gem} = 2.0 Hz, vinyl-C*H2*$_b$″), 6.37 (1H, dd, J_{trans} = 17.7 and J_{cis} = 11.5 Hz, vinyl-C*H1*″), 8.20 (1H, s, 6-C*H*); 11.42 (1H, bs, N*H*) ppm.

^{13}C NMR (75 MHz, DMSO-d_6): δ = 61.50 (5′-CH_2), 69.12 (3′-CH), 73.56 (2′-CH), 84.37 (4′-CH), 87.71 (1′-CH), 111.11 (5-*C*-vinyl), 114.16 (HC=CH_2), 128.89 (HC=CH_2), 138.03 (6-*C*H), 150.22 (2-*C*O), 162.41 (4-*C*O) ppm.

ESI(−)-MS (m/z) calcd, 270.09 [$C_{11}H_{14}N_2O_6$]; found, 269.1 [M − H]$^-$.

2-Fur(2-yl)adenosine[67] (5). Compound **5** was prepared, isolated, and purified following method B 1 (99% yield) as well as method B 2 (99% yield) in a reaction time of 4 h (control via TLC, eluent 9:1 DCM/MeOH) for both procedures.

R_F = 0.18 (CH_2Cl_2/MeOH = 9:1). ^{1}H NMR (250 MHz, DMSO-d_6,): δ = 3.53−3.62 (1H, m, 5′-C*H*), 3.66−3.74 (1H, m, 5′-C*H*), 3.95−3.99 (1H, m, 4′-C*H*), 4.16−4.21 (1H, m, 3′-C*H*), 4.67 (1H, dt, J = 5.9 and 5.4 Hz, 2′-C*H*), 5.11 (1H, dd, J = 6.3 and 5.1 Hz, 5′-O*H*), 5.20 (1H, d, J = 4.5 Hz, 3′-O*H*), 5.44 (1H, d, J = 6.1 Hz, 2′-O*H*), 5.92 (1H, d, J = 6.3 Hz, 1′-C*H*), 6.62 (1H, dd, J = 3.2 and 1.7 Hz, Furyl-*H4*″), 7.12 (1H, d, J = 3.2 Hz, Furyl-*H3*″),7.40 (2H, bs, N*H*$_2$), 7.79 (1H, bs, Furyl-*H5*″), 8.35 (1H, s, 8-C*H*) ppm.

^{13}C NMR (100 MHz, DMSO-d_6): δ = 61.46 (5′-CH_2), 70.46 (3′-CH), 73.01 (2′-CH), 85.54 (4′-CH), 87.11 (1′-CH), 110.87 (furyl-*C3*″), 111.56 (furyl-*C4*″), 117.83 (5-*C*), 139.75 (8-CH), 144.11 (furyl-*C5*″), 149.67 (4-*C*), 151.14 (2-*C*-furyl), 152.37 (furyl-*C2*″), 155.68 (6-*C*NH$_2$) ppm.

ESI(+)-MS (m/z) calcd, 333.11 [$C_{14}H_{15}N_5O_5$]; found, 334.3 [M + H]$^+$, 356.4 [M + Na]$^+$.

HRMS (M + H): calcd, 334.11460 and 335.11795; found, 334.11488 and 335.11821.

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2-Thien(2-yl)adenosine[67] (6). Compound **6** was prepared, isolated, and purified following method B 2 (98% yield) in a reaction time of 6 h (control via TLC, eluent 9:1 DCM/MeOH).

R_F = 0.20 (CH_2Cl_2/MeOH = 9:1). ^{1}H NMR (250 MHz, DMSO-d_6): δ = 3.53−3.62 (1H, m, 5′-C*H*), 3.67−3.75 (1H, m, 5′-C*H*), 3.94−3.99 (1H, m, 4′-C*H*), 4.20−4.56 (1H, m, 3′-C*H*), 4.72 (1H, dt, *J* = 5.8 and 5.4 Hz, 2′-C*H*), 5.05 (1H, t, *J* = 5.7 Hz, 5′-O*H*), 5.22 (1H, d, *J* = 4.6 Hz, 3′-O*H*), 5.46 (1H, d, *J* = 6.1 Hz, 2′-O*H*), 5.91 (1H, d, *J* = 6.1 Hz, 1′-C*H*), 7.14 (1H, dd, *J* = 3.6 and 4.8 Hz, thienyl-*H4*″), 7.37 (2H, bs, NH_2), 7.62 (1H, d, *J* = 4.7 Hz, thienyl-*H5*″), 7.82 (1H, d, *J* = 3.3 Hz, thienyl-*H3*″), 8.33 (1H, s, 8-C*H*) ppm.

^{13}C NMR (62.9 MHz, DMSO-d_6): δ = 61.65 (5′-CH_2), 70.71 (3′-CH), 73.06 (2′-CH), 85.54 (4′-CH), 87.41 (1′-CH), 118.07 (5-*C*), 127.04 (thienyl-*C3*″), 127.79 (thienyl-*C5*″), 128.74 (thienyl-*C4*″), 140.13 (8-CH), 144.20 (thienyl-*C2*″), 149.80 (4-*C*), 154.78 (2-*C*-thienyl), 155.61 (6-CNH_2) ppm.

ESI(+)-MS (*m*/*z*) calcd, 349.08 [$C_{14}H_{15}N_5O_4S$]; found, 350.3 [M + H]$^+$, 372.5 [M + Na]$^+$.

HRMS (M + H): calcd, 350.09175, 351.09511 and 352.08755; found, 350.09203, 351.09465 and 352.08778.

2-Benzo[*b*]thienyladenosine (7). Compound **7** was prepared, isolated, and purified following method B 2 (90% yield after several purification steps) in a reaction time of 6 h (control via TLC, eluent 9:1 DCM/MeOH). In this experiment, the crude product was treated with numerous washes by hexane to remove the remaining tributyltin iodide. To accomplish sufficient purity, an analytical amount of product was furthermore purified via RP-HPLC [preparative Phenomenex Jupiter Proteo C12 column (250 × 15 mm) using the gradient 3−60% C within 45 min, 10% A constantly (eluent A, 1 M TEAA buffer, pH 6.5; eluent B, Millipore water; eluent C, acetonitrile; 6 mL/min flow)], and a pure product of high quality in 90% could be recovered.

R_F = 0.22 (CH_2Cl_2/MeOH = 9:1). ^{1}H NMR (250 MHz, DMSO-d_6): δ = 3.55−3.64 (1H, m, 5′-C*H*), 3.69−3.77 (1H, m, 5′-C*H*), 3.95−3.99 (1H, m, 4′-C*H*), 4.21−4.26 (1H, m, 3′-C*H*), 4.73 (1H, q, *J* = 5.8 Hz, 2′-C*H*), 5.04 (1H, t, *J* = 5.7 Hz, 5′-O*H*), 5.24 (1H, d, *J* = 4.7 Hz, 3′-O*H*), 5.49 (1H, d, *J* = 6.1 Hz, 2′-O*H*), 5.96 (1H, d, *J* = 6.0 Hz, 1′-C*H*), 7.39 (2H, dd, *J* = 3.1 and 6.0 Hz, benzothienyl-*H6*″ *and H7*″), 7.47 (2H, bs, NH_2), 7.91−7.98 (2H, m, benzothienyl-*H5*″ *and H8*″), 8.15 (1H, s, benzothienyl-*H3*″), 8.40 (1H, s, 8-C*H*) ppm.

^{13}C NMR (100 MHz, DMSO-d_6): δ = 61.61 (5′-CH_2), 70.58 (3′-CH), 73.18 (2′-CH), 85.48 (4′-CH), 87.24 (1′-CH), 118.39 (5-*C*), 122.45 (benzothienyl-*C8*″), 123.57 (benzothienyl-*C3*″), 124.46 (benzothienyl-*C5*″ *and C6*″), 125.22 (benzothienyl-*C7*″), 139.76 (benzothienyl-*C9*″), 140.32 (benzothienyl-*C4*″), 140.42 (8-CH), 144.33 (benzothienyl-*C2*″), 149.87 (4-*C*), 154.67 (2-*C*-benzothienyl), 155.65 (6-CNH_2) ppm.

ESI(−)-MS (*m*/*z*) calcd, 399.10 [$C_{18}H_{17}N_5O_4S$]; found, 398.4 [M − H]$^-$.

HRMS (M + H): calcd, 400.10740, 401.11076, and 402.10320; found, 400.10708, 401.10931 and 402.10313

5-Fur(2-yl)uridine[68] (8). Compound **8** was prepared, isolated, and purified following method B 2 in a reaction time of 4 h (control via TLC, eluent 9:1 DCM/MeOH). The product could be obtained in 99% yield as an off-white solid but is light-sensitive (color turns to orange if the compound is disposed to light).

R_F = 0.21 (CH_2Cl_2/MeOH = 9:1). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.57−3.70 (2H, m, 5′-CH_2), 3.88−3.91 (1H, m, 4′-C*H*), 4.01 (1H, pt, *J* = 4.6 Hz, 3′-C*H*), 4.11 (1H, pt, *J* = 5.0 Hz, 2′-C*H*), 5.10 (1H, bs, 3′-O*H*), 5.18 (1H, bs, 5′-O*H*), 5.42 (1H, bs, 2′-O*H*), 5.86 (1H, d, *J* = 5.1 Hz, 1′-C*H*), 6.52 (1H, dd, *J* = 1.8 and 3.3 Hz, furyl-*H4*″), 6.85 (1H, d, *J* = 3.1 Hz, furyl-*H3*″), 7.60 (1H, d, *J* = 1.7 Hz, furyl-*H5*″), 8.41 (1H, s, 6-C*H*); 11.65 (1H, bs, N*H*) ppm.

^{13}C NMR (100 MHz, DMSO-d_6): δ = 61.50 (5′-CH_2), 70.56 (3′-CH), 74.45 (2′-CH), 85.66 (4′-CH), 89.11 (1′-CH),105.57 (5-*C*-furyl), 107.86 (furyl-*C3*″), 111.46 (furyl-*C4*″), 134.83 (6-CH), 141.47 (furyl-*C5*″), 146.29 (furyl-*C2*″), 149.60 (2-CO), 160.02 (4-CO) ppm.

ESI(−)-MS (*m*/*z*) calcd, 310.08 [$C_{13}H_{14}N_2O_7$]; found, 309.3 [M − H]$^-$.

HRMS (M + Na): calcd, 333.06932 and 334.07268; found, 333.06950 and 334.07116.

5-Thien(2-yl)uridine[69] (9). Compound **9** was prepared, isolated, and purified following method B 2 (99% yield) in a reaction time of 3 h (control via TLC, eluent 9:1 DCM/MeOH).

R_F = 0.27 (CH_2Cl_2/MeOH = 9:1). ^{1}H NMR (250 MHz, DMSO-d_6): δ = 3.59−3.67 (1H, m, 5′-C*H*), 3.71−3.79 (1H, m, 5′-C*H*), 3.90−3.92 (1H, m, 4′-C*H*), 4.03−4.15 (2H, m, 2′- and 3′-C*H*), 5.07 (1H, d, *J* = 5.4 Hz, 3′-O*H*), 5.41 (1H, t, *J* = 4.6 Hz, 5′-O*H*), 5.45 (1H, d, *J* = 5.2 Hz, 2′-O*H*), 5.83 (1H, d, *J* = 4.1 Hz, 1′-C*H*), 7.04 (1H, dd, *J* = 3.7 and 5.1 Hz, thienyl-*H4*″), 7.40 (1H, dd, *J* = 3.7 and 1.0 Hz, thienyl-*H3*″), 7.45 (1H, dd, *J* = 5.1 and 1.0 Hz, thienyl-*H5*″), 8.64 (1H, s, 6-C*H*); 11.68 (1H, bs, N*H*) ppm.

^{13}C NMR (62.9 MHz, DMSO-d_6): δ = 60.07 (5′-CH_2), 69.25 (3′-CH), 74.16 (2′-CH), 84.54 (4′-CH), 88.58 (1′-CH), 108.16 (5-*C*-thienyl), 122.42 (thienyl-*C3*″), 125.54 (thienyl-*C5*″), 126.25 (thienyl-*C4*″), 133.79 (thienyl-*C2*″), 135.59 (6-CH), 149.43 (2-CO), 161.15 (4-CO) ppm.

ESI(−)-MS (*m*/*z*) calcd, 326.06 [$C_{13}H_{14}N_2O_6S$]; found, 325.2 [M − H]$^-$.

HRMS (M + Na): calcd, 349.04648, 350.04983 and 351.04227; found, 349.04693, 350.05079, and 351.06179.

5-Benzo[*b*]thienyluridine[70] (10). Compound **10** was prepared, isolated, and purified following method B 2 (95% yield) in a reaction time of 3 h (control via TLC, eluent 9:1 DCM/MeOH). In this experiment, the crude product was additionally extracted between aqueous potassium fluoride solution and ethyl acetate to remove the remaining tributyltin iodide.

R_F = 0.34 (CH_2Cl_2/MeOH = 9:1). ^{1}H NMR (250 MHz, DMSO-d_6): δ = 3.65−3.72 (1H, m, 5′-C*H*), 3.78−3.86 (1H, m, 5′-C*H*), 3.94−3.97 (1H, m, 4′-C*H*), 4.07−4.19 (2H, m, 2′- and 3′-C*H*), 5.12 (1H, d, *J* = 5.3 Hz, 3′-O*H*), 5.49−5.54 (2H, m, 2′-O*H* and 5′-O*H*), 5.85 (1H, d, *J* = 3.7 Hz, 1′-C*H*), 7.26−7.38 (2H, m, benzothienyl-*H6*″ *and H7*″), 7.78 (1H, s, benzothienyl-*H3*″), 7.79 (1H, m, benzothienyl-*H5*″), 7.90 (1H, d, *J* = 7.1 Hz, benzothienyl-*H8*″), 8.83 (1H, s, 6-C*H*); 11.78 (1H, bs, N*H*) ppm.

^{13}C NMR (63 MHz, DMSO-d_6): δ = 59.91 (5′-CH_2), 69.08 (3′-CH), 74.28 (2′-CH), 84.44 (4′-CH), 88.82 (1′-CH), 107.73 (5-*C*-benzothienyl), 119.01(benzothienyl-*C3*″), 121.77 (benzothienyl-*C8*″), 122.94 (benzothienyl-*C5*″), 123.87 (benzothienyl-*C6*″), 124.21 (benzothienyl-*C7*″), 135.01 (benzothienyl-*C2*″), 137.46 (6-CH), 138.73 (benzothienyl-*C4*″), 138.94 (benzothienyl-*C9*″) 149.42 (2-CO), 161.22 (4-CO) ppm.

ESI(−)-MS (*m*/*z*) calcd, 376.07 [$C_{17}H_{16}N_2O_6S$]; found, 375.3 [M − H]$^-$, 411.3 [M + Cl]$^-$.

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HRMS (M + Na): calcd, 399.06213, 400.06548, and 401.05792; found, 399.06232, 400.06669, and 401.07729.

Preparation of TC Protected 5-Iodo Uridine Phosphoramidite. Synthesis of 5-iodo uridine phosphoramidite via TC protection was performed according to the procedure reported by Caruthers et al.[71] for natural nucleosides. Marckiewicz protected 5-iodo uridine **11** was synthesized via standard methods (see, e.g., Matsuda et al.[72]).

2′-*O*-(Thiocarbonylimidazolyl)-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-5-iodo Uridine (12). Silyl protected 5-iodo uridine **11** (1.32 g, 2.15 mmol) was dissolved in 13.2 mL of dry MeCN under argon. Subsequently, 0.58 g (3.23 mmol) of 1,1′-thiocarbonyldiimidazole and 15.9 mg (0.13 mmol) of DMAP were added, and the reaction was stirred for 6 h at 30 °C. After cooling for 2 h at −20 °C, the product had crystallized to a white solid and was isolated by filtration. Dried under vacuum, 1.17 g (1.62 mmol, 75%) of the 2′-thiocarbonylimidazole product **12** was obtained.

^{1}H NMR (250 MHz, DMSO-d_6): δ = 0.80−1.09 (28 H, m, 2 × Si(C*H*(CH_3)$_2$)$_2$); 3.94−4.02 (1H, m, 4′-C*H*), 4.10−4.13 (2H, m, 5′-CH_2), 4.77−4.83 (1H, m, 3′-C*H*), 5.91−5.93 (1H, d, *J* = 1.4 Hz 1′-C*H*), 6.35 (1H, dd, *J* = 5.7 Hz, *J* = 1.4 Hz, 2′-C*H*), 7.11−7.13 (1H, m, imidazolyl), 7.86−7.88 (1H, m, imidazolyl), 8.05 (1H, s, imidazolyl), 8.55 (1H, s, 6-C*H*); 11.82 (1H, bs, N*H*) ppm.

^{13}C NMR (75 MHz, DMSO-d_6): δ = 11.97, 12.03, 12.26, 12.55, 16.43, 16.80, 16.87, 17.02, 17.20, 17.30, 60.34, 69.34, 69.55, 81.26, 82.72, 88.94, 118.55, 130.88, 136.94, 145.84, 149.72, 160.52, 183.07 ppm.

ESI(+)-MS (*m*/*z*) calcd, 722,11 [$C_{25}H_{39}IN_4O_7SSi_2$]; found, 724.2 [M + H]$^+$.

2′-*O*-(1,1-Dioxo-1λ^6-thiomorpholine-4-carbothioate)-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)-5-iodo Uridine (13). Compound **12** (1.75 g (2.42 mmol)) was dissolved in 20 mL of dry MeCN under argon by heating with a heatgun. While the reaction mixture was still hot, 0.36 g (2.66 mmol) of thiomorpholine-1,1-dioxide was added and the reaction stirred for 6.5 h at 30 °C. The reaction was controlled via TLC (95:5 DCM/isopropanol), and after completion, the solvent was removed. The crude product was subsequently purified by column chromatography (gradient 99:1 DCM/isopropanol) and dried under vacuum yielding in 1.72 g (2.18 mmol, 90%) of product **13**.

R_F = 0.54 (DCM/isopropanol = 95:5). ^{1}H NMR (400 MHz, DMSO-d_6): δ = 0.97−1.08 (28 H, m, 2 × Si(C*H*(CH_3)$_2$)$_2$); 3.09−3.16 (2H, m, thiomorpholine); 3.31−3.39 (2H, m, thiomorpholine); 3.89−4.48 (7H, m, 4′-C*H*, 5′-CH_2, thiomorpholine), 4.70−4.74 (1H, m, 3′-C*H*), 5.73 (1H, d, *J* = 1.7 Hz, 1′-C*H*), 6.11 (1H, dd, *J* = 5.9 Hz, *J* = 5.9 Hz, 2′-C*H*), 8.07 (1H, s, 6-C*H*); 11.82 (1H, bs, N*H*) ppm.

^{13}C NMR (75 MHz, DMSO-d_6): δ = 11.83, 12.05, 12.31, 12.57 (2 × Si(CH(CH_3)$_2$)$_2$); 16.64, 16.73, 16.80, 16.82, 17.03, 17.20, 17.22, 17.30 (2 × Si(CH(CH_3)$_2$)$_2$); 43.47, 48.05, 50.52, 50.97 (thiomorpholine); 60.42 (5′-CH_2); 68.91 (3′-CH), 69.62 (6-*C*-I), 80.66 (2′-CH), 81.38 (4′-CH), 90.01 (1′-CH), 146.30 (6-CH), 149.72 (2-CO), 160.52 (4-CO), 183.07 (C═S) ppm.

HRMS (M + Na): calcd, 812.09944, 813.10279, 814.09523, and 815.09859; found, 812.10108, 813.10375, 814.09516, and 815.10020.

2′-*O*-(1,1-Dioxo-1λ^6-thiomorpholine-4-carbothioate)-5-iodo Uridine (15). Compound **13** (1.98 g (2.51 mmol)) was dissolved in 20 mL of 2-MeTHF under argon, and 1.22 mL (15.06 mmol) pyridine was added. The reaction mixture was then cooled to 0 °C, and 0.78 mL of 70% HF in pyridine was added dropwise with stirring. After addition, the reaction was stirred for 22 h at room temperature. Reaction control was performed via TLC (95:5 DCM/isopropanol). The reaction mixture was extracted with water twice, the combined aqueous layers were re-extracted with 2-MeTHF, and the combined organic layers were dried over anhydrous sodium sulfate. Sodium sulfate was removed by filtration and the solvent evaporated under vacuum (keeping the water bath temperature less than 40 °C). For further purification, column chromatography with DCM and 5−10% isopropanol was carried out, and after drying under high vacuum, product **15** was obtained in 85% (1.17 g, 2.13 mmol) yield.

R_F = 0.12 (DCM/isopropanol = 95:5). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.20−3.31 (4H, m, thiomorpholine); 3.62−3.68 (2H, m, 5′-CH_2), 4.01−4.50 (6H, m, 4′-C*H*, 3′-C*H*, thiomorpholine), 5.34 (1H, t, *J* = 4.5 Hz, 5′-O*H*), 5.62 (1H, d, *J* = 5.2 Hz, 3′-O*H*), 5.66 (1H, dd, *J* = 5.4 Hz, *J* = 6.4 Hz, 2′-C*H*), 6.06 (1H, d, *J* = 6.5 Hz, 1′-C*H*), 8.41 (1H, s, 6-C*H*); 11.72 (1H, bs, N*H*) ppm.

^{13}C NMR (75 MHz, DMSO-d_6): δ = 43.47, 48.12, 50.39, 50.63 (thiomorpholine); 60.98 (5′-CH_2); 69.27 (3′-CH), 70.16 (6-*C*-I), 81.59 (2′-CH), 85.68 (1′-CH), 86.51 (4′-CH), 144.48 (6-CH), 150.68 (2-CO), 160.35 (4-CO), 186.34 (*C*═S) ppm.

HRMS (M + Na): calcd, 547.96528, 548.96863 and 549.96107; found, 547.96419, 548.96757 549.95986.

2′-*O*-(1,1-Dioxo-1λ^6-thiomorpholine-4-carbothioate)-5′-*O*-(4,4′-dimethoxytrityl)-5-iodo Uridine (16). Compound **15** (1.77 g (2.51 mmol)) was dissolved in 40 mL of dry DCM under argon and cooled to 0 °C. In the first instance, 0.65 equiv (0.18 mL, 1.63 mmol) of NMM and 0.6 equiv (0.51 g, 1.51 mmol) of DmTrCl were added to the reaction mixture with stirring at 0 °C. More NMM (0.65 equiv) and DmTrCl (0.6 equiv) were added in portions while the reaction was controlled by TLC (95:5 DCM/isopropanol). The conversion was complete after 4 h, and the reaction mixture was quenched with MeOH, extracted with saturated $NaHCO_3$ and DCM, and the combined organic fractions dried over sodium sulfate. After evaporation of the solvent, the crude product was purified by column chromatography (gradient: DCM with 1−5% isopropanol) yielding in 70% (1.49 g, 1.76 mmol) product **16**.

R_F = 0.49 (DCM/isopropanol = 95:5). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.16−3.31 (6H, m, thiomorpholine,, 5′-CH_2); 3.74 (6H, s, 2 × OCH_3) 4.11−4.51 (6H, m, 4′-C*H*, 3′-C*H*, thiomorpholine), 5.70 (1H, d, *J* = 5.4 Hz, 3′-O*H*), 5.91 (1H, t, *J* = 6.2 Hz, 2′-C*H*), 6.09 (1H, d, *J* = 6.4 Hz, 1′-C*H*), 6.88−6.91 (4H, m, DmTr); 7.21−7.36 (7H, m, DmTr); 7.45−7.47 (2H, m, DmTr); 8.10 (1H, s, 6-C*H*); 11.81 (1H, bs, N*H*) ppm.

^{13}C NMR (75 MHz, DMSO-d_6): δ = 43.49, 48.19, 50.39, 50.61 (thiomorpholine); 54.97 (2 × OCH_3); 63.41 (5′-CH_2); 68.99 (3′-CH), 70.59 (6-*C*-I), 81.04 (2′-CH), 84.22 (4′-CH), 85.88 (1′-CH), 86.13 (OC-triphenyl); 113.22, 126.69, 127.60, 127.88, 129.73, 135.00, 135.18 (DmTr); 144.42 (6-CH), 150.11 (2-CO), 158.06 (DmTr); 160.32 (4-CO), 186.36 (*C*═S) ppm.

ESI(+)-MS (*m*/*z*) calcd, 872,08 [$C_{35}H_{36}IN_3O_{10}S_2Na$]; found, 872.26 [M + Na]$^+$.

2′-*O*-(1,1-Dioxo-1λ^6-thiomorpholine-4-carbothioate)-5′-*O*-(4,4′-dimethoxytrityl)-5-iodo uridine-3′-*O*-(β-cyanoethyl)-*N*,*N*-diisopropyl-phosphoramidite (14). Nucleoside **16** (1.51 g (1.78 mmol)) was dissolved in 40 mL of dry DCM under argon and 0.29 mL (2.67 mmol) of NMM and 0.48 mL (2.14 mmol) of 2-cyanoethyl-*N*,*N*-diisopropylchloro-

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phosphoramidite were added. After 1 h, a precipitate formed and was dissolved by the addition of 10 mL of DCM with stirring. The reaction was controlled via TLC (95:5 DCM/isopropanol); after 2 h, the conversion was still incomplete, and thus, another 0.08 mL (0.71 mmol) of NMM and 0.12 mL (0.53 mmol) of 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite were added. The reaction showed to be complete after 3.5 h and was stopped by the addition of DCM and saturated $NaHCO_3$. After separation, the organic phase was extracted with saturated NaCl and subsequently dried over anhydrous sodium sulfate. Finally, the DCM layer was filtered and evaporated. The resulting oil was dissolved in DCM and purified by chromatography on a column pre-equilibrated with 10% acetone and 1% TEA in hexanes. After neutralization of the silica gel, the excess TEA was removed by flushing the column with a volume of 35% acetone in hexanes. Separation was performed using the gradient 35−40% acetone in hexane, and product **14** was obtained as colorless foam in 75% (1.4 g, 1.34 mmol) yield. To remove the remaining phosphitylation reagent, the phosphoramidite was precipitated from DCM/hexane giving a white solid.

R_F = 0.25 (hexane/acetone = 6:4). ^{1}H NMR (400 MHz, DMSO-d_6): 0.95−1.20 (12H, m, 2 × CH(CH_3)$_2$); 2.76 and 2.89 (2H, 2 × t, J = 5.9 Hz, OCH_2CH_2CN); 3.13−3.59 (8H, m, thiomorpholine, 2 × $CH(CH_3)_2$, 5′-CH_2); 3.65−3.71 (1H, m, OCH_2CH_2CN); 3.73 and 3.74 (6H, 2 × s, 2 × OCH_3); 3.84−3.94 (1H, m, OCH_2CH_2CN); 3.98−4.10 (2H, m, thiomorpholine); 4.24−4.31 (1H, m, 4′-C*H*); 4.47−4.50 (1H, m, thiomorpholine); 4.68−4.82 (2H, m, 3′-C*H*, thiomorpholine), 5.88 and 5.94 (1H, 2 × t, J = 5.9 Hz, 2′-C*H*); 6.06−6.08 (1H, m, 1′-C*H*), 6.86−6.90 (4H, m, DmTr); 7.23−7.36 (7H, m, DmTr); 7.45−7.47 (2H, m, DmTr); 8.09 and 8.11 (1H, 2 × s, 6-C*H*); 11.83 (1H, bs, N*H*) ppm.

^{31}P NMR (400 MHz, DMSO-d_6): δ = 149.11 and 149.80 (diasteromers, relation 6:4) ppm.

ESI(+)-MS (m/z) calcd, 1072,19 [$C_{44}H_{53}IN_5O_{11}PS_2Na$]; found, 1072.32 [M + Na]$^+$.

Synthesis, Postsynthetic Stille Cross-Coupling, Purification, and Characterization of Oligonucleotides. Oligonucleotide synthesis via TBDMS- or TC[71]-chemistry was accomplished using an Expedite Nucleic Acid Synthesis System from PerSeptive Biosystems in 1 μmol scale using standard protocols. Nucleotide building blocks and cpg-solid support were purchased from SAFC-Proligo (Sigma-Aldrich) for TBDMS strategy or Link technology for TC protected building blocks. Oligonucleotide synthesis via ACE chemistry[73] in 0.2 or 1 μmol scale was performed on a rebuilt ABI 392 synthesizer (Applied Biosystems) with phosphoramidites and polystyrene solid support purchased from Thermo Fisher (Dharmacon). Besides the unmodified nucleotide building blocks, also 5-iodo uridine phosphoramidites for TBDMS and ACE strategy were commercially available, and TC-protected 5-iodo uridine phosphoramidite was prepared as described in the previous section according to the procedure for unmodified building blocks reported by Caruthers et al.[71] 2-Iodo adenosine phosphoramidite via ACE strategy was synthesized as described before.[52,53] Every RNA synthesis was carried out in DMTr-off modus.

General Procedure for the Pd(0) Catalyzed Stille Cross-Coupling of Iodo Modified Oligonucleotides with Tributyl Organo Stannanes Following Method B 2. After completion of automated RNA synthesis on solid support, the columns with fully assembled RNA were dried under vacuum, flushed with argon, and a solution of 14 mg (15 equiv) of $Pd_2(dba)_3$ and 7 mg (30 equiv) of P(furyl)$_3$ in 150 μL of absolute DMF was added and dispersed under exclusion of air via syringes placed on both ends of the column and allowed to sit for 5 min. Meanwhile, a solution of 60 equiv tributyl organostannane in 100 μL of absolute DMF was prepared, subsequently added to the column−syringe system, thoroughly mixed, and reacted in the oven at 60 °C for 2−18 h with occasional moving of the syringes back and forth.

When the reaction had proceeded, the reaction solution was removed, and the RNA on column was washed consecutively with DMF, DCM, hexane, DCM, MeCN, 0.1 M aqueous EDTA, Millipore water, and MeCN. After drying the solid support, the RNA was cleaved, deprotected, and purified as usually performed for unmodified RNA.

General Procedure for Deprotection and Cleavage of TBDMS Protected RNA from cpg Following a Standard Procedure. For deprotection and cleavage of base-labile groups, the resin loaded with modified RNA was removed from the column, suspended in 1 mL of a 3:1 mixture of 37% aq ammonia/ethanol and incubated at 35 °C for 18−24 h. Afterward, the supernatant was separated, the cpg material was washed for 3 times with DEPC water, and the combined fractions were evaporated to dryness. For removing the 2′-TBDMS group, the oligonucleotide was dissolved in 0.3 mL of a 6:3:4 mixture of 1-methyl-2-pyrrolidon/triethylamine/triethylamine trihydrogenfluoride complex, vortexed, and incubated for 90 min at 60 °C. Subsequently, 1.2 mL *n*-butanol was added to the clear solution, mixed, and left for 1 h at −75 °C. To isolate the precipitated RNA, the mixture was centrifuged at 0 °C and 12500 rpm for 30 min, and after decanting supernatant, the residue presented the completely deprotected RNA pellet ready for purification.

General Procedure for Deprotection and Cleavage of TC Protected RNA from cpg, Based on the Procedure Described by Caruthers[71] and Following the Recommendations of Link Technology. The cpg material was left in the synthesizer column, dried under vacuum, and flushed with argon. The resin was washed for 3 min with 20% diethylamine in dry acetonitrile, washed with acetonitrile, and dried. Afterward, the cpg material was treated with 0.5 mL 1:1 ethylene diamine/dry toluene for 2 h. After washing with abs. acetonitrile and drying the resin still in column, the RNA was eluted by the addition of 2 mL 0.1 M TEAA buffer (sterile). When the solution was concentrated, the residue exhibited the deprotected crude RNA pellet.

General Procedure for Deprotection and Cleavage of ACE Protected RNA from Polystyrene Following the Reported Procedure.[74] First, the phosphate protecting groups were cleaved on solid support by treatment with 2 mL of a 0.4 M solution of disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate-trihydrate (S_2Na_2) in DMF/H_2O = 98:2 within 20 min. Deprotection of the exocyclic protection groups and cleavage of acetate of the 2′-ACE group as well as cleavage of the oligonucleotides from solid support was effected by treatment with a 40% aqueous solution of methylamine for 10 min at 55 °C. The final cleavage of the 2′-ACE protecting group was performed under sterile conditions with a TEMED/acetic acid buffer adjusted to pH 3.8 for 30 min at 60 °C.

General Procedure for Separation and Purification of Oligonucleotides. The crude RNA was purified by AE-HPLC (A, sterile DEPC water; B, 1 M LiCl sterile; gradient for 12-mers, 0−80% B within 40 min; gradient for 5-mers, 0−50% B

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within 40 min) with a semipreparative Dionex DNAPac PA-100 column (9 × 250 mm) and 5 mL/min flow. Further purification as well as concurrent desalting was accomplished by RP-HPLC using a preparative (250 × 15 mm) Phenomenex C12 column with the following gradient (eluent A, 1 M TEAA buffer sterile, pH 6.5; eluent B, DEPC water; eluent C, acetonitrile; constant 10% A, 0−3 min, 0% C; and 3−26.5 min, 0−45% C, 6 mL/min flow). For small quantities, an analytical (250 × 4.6 mm) Phenomenex C12 column was used (gradient: A, 1 M TEAA buffer sterile, pH 6.5; B, DEPC water; C, acetonitrile; constant 10% A, 0−2 min, 0% C; and 2−25 min, 0−30% C, 1 mL/min flow).

Concentrated RNA fractions were analyzed via ESI mass spectrometry on a LCMS system.

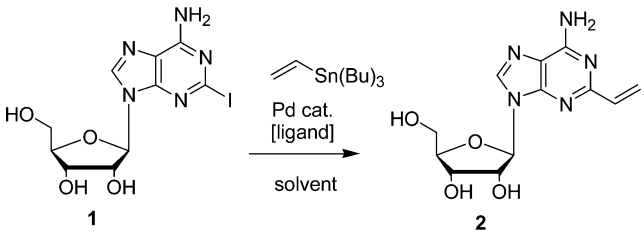
The masses for the synthesized RNAs **ON 1−ON 18** are presented in Tables 6 and 7 in the Results and Discussion section and were found to be in agreement with calculated values.

RESULTS AND DISCUSSION

Test Reactions on Monomer Model Compounds. Stille cross-coupling is a completely new approach in combination with nucleic acids on solid support; thus, model studies on the monomer level prior to transfer to oligonucleotides were required. To find proper conditions, stability and reactivity screenings were investigated with respect to the commonly reported reflux temperature preferred for Stille cross-couplings. This should be alleviated in a still reasonable reaction time to obtain a suitable method compatible with RNA on column. Some examples of Stille cross-couplings on monomeric purines and pyrimidines are described, and first studies of palladium-catalyzed cross-couplings involving nucleosides are presented by Nair et al.[60−62,64] They reported the reaction of inosine, nebularine, and adenosine derivatives toward alkylation in the rarely explored 2-position. In a general procedure for Stille cross-coupling, they used vinyl(tributyl)stannane at 90 °C in toluene for ribose-protected and DMF for the unprotected nucleoside with $Pd(MeCN)_2Cl_2$ as catalyst and optionally the ligand P(*o*-tol)$_3$.

According to this, our first experimental series included test reactions with unprotected 2-iodo adenosine **1** and commercially available vinyl(tributyl)stannane (Scheme 1). We

Scheme 1. Stille Cross-Coupling on Model Compound 2-Iodo Adenosine with Vinyl(tributyl)stannane as the Test Reaction



followed the mentioned procedure with $Pd(MeCN)_2Cl_2$ to estimate the feasibility for reactions on RNA (Table 1). After accomplishing excellent results under the reported conditions ($Pd(MeCN)_2Cl_2$, 90 °C, 3 h) with and without using the ligand P(*o*-tol)$_3$ (exp. nos. 1 and 2), we further explored prolonged reaction times at room temperature intending a setting that might be more suitable for the RNA oligonucleotide. As shown

Table 1. First Experiments on Stille Cross-Couplings for the Reaction of 2-Iodo Adenosine 1 to 2-Vinyl Adenosine 2 Using a Catalyst/Ligand System Based on the Procedure Described by Nair et al.[60−62]

exp. no.	cat.	lig.	temp.	time	conversion
1[a]	$Pd(MeCN)_2Cl_2$	P(tol)$_3$	90 °C	3 h	99%
2[a]	$Pd(MeCN)_2Cl_2$	no	90 °C	3 h	99%
3[a]	$Pd(MeCN)_2Cl_2$	no	r.t.	72 h	75%
4[a]	$Pd(MeCN)_2Cl_2$	P(tol)$_3$	r.t.	72 h	34%
5[a]	$Pd(MeCN)_2Cl_2$	no	r.t.	18 h	75%
6[b]	$Pd(MeCN)_2Cl_2$	no	r.t.	18 h	72%
				350 h	78%
7[b]	$Pd(MeCN)_2Cl_2$	no	60 °C	2.5 h	66%
				72 h	85%
				350 h	95%
8[b,c]	$Pd(MeCN)_2Cl_2$	no	60 °C, MW	6 h	99%
9[b,d]	$Pd(MeCN)_2Cl_2$	no	60 °C, MW	2 min	99%

[a]Product **2** was isolated and purified, and the conversion was determined via the integration of proton NMR spectra. [b]The reaction mixture was not worked up, but conversion rates were monitored by the integration of HPLC chromatograms. [c]Microwave induced reaction was performed in an open reflux system with 100 W. [d]Microwave induced reaction was performed in a sealed tube with 70 W, 99% conversion considering that no compound **1** could be detected anymore but that traces of a side-product was formed.

in Table 1, we could only isolate the product 2-vinyl adenosine **2** in 75% yield (exp. nos. 3 and 5) and were not able to observe conversion higher than 78% via RP-HPLC control even after several days (exp. no. 6). The use of ligand P(*o*-tol)$_3$ (exp. no. 4) seemed not to have any accelerating but rather a negative effect on the conversion rate and was therefore omitted for further tests. Obviously, there was no benefit in intensely prolonged reaction times because a sort of steady state arose after one day. This phenomenon could be due to decomposition of the catalyst $Pd(MeCN)_2Cl_2$, which would be in analogy to what Farina et al.[63] suggested in their studies on Stille cross-couplings on 5-iodo uracil with this particular catalyst. Although tests with slightly increased temperatures of 50−60 °C (Table 1, exp no. 7) turned out to be an improvement, there was a need for better conversion rates in a reasonable time period at least for the monomer model. Therefore, we exposed our reaction mixture to microwave irradiation (Table 1, exp. nos. 8 and 9) resulting in accelerated conversion either in an open system under reflux conditions (60 °C, 100 W, complete conversion after 6 h) or in a sealed tube (100% conversion after 2 min at 60 °C and 70 W, but a side product apart of the desired product **2** occurred as a shoulder in HPLC control). As we could neither separate nor characterize the side product under these rather harsh and thus not necessarily suitable conditions, we did not follow this method anymore. According to Aucagne et al.,[59] who reported the need for double-coupling for nearly complete conversion when working with Stille-cross-couplings on monomeric uridines on solid support, and also according to our experience with Sonogashira cross-couplings on oligonucleotides,[51] we tested here the benefit of double-couplings for the Stille reaction on the monomer level but could not determine a significant acceleration.

In summary, the particular conditions for Stille cross-coupling with $Pd_2(MeCN)_2Cl_2$ did not succeed at room temperature or slightly increased temperature in an adequate

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time range but showed promising results either at 90 °C in 3 h or at 60 °C and 100 W for 6 h under microwave irradiation in an open flask.

We did not deepen tests under the described procedure but switched to another catalyst system permitting milder conditions. For that purpose, we followed the less commonly used method described by Farina et al.[63] who applied Stille cross-couplings resulting in 5-substituted uracil and uridine derivatives with $Pd_2(dba)_3$ and the ligand $P(furyl)_3$. The priority of Farina's work was to investigate approaches for substituted uracils; thus, they used for reasons of solubility *N*-methyl-pyrrolidone and focused on exploring the palladium ligand. They found $Pd(MeCN)_2Cl_2$ and $Pd(PPh_3)_4$ [tetrakis-(triphenylphosphine)] to be unsatisfactory for their systems. But conversion of 5-iodoridine to 5-vinyluridine in 16 h at room temperature with the aforementioned $(Pd)_2(dba)_3$/$P(furyl)_3$ in 89% yield turned out to be successful. They could extend these results to the ribose-protected uridine derivative only by a longer reaction time of 40 h, increasing temperature to 50 °C and use of THF. On the basis of this, our next experimental series (Table 2) first comprised reactions of

Table 2. Further Experiments on Stille Cross-Couplings for Reaction of 2-Iodo Adenosine 1 to 2-Vinyl Adenosine 2 Using a Catalyst/Ligand System Based on the Procedure Described by Farina et al.[63] as well as Additional Catalyst Tests

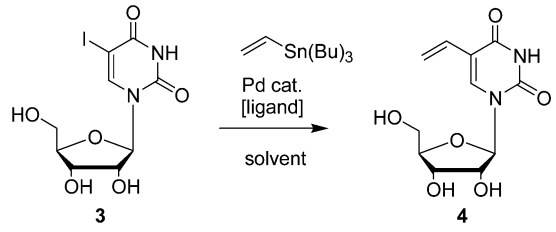
exp. no.	cat.	lig.	temp.	time	conver sion
10[a]	$Pd_2(dba)_3$	$P(fur)_3$	r.t.	168 h	5%
11[b]	$Pd_2(dba)_3$	$P(fur)_3$	r.t.	18 h	10%
				120 h	30%
12[c]	$Pd_2(dba)_3$	$P(fur)_3$	r.t.	18 h	10%
				120 h	27%
13[b,d]	$Pd_2(dba)_3$	$P(fur)_3$	r.t.	18 h	25%
				72 h	50%
14[c,d]	$Pd_2(dba)_3$	$P(fur)_3$	r.t.	18 h	20%
				72 h	60%
15[c]	$Pd(PPh_3)_2Cl_2$	no	r.t.	23 h	27%
				48 h	45%
16[c]	$Pd(PPh_3)_4$	no	r.t.	27 h	22%
17[c,d]	$Pd_2(dba)_3$	no	r.t.	23 h	88%
				350 h	90%
18[c,d]	$Pd_2(dba)_3$	no	60 °C	3 h	99%
19[c,d]	$Pd_2(dba)_3$	$P(fur)_3$	60 °C	3 h	99%
20[c,d,e]	$Pd_2(dba)_3$	no	50 °C	3 h	>95%

[a]The reaction was carried out in THF as the solvent. [b]The reaction was carried out in NMP as the solvent. [c]The reaction was carried out in DMF as the solvent. [d]Compared to the original procedure (2 mol % cat. and 4 mol % ligand), we increased the loading of catalyst (5.4 mol %) and ligand (12 mol %). [e]Complete conversion was only reproducible if Stille coupling was performed twice.

our model 2-iodo adenosine **1** to 2-vinyl adenosine **2** (Scheme 1) under the originally presented conditions of Farina et al.[63] with $Pd_2(dba)_3$/$P(furyl)_3$ at room temperature. To also cover solvent effects for this particular catalyst/ligand system, we did not only use DMF but also NMP and THF. Not unexpectedly since the solubility of **1** is poor in THF, this test afforded almost no conversion (exp. no. 10). However, the coupling reaction in NMP and DMF proceeded but turned out to be very slow (Table 2, exp. nos. 11 and 12). In contrast to the results of Farina, the conversion here was in both cases only 10% after 18 h representing an immensely differing behavior of the purine nucleoside **1**. Higher loading of catalyst and ligand showed slightly better reactivity (3 days: 60% conversion in DMF, 50% in NMP, Table 2, exp. nos. 13 and 14) but were not sufficient for our purposes. Considering the strong dependence and sensibility of altered conditions and types of nucleobases, other Pd catalysts such as $Pd(PPh_3)_2Cl_2$ [bis-(triphenylphosphine) palladiumdichloride], were applied for the reaction of 2-iodo adenosine **1** with vinyl(tributyl)stannane. $Pd(PPh_3)_2Cl_2$ was also introduced more recently for Stille cross-couplings of, e.g., furane to purines by Persson et al.[67] and to uridine by Tor et al.[68] under reflux conditions in THF or dioxane. In our case, the reaction was carried out in DMF at room temperature yielding in 27% product **2** after 23 h and 45% **2** after 48 h (Table 2, exp. no. 15), which could not be increased while prolonging the time. Also the alternative of using $Pd(PPh_3)_4$ at room temperature showed only a conversion of 22% after 27 h (Table 2, exp. no. 16) and therefore did not meet our requirements. On the basis of the observation that the kind of ligand and also the fact of whether it is added at all play an eminent role (discussed by Farina et al.[63] and also noted by us in our first model reactions with $Pd(MeCN)_2Cl_2$), the next investigations were performed with $Pd_2(dba)_3$ without utilizing $P(furyl)_3$ as ligand. Surprisingly, the relationship of converted product **2** to starting material **1** was almost reciprocal in comparison to the earlier test reactions: after 23 h, already 88% of product **2** had formed (Table 2, exp. no. 17). Unfortunately, again a kind of steady state seemed to appear when conversion came up to 90%; no complete conversion could be accomplished even after two weeks. Nevertheless, these conditions were almost optimal and were extended to slightly increased temperatures. These experiments presented the breakthrough as full conversion could be detected after 3 h at 60 °C (exp. no. 18). Interestingly, higher temperature also allowed the addition of ligand showing the same result of completion after 3 h (exp. no. 19). Furthermore, we expanded that discovery to test the same setup at only 50 °C (exp. no. 20). The findings were in principle consistent with the ones at 60 °C but were only reproducible when double coupling was performed.

After establishing successful conditions suitable for RNA on solid support for 2-iodo adenosine **1**, the next step was to extend the studies to 5-iodo uridine **3** (Scheme 2 and Table 3).

Scheme 2. Stille Cross-Coupling on Model Compound 5-Iodo Uridine with Vinyl(tributyl)stannane as the Test Reaction



We performed a similar test series applying HPLC control, beginning with Stille cross-couplings of unprotected 5-iodo uridine **3** and vinyl(tributyl)stannane under the now optimized conditions using the catalyst $Pd_2(dba)_3$ at 60 °C. When we employed $P(furyl)_3$ as ligand, complete conversion could be observed after 3 h (Table 3, exp. no. 21) in analogy to the

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Table 3. Stille Cross-Couplings on 5-Iodo Uridine 3 to 5-Vinyl Uridine 4 Using the Setup That Was Optimized for the Adenosine Derivative (compare Table 2, Exp. No. 18−20) and Comparison with Another Catalyst System

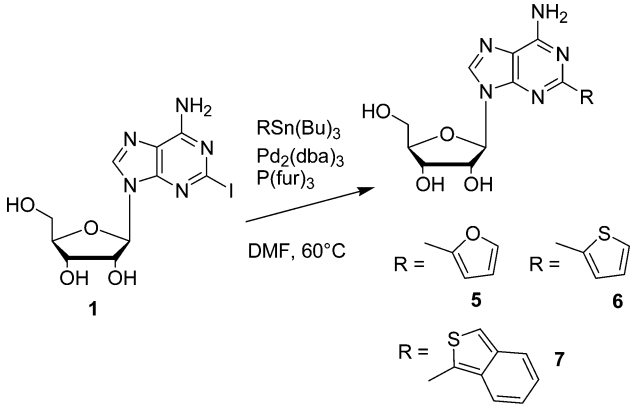
exp. no.	cat.	lig.	temp.	time	conver sion
21	$Pd_2(dba)_3$	$P(fur)_3$	60 °C	3 h	99%
22	$Pd_2(dba)_3$	no	60 °C	3 h	41%
				72 h	79%
23	$Pd_2(dba)_3$	$P(fur)_3$	50 °C	3 h	99%
24	$Pd(MeCN)_2Cl_2$	no	90 °C	3 h	<30%

adenosine derivative. Amazingly, this was not possible without utilizing the ligand, and in 3 h at 60 °C, only 41% 5-vinyluridine **4** formed and could be only increased to 79% product **4** after 3 days of reaction time (Table 3, exp. no. 22). This means, that contrary to adenosine **1** that reacted with and without ligand, the presence of $P(furyl)_3$ is obligatory for Stille cross-coupling of 5-iodo uridine **3**. However, further tests on 5-iodo uridine **3** utilizing the ligand at 50 °C and only 2 h of reaction time lead to complete conversion (Table 3, exp. no. 23). This indicates that uridine **3** is even more reactive than 2-iodo adenosine **1** if optimized conditions are applied. These results agree with those of Agrofoglio et al.[58] and Farina et al.[63] who emphasized the importance of the kind of ligand in Stille cross-couplings with uracil/uridine derivatives and basically found similar trends. Our reactions with higher catalyst loading and the use of unprotected 5-iodo uridine **3** in DMF showed even better conversion rates.

Overall, it was a challenging task to find a general setup (e.g., the mentioned influence of applying ligand and excellent results for adenosine in Table 1, exp. nos. 1 and 2 compared to Table 3, exp. no. 24 showing the incompatibility with uridine under identical conditions), but in the end, we successfully developed conditions available for both nucleosides.

Nevertheless, it was our intention to enlarge the scope of feasible Stille cross-coupling partners for our methodology. Therefore, we investigated the coupling with 2-furyl-thienyl- and benzothienyl organostannanes resulting in potentially fluorescent adenosine and uridine derivatives (compounds **5**−**10**) with our established procedure using $Pd_2(dba)_3/P(furyl)_3$ in DMF at 60 °C (Schemes 3 and 4 and Table 4). The

Scheme 3. Synthesis of Heteroaryl Modified Adenosine Derivatives via Stille Cross-Coupling under Optimized Conditions



conversion could be monitored via TLC, and the reaction in case of furane was complete after 3 h for 2-iodo adenosine **1**

Scheme 4. Synthesis of Heteroaryl Modified Uridine Derivatives via Stille Cross-Coupling under Optimized Conditions

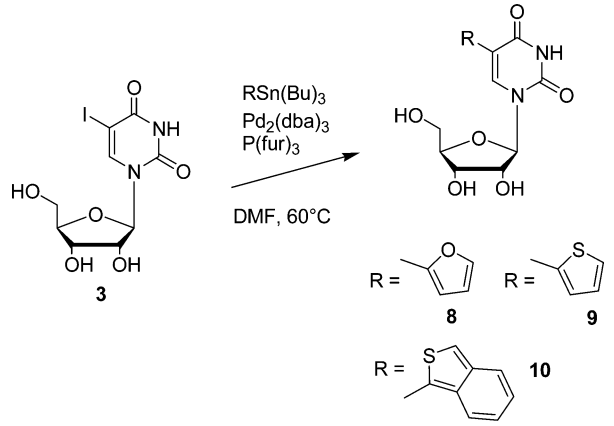


Table 4. Stille Cross-Couplings with Heterocyclic Aryl Compounds on 2-Iodo Adenosine 1 and 5-Iodo Uridine 3 Following the Established Procedure

exp. no.	starting material	coupling partner	time	product
25	**1**	2-furyl-(tributyl)stannane	3 h	**5**
26	**1**	2-thienyl-(tributyl)stannane	6 h	**6**
27	**1**	2-benzothienyl-(tributyl) stannane	8 h	**7**
28	**3**	2-furyl-(tributyl)stannane	3 h	**8**
29	**3**	2-thienyl-(tributyl)stannane	3 h	**9**
30	**3**	2-benzothienyl-(tributyl) stannane	3 h	**10**

and 5-iodo uridine **3** as well (Table 4, exp. nos. 25 and 28). However, this was a convenient result concerning the application to RNA as this was comparable to the tests with vinyl organostannane. Furthermore, we obtained under mild conditions the same excellent yields of over 95% after purification similar to that of Persson et al.[67] in refluxing THF after 72 h with respect to the protected adenosine derivative and such as Tor et al.[68] who treated 5-iodo uridine for only 2 h but at 90 °C. For the coupling on iodo uridine **3**, we found the same fast conversion yielding 5-thienyl and 5-benzothienyl uridine **9** and **10** after 3 h (Table 4, exp. nos. 29 and 30). Again, the already published procedures[69,70] reported longer reaction times or higher temperatures as well as decreased yields of only 66% in the case of attaching benzothiophene. The adenosine derivative **1** required in our hands a slightly prolonged reaction time of 6 h for thiophene (opposite to the published method[67] of 50 h refluxing in THF) and 8 h for Stille cross-coupling with the benzo[*b*]thienyl stannane presenting the new compound **7** (Table 4, exp. nos. 29 and 30). Although the reactivity seemed to be reduced in comparison to experiments with vinyl- and furyl stannane, the reaction time should still qualify also for applications on RNA. Prolonged coupling times for 2-iodo purine derivatives have also been reported in two diploma theses[75,76] performed in our working group that evaluated Suzuki cross-couplings on protected or unprotected 2-iodo inosines with 3-furyl, -thienyl, and -benzothioenyl at 100 °C in 93 h. In agreement with our finding of lower reactivity for the Stille cross-coupling with benzothiophene (Table 4, exp. no. 27), defined conditions in the case of Suzuki cross-coupling[75] had to be applied for

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conversion, and the resulting yield was worse compared to that of 3-furane and 3-thiophene. This might be due to higher sterical hindrance and additionally to an electronically more complex structure. Although the reactivity trends show similar behavior for both cross-coupling types, it must be pointed out that the Stille cross-coupling following our optimized method proceeded under much milder conditions in a still reasonable time (60 °C in 8 h instead of 100 °C in 93 h). In conclusions, we were able to establish reaction conditions permitting Stille cross-coupling on 2-iodo adenosine **1** and 5-iodo uridine **3** with different coupling partners that should be compatible with RNA on solid support.

Furthermore, the heterocyclic-modified nucleosides (**5**−**10**) that had been synthesized were subsequently characterized with respect to their photophysical properties (Table 5). The

Table 5. Photophysical Properties in Water of Furyl, Thienyl, and Benzothienyl Modified Adenosine and Uridine Derivatives 5−10[a]

compd	absorption λ_{max} [nm]	emission λ_{max} [nm]
2-(fur-2-yl) adenosine **5**	301	385
2-(thien-2-yl) adenosine **6**	310	410
2-(benzothien-2-yl) adenosine **7**	319	423
5-(fur-2-yl) uridine **8**	317	444
5-(thien-2-yl) uridine **9**	315	446
5-(benzothien-2-yl) uridine **10**	319	459

[a]The nucleoside compounds were measured at room temperature in water containing 0.1% DMSO as solvent maintaining a concentration of 10 μM.

maxima of absorption and emission were determined in water because enhanced emission can be observed in an aqueous environment[68−70] and due to the option of comparing the data with the later described RNA also measured in water. The spectra show equivalent results in the case of the uridine set (**8**−**10**) that was already examined by Tor et al.[68−70]. To the best of our knowledge, no fluorescent studies about the adenosine derivatives (**5**−**7**) exist so far; they are only described in the literature as potential antiviral agents.[67] This might be due to the low fluorescent intensity (especially compared to the uridine derivatives), even if the emission can be found in the visible range.

Stille Cross-Coupling on RNA. The now optimized conditions for the monomers were transferred to RNA with the difference that higher excesses of reagents and catalyst loading were used. The reasons for this were, on the one hand, of practical origin as handling of catalytic amounts with respect to a scale of 1 μmol or less oligonucleotide is difficult. Also, the overload should compensate for the fact that the RNA is bound on solid-phase, and the reaction, therefore, is heterogeneous. Furthermore, employing high excess in reactions on solid support is in general widely used and particularly applied for the derivatization of oligonucleotides.[20,46,51]

Our first experiments on oligonucleotides are presented under conditions we investigated on monomers at the beginning (compare Scheme 1 and Table 1, entries 7 and 8). Thus, we performed Stille cross-couplings on RNA with $Pd(MeCN)_2Cl_2$ as catalyst at 90 °C in 3 h and at 60 °C under microwave irradiation for 6 h, respectively. That setup intended to check the stability of RNA under this rather harsh setting and to confirm the need for further monomer test reactions to find milder conditions. Not unexpectedly, these trials on mixed 12-mer RNAs with incorporated 2-iodo adenosine did not result in the desired product, but besides traces of breakdown products, unreacted 12-mer, and unidentified compound with a higher mass than the starting material or the product, the main material could not be isolated anymore.

For that reason, we switched to our alternative method proceeding under milder conditions ($Pd_2(dba)_3$/P(fur)$_3$ at 60 °C, presented in Table 2, exp. no. 19). The first studies following this approach consisted of Stille couplings of mixed sequences containing 2-iodo adenosine and 5-iodo uridine with vinyl(tributyl)stannane. The results were promising as traces of product could be observed in mass spectroscopy, but still the isolation of the product in good yield had not been accomplished. To simplify the setup and subsequent purification as well as characterization, we decided to use less complex sequences and carried out Stille cross-coupling with poly uridine 5- and 12-mers (Figure 1). For that, we

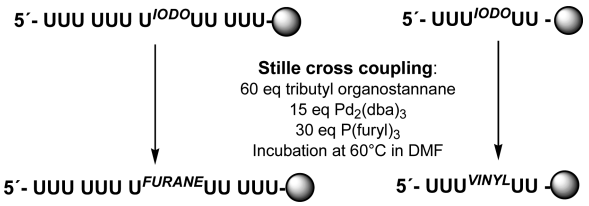

Figure 1. Stille cross-couplings on poly uridine model RNAs with vinyl- and furyl(tributyl)stannane under defined conditions.

incorporated commercially available 5-iodo uridine phosphoramidite and synthesized 2-iodo adenosine phosphoramidite[52,53] in the center of the RNA strand as well as at the 5′-end. We performed the coupling reaction with 60 equiv of vinyl- or furyl(tributyl)-stannane under optimized conditions: 15 equiv of $Pd_2(dba)_3$ as a catalyst, 30 equiv of P(furyl) as a ligand for 12−18 h in DMF at 60 °C. Additionally, we carried out double coupling under identical conditions for 6−10 h with washing-off of the reagents in between to ensure complete conversion. This proceedure turned out to be successful, and the desired site-specifically labeled oligonucleotides could be isolated after deprotection, cleavage from solid support, separation on anion exchange HPLC, and desalting. Coupling yields of separated RNAs were found to be comparable to those of unmodified reference oligonucleotides in automated standard synthesis. All modified RNAs were characterized via mass spectrometry (Table 6, **ON 1**−**ON 7**).

Conversion was found to be complete, and no starting material containing iodo modified RNA was isolated. This represents a remarkable result, in particular regarding the fact that postsynthetic Stille cross-couplings at intrastrand positions succeeded in the same degree as those on 5′-terminal nucleotides. The efficiency of that totally postsynthetic strategy highlights our method compared to other postsynthetic reactions such as the above-noted Sonogashira cross-couplings described by Khan et al.[46] and Wagenknecht et al.,[47] which were limited by the fact that conversion only proceeded on the free 5′-end requiring interruption of automated oligonucleotide synthesis.

Since we explored not only the commonly used TBDMS but also the ACE strategy[73] to take the same advantages such as the use of mild reagents and excellent yields evaluated by us for Sonogashira cross-couplings,[52] we discovered that applying

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Table 6. Synthesis of Modified RNA via Stille Cross-Couplings under Conditions Presented in Figure 1 with Double-Coupling: Results of Mass Spectrometry[a]

name	sequence	modification	exact mass calculated	mass found [Da]
ON 1[b]	5′- U*UU UU	vinyl	1494.19	1494.2
ON 2[b]	5′- UUU* UU	vinyl	1494.19	1494.2
ON 3[b]	5′- UUA* UU	vinyl	1517.22	1517.2
ON 4[b]	5′- UUU UUU U*UU UUU	furyl	3676.37	3676.4
ON 5[c]	5′- UUU* UU	vinyl	1494.19	1494.2
ON 6[c]	5′- UUU* UU	furyl	1534.19	1534.1
ON 7[c]	5′- UUU UUU U*UU UUU	furyl	3676.37	3676.5
ON 8[c]	5′- UUU* UU	thienyl	1550.16	1550.2
ON 9[c]	5′- UUU* UU	benzothienyl	1600.17	1600.2
ON 10[c]	5′- UUU UUU U*UU UUU	thienyl	3692.34	3692.4
ON 11[c]	5′- UUU UUU U*UU UUU	benzothienyl	3742.37	3742.4

[a]In the experimental series presented in this table, also fractions containing the mass of reduced deiodinated RNA could be isolated, but no starting material was found. [b]Synthesis was performed via ACE protecting group strategy; besides, the desired modified RNA single and multiple methylated product (+14) could be also found. [c]Synthesis was performed via the TBDMS protecting group strategy.

Stille cross-coupling on ACE protected RNA containing uridine yields in methylated side products besides the desired product (Table 6, **ON 1**−**ON 4**). This will be discussed in detail elsewhere (manuscript in preparation).

Because of finding the ACE strategy not completely satisfactory, further expanding the scope of Stille cross-coupling with more coupling partners was only performed with TBDMS protected RNA. This was achieved by attaching thiophene and benzothiophene to 5- and 12-mer poly uridines (Table 6, **ON 8**−**ON 11**) providing not just the furyl-modified (**ON 6** and **ON 4**/**7**) but a complete set of aryl-substituted poly uridine oligonucleotides possibly exhibiting fluorescent properties.

Actually, in the case of RNA synthesis via the TBDMS strategy and subsequent Stille cross-coupling (**ON 8**−**ON 11**), the only isolated fraction not identical with the product (20−30%, determined via integration of RP-HPLC peaks, conform to the relationship of isolated amounts of side product and desired derivatized RNA) had been characterized as reduced, deiodinated species in full length. This electrophilic reduction during the catalytic cycle is in general known for palladium-catalyzed cross-couplings, and most notably Stille couplings using $Pd_2(dba)_3$ were reported to show particular side products.[63,77] The origin of the side reaction is uncertain; an alkyl transfer with β-elimination might induce the reductive elimination, but a radical mechanism could also be involved.[77] While Farina et al.[63] described uracil as the side product in Stille cross-couplings of 5-iodo uracil with vinyl(tributyl)-stannane in the presence of $Pd_2(dba)_3$, we have never observed this under our conditions in 3 h at 60 °C on a nucleoside level. It could be interpreted as a kind of "overloading" for treating the oligonucleotide with respect to a high excess of catalyst, long reaction time, and double-coupling. In fact, if exposure of RNA to conditions of Stille cross-coupling had been diminished, deiodination resulting from reduction could be prevented. This we proved by employing Stille cross-coupling with furyl(tributyl)stannane on TBDMS protected poly uridine 11-mer for only 10 h in a single instead of double coupling leading to full conversion without any side product (Table 7, **ON 12**, and Figure 2).

Table 7. Further Synthesis of Modified RNA via Stille Cross-Couplings under Conditions Presented in Figure 1 in a Single Coupling and Decreased Reaction Time: Results of Mass Spectrometry[a]

name	sequence	modification	reaction time	exact mass calculated	mass found [Da]
ON 12[b]	5′- UUU UUU*UU UUU	furyl	10 h	3370.34	3370.4
ON 13[c]	5′- UUU UUU U*UU UUU	furyl	13 h	3676.37	3676.4
ON 14[d]	5′- AAA AAA* AAA AAA	thienyl	7 h	3968.66	3968.9
ON 15[d]	5′- AAA AAA* AAA AAA	benzothienyl	7 h	4018.67	4018.9
ON 16[d]	5′- AAA AAA* AAA AAA	furyl	2 h	3952.69	3952.9
ON 17[d]	5′- CUU UUC A*UU CUU	furyl	2 h	3696.44	3696.7
ON 18[b]	5′- CGA CU*A UAG UCG	furyl	7 h	3859.56	3859.7

[a]In the experimental series presented in this table, no starting material and only traces under 5% of reduced deiodinated RNA could be isolated. [b]Synthesis was performed via the TBDMS protecting group strategy. [c]Synthesis was performed via the TC protecting group strategy. [d]Synthesis was performed via the ACE protecting group strategy; for **ON 14**−**ON 16**, only desired modified RNA was obtained, and for **ON 17** containing U, traces of single and multiple methylated product (+14) were also found but could be separated via RP-HPLC.

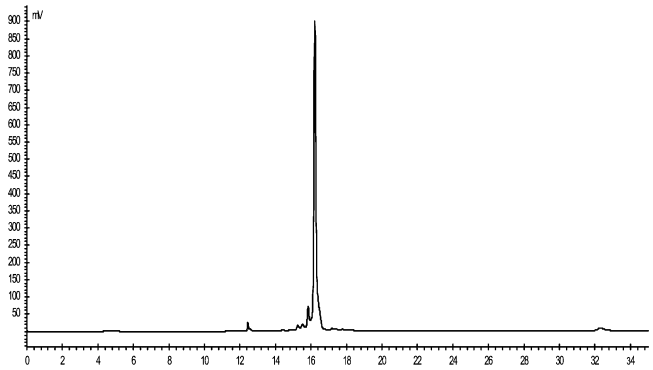

Figure 2. Reversed phase HPLC chromatogram of furyl-modified 11mer **ON 12**. The TBDMS protected RNA was synthesized as described in the text and Table 7. After automated solid phase synthesis, derivatization by Stille cross-coupling under optimized conditions (decreased reaction time and single coupling) was performed. Separation of the 11-mer from natural breakdown sequences via ion exchange HPLC showed a similar pattern compared to unmodified samples, and subsequent desalting and purification on preparative RP HPLC (shown here) result in one main peak (retention time 16.2 min) identified as pure furyl-coupled RNA. The small signal at 15.8 min retention time (<5% of all material) presents traces of deiodinated, reduced side-product and can be separated from the main fraction.

The same result with excellent conversion rate and very good overall yield of site-specifically furyl-labeled RNA (Table 7, **ON 13**) could be found when we expanded our protecting group

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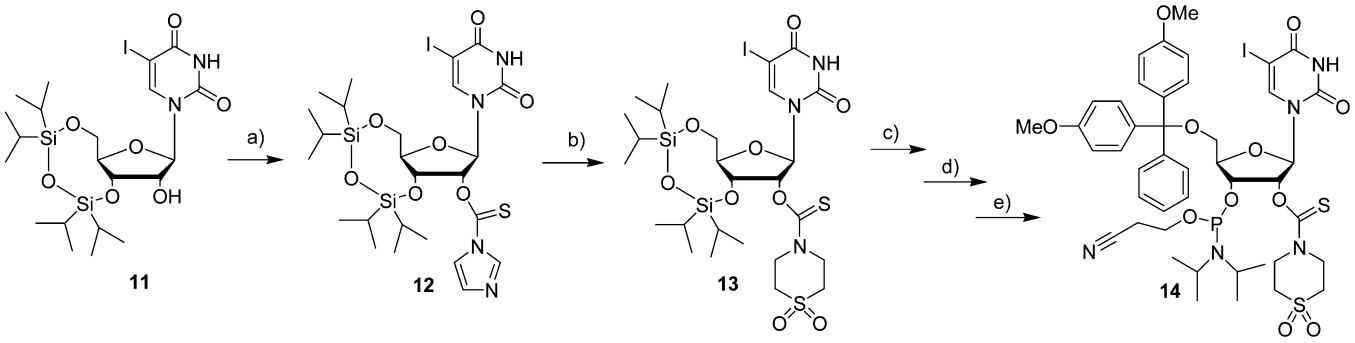
Scheme 5. Synthesis of TC Protected 5-Iodo Uridine Phosphoramidite 14[a]



[a](a) 1,1′-Thiocarbonylimidazole, DMAP, MeCN, r.t., 6 h; (b) thiomorpholine-1,1′-dioxide, MeCN, r.t., 6 h; (c) HF/pyridine, 2-MeTHF, 0 °C-r.t., 22 h; (d) DmTrCl, NMM, DCM, 0 °C, 4 h; (e) 2-cyanoethyl-*N*,*N*-diisopropylchloro-phosphoramidite, NMM, DCM, r.t., 3.5 h.

strategies to the TC chemistry recently described by Caruthers et al.[71] In order to investigate this experiment, we prepared the TC protected 5-iodo uridine phosphoramidite **14** (Scheme 5) following the procedure reported by Caruthers et al.[71] on natural nucleosides for subsequent incorporation to RNA. Applying the TC strategy turned out to be fully compatible with our Stille cross-coupling approach in a single reaction overnight.

Hence, additional derivatizations by Stille cross-coupling to further elaborate the universality of our method were performed under decreased reaction times. These experiments showed the versatility for adenosine rich sequences that were reported to effect complications[56] and moreover to complete the analogue set with furyl-, thienyl-, and benzothienyl-modified adenosine building blocks (Table 7, **ON 14**−**ON 16**). In order to prove further applicability for our approach, we also applied Stille cross-coupling to more complex mixed RNA sequences on iodo uridine as well as on iodo adenosine with furane (Table 7, **ON 17** and **ON 18**). All modified RNAs could be successfully synthesized and isolated, and the selected conditions and also reduced reaction time was even satisfactory for the self-complementary sequence being sterically more hindered as well as for the less reactive iodo adenosine. At least coupling with furyl(tributyl)stannane on 2-iodo adenosine afforded fully modified **ON 16** and **ON 17** in only 2 h. In particular, noteworthy is the ease of attaching benzothiophene to iodo adenosine in RNA in excellent yield in 7 h despite the necessity for prolonged reaction times for the nucleoside monomers. Thus, it could be shown that this approach for the Stille cross-coupling seems general for different coupling partners. It provides therefore another benefit compared to Suzuki cross-coupling studies on DNA by Manderville et al.[56] who observed a significant amount of unreacted starting material in the coupling of 8-bromo desoxyadenosine with benzothienyl boronic acid.

Besides the fact that modifications on the 2-position of adenosine inserted into RNA have been in general only rarely explored, our particular 2-aryl purine modifications in RNA (**ON 3**; **ON 14**−**ON 17**) present new compounds. To the best of our knowledge, 2-functionalized riboadenosines reported so far are only established for pyrene and radical spin label modifications used by us in an A-form RNA double helix in the minor groove.[50−54] Since the linkage to the label had been acetylenic resulting from Sonogashira cross-coupling on RNA so far, we expanded here the substitution pattern to aryls via Stille cross-coupling.

The convenience of our method is clearly demonstrated by the fact that we are able to generate a set of differently labeled RNAs deriving from just one precursor. That way, both series of modified uridine (**ON 7**, **ON 10**, and **ON 11**) and adenosine (**ON 14**−**ON 16**) 12-mer oligonucleotides could be easily synthesized from iodo substituted RNAs that were prepared in good yield during automated solid-phase synthesis. Afterward, the solid support was divided and Stille cross-coupling was performed in parallel synthesis with furyl, thienyl, and benzothienyl for each RNA. The common way of inserting modified phosphoramidites such as, e.g., a 3-furyl inosine derivative that was incorporated into model RNA in our working group,[76] resulted in the corresponding modified oligonucleotide but turned out to be much more time-consuming and less efficient in preparation and yield when compared to the divergent postsynthetic strategy described herein.

Tor et al.[68−70,78,79] presented one more alternative to synthesize modified RNA via enzymatic incorporation of a particular modified triphosphate. This technique was used for 5-uridine functionalization by furyl, thienyl, and benzothienyl to build up RNA containing the so-called responsive fluorescent nucleoside analogues that are sensitive to changes in the microenvironment. This basically represents the same class of target RNA we synthesized to show the versatility of our approach. According to Tor et al.,[68] the transcription process using modified nucleoside triphosphates accepted by T7 RNA polymerase is prior to the complicated synthesis up to modified phosphoramidites because of only two synthesis steps and more effective incorporation. However, while Tor et al. synthesized defined RNA strands (e.g., HIV-1 TAR construct[78]) with particularly emissive pyrimidine nucleosides to monitor RNA−ligand interactions by fluorescence measurements, we primarily focused on developing a new, reliable method allowing efficient attachment of a variety of labels to easily access iodo modified RNA, also avoiding the synthesis of complexly modified phosphoramidites. As result, a universal strategy to site-specifically modified oligonucleotides that can be applied independent of sequence, position of modification, kind of nucleobase, and sort of conjugate is provided.

In analogy to earlier examined monomers (compare Table 5), we attained the same typical absorption and emission maxima for 5-fur(2-yl), thien(2-yl) and benzothien(2-yl) uridine 5-mer and 12-mer RNA in water (Table 8, **ON 6**−**ON 11**). The almost identical emission bands of the modified single-stranded RNA compared to the free nucleoside suggest

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Table 8. Photophysical Properties of a Set of 2-Furyl, Thienyl, and Benzothienyl Modified 5-mer and 12-mer RNA[a]

name	sequence	modification	absorption λ_{max} [nm]	emission λ_{max} [nm]
ON 6	5′- UUU* UU	furyl	320	444
ON 8	5′- UUU* UU	thienyl	317	449
ON 9	5′- UUU* UU	benzothienyl	322	470
ON 7	5′- UUU UUU U*UU UUU	furyl	320	444
ON 10	5′- UUU UUU U*UU UUU	thienyl	319	450
ON 11	5′- UUU UUU U*UU UUU	benzothienyl	322	470
ON 14	5′- AAA AAA* AAA AAA	furyl	310	405
ON 15	5′- AAA AAA* AAA AAA	thienyl	312	405
ON 16	5′- AAA AAA* AAA AAA	benzothienyl	325	407
ON 17	5′- CUU UUC A*UU CUU	furyl	312	406
ON 18	5′- CGA CU*A UAG UCG	furyl	322	435

[a]The oligonucleotides were measured at room temperature in sterile water as solvent maintaining a concentration of 10 μM.

that the modified nucleosides remain also in the oligomer in a polar environment, which is in full agreement with results of Tor et al.[68] Significant in the first instance is the very high intensity and bathochromic shift of benzothienyl modified RNA (**ON 8** and **ON 11**) that helps in investigating nucleic acid dynamics and recognition processes.[70]

Also, the aryl-modified adenosine sequences were characterized with respect to their fluorescence properties. Contrary to the uridine series, there is no significant bathochromic shift from the furyl- over thienyl- up to benzothienyl-modified RNA (Table 8, **ON 14**–**ON 16**). As expected from the results of the nucleoside monomers, the fluorescence intensity of adenosine modifications is decreased compared to the uridine ones. For mixed sequences, the same trend in a hyperchromic effect for furyl modified uridine (**ON 18**) and an emission in the visible range but with low intensity for furyl modified adenosine (**ON 17**) could be observed. In addition, the self-complementary RNA **ON 18** shows a slightly hypochromic emission (435 nm) compared to the free nucleoside **8** (444 nm) suggesting that the secondary structure can change the polar environment to some extent.

CONCLUSIONS

In conclusion, we have demonstrated the proof of principle for the palladium-mediated Stille cross-coupling as a convenient strategy for postsynthetic modification of RNA on column. Our concept of postsynthetic derivatization combines the advantages of solid support approaches with respect to high conversion and excellent yields while using only economical amounts of reagents. An additional benefit is easy access to diversely modified RNAs arising from just one oligonucleotide precursor.

To the best of our knowledge, this is the first time that Stille cross-coupling on oligonucleotides has been described. Hence, our studies broadened the range of available metal-catalyzed methods (that facilitate the use of well accessible halogenated nucleotide precursors) such as Sonogashira cross-coupling,[46,47,51,53] copper-catalyzed oxidative acetylenic coupling,[20] and Suzuki–Miyaura cross-coupling[56] that were reported so far for modifying oligonucleotides postsynthetically. Particularly, with regard to the wide range of studies requiring labeled RNA, which is less stable and therefore synthetically more challenging than DNA, our Stille cross-coupling presents a reliable and mild approach. Hence, the scope and linker pattern of the Sonogashira coupling as the only existing technique qualified for RNA so far is extended.

The Stille cross-coupling was found to show large differences in reactivity at decreased temperatures with respect to the catalyst/ligand system for purine or pyrimidine nucleosides even on a monomer level. Nevertheless, we could successfully transfer the on nucleoside optimized reaction conditions now to Stille cross-coupling on RNA. We confirmed the eligibility and universality of the established setup for intrastrand or at terminal position inserted 5-iodo uridine as well as 2-iodo adenosine with various coupling partners using different sequences.

Moreover, we were able to show that Stille cross-coupling can be in principle applied for RNA synthesized via different protecting group strategies (TBDMS, TC, and ACE). Whereas the applicability of our method concerning ACE-protected RNA containing uridine is limited due to the formation of methylated side-products, the compatibility with TBDMS as well as with TC chemistry independently of the sequence is shown to be excellent.

As Nair et al.[60] indicated for monomers, it is noteworthy to mention that the vinyl-coupled RNAs are key precursors for further functionalization. By dihydroxylation and periodate cleavage, an aldehyde function is created. These aldehydes can be used as precursors for building up benzimidazoles known as important pharmacophores. Recently, an efficient microwave-assisted synthesis of 5-benzimidazolyl-2′-desoxyuridines resulting in high fluorescent activity was developed in our group.[80] Both aspects of Stille cross-coupling directly resulting in functionally modified RNA or affording a precursor qualified for further derivatization open the opportunity for convenient synthesis of modified RNA.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

cpg, controlled pore glass; DCM, dichloromethane; DEPC, diethylpyrocarbonate; DMAP, 4-(dimethylamino)pyridine; DMF, *N*,*N*-dimethyl formamide; DmTrCl, 4,4′-dimethoxytriphenylmethyl chloride; EDTA, ethylenediaminetetraacetate; MeCN, acetonitrile; NMM, *N*-methylmorpholine; $Pd_2(dba)_3$, tris(dibenzylideneacetone)dipalladium(0); $Pd(MeCN)_2Cl_2$, palladium(II) chloride diacetonitrile complex; $P(fur)_3$, tri-2-

furylphosphine; P(*o*-tol)$_3$, tri(*o*-toluyl)phosphine; TEA, triethylamine; TEAA, triethylammoniumacetate; TEMED, tetramethylethylenediamine; THF, tetrahydrofuran

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