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Spin-Labeled RNA

Synthesis of a Cytidine Phosphoramidite with Protected Nitroxide Spin Label for EPR Experiments with RNA

Timo Weinrich,[a] Markus Gränz,[b] Christian Grünewald,[a] Thomas F. Prisner,[b] and Michael W. Göbel*[a]

Abstract: Spin labeling of oligonucleotides with nitroxides is hampered by their intrinsic instability under conditions of solid-phase synthesis and enzymatic ligation. Although nitroxide decomposition can be avoided in some cases by postsynthetic introduction or by special reaction conditions, a more general solution would be reversible protection of the radical. We have recently developed such a method based on photolabile protection groups for DNA oligonucleotides and demonstrated their application in EPR spectroscopy. Here, we extend this method to RNA oligonucleotides. By improving the synthetic procedures, the yield of the coumarin-protected phosphoramidite could be increased by a factor of 12. Effective recovery of the nitroxides on a duplex RNA enables pulsed EPR experiments to be performed directly after irradiation and air oxidation. Data at Q-band frequency is shown and distances measured with PELDOR (pulsed electron-electron double resonance) spectroscopy agree well with the calculated values.

Introduction

Labeling of RNA with persistent nitroxide radicals[1] such as 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) enables the use of EPR spectroscopy to study the local dynamics of oligonucleotides and of complexes they form.[2] PELDOR, pulsed electron-electron double resonance (likewise known as double electron electron resonance; DEER), can also determine the distance and relative orientation between two nitroxides.[3] However, access to large and multiple-labeled RNA samples may become a limiting factor for PELDOR applications. Although unprotected nitroxides can be incorporated into RNA directly through phosphoramidite chemistry,[4] special precautions are required to avoid decomposition.[1a–d,1f,4] Furthermore, the need to purify products by HPLC is a limitation to oligonucleotide lengths.[5] Longer RNA strands can be prepared by enzymatic ligation of several fragments. However, significant degradation of spin labels is typically observed,[5] presumably because of the thiol components of the buffer that are required to keep the enzyme active. Höbartner has introduced a gentle method for the ligation of spin-labeled RNA based on deoxyribozymes.[5] Given that nitroxide instability is a general problem in many steps of oligonucleotide synthesis, we preferred a more fundamental solution: nitroxides when reduced and alkylated with a light-sensitive protective group may become stable against all critical conditions of sample preparation.[6] Photolytic removal of such groups by irradiation at 366 or 405 nm is

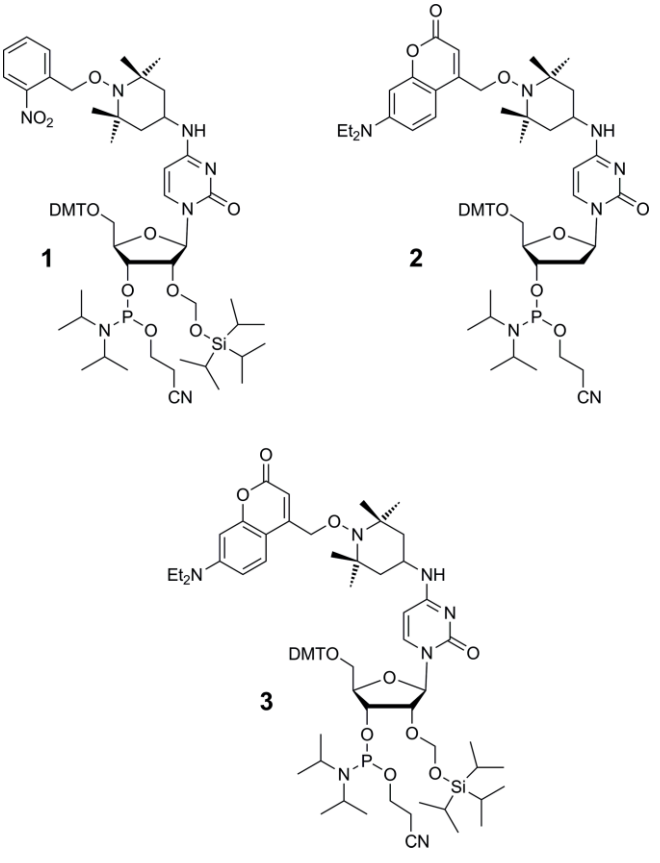


Figure 1. Structures of the protected phosphoramidites **1**, **2**, and **3**.

[a] *Institute of Organic Chemistry and Chemical Biology, Goethe-University Frankfurt,*
Max-von-Laue-Str. 7, 60438 Frankfurt am Main, Germany
E-mail: M.Goebel@chemie.uni-frankfurt.de
http://web.uni-frankfurt.de/fb14/goebel/
[b] *Institute of Physical and Theoretical Chemistry and Center for Biomolecular Magnetic Resonance, Goethe-University Frankfurt, Max-von-Laue-Str. 7, 60438 Frankfurt am Main, Germany*
Supporting information and ORCID(s) from the author(s) for this article are available on the WWW under http://dx.doi.org/10.1002/ejoc.201601174.

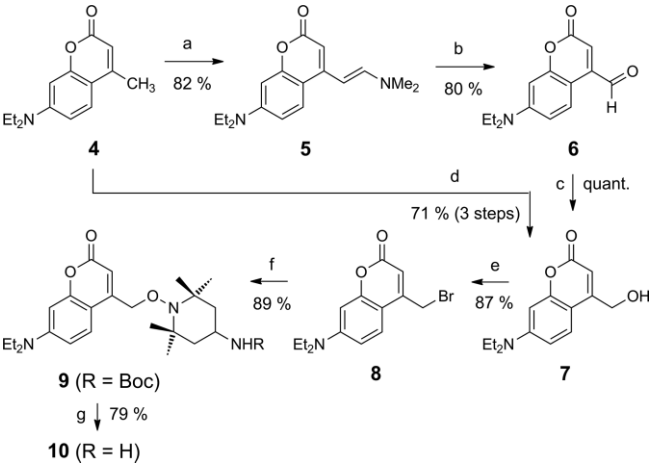
known not to affect ribonucleic acids.[7] The resulting hydroxylamine is oxidized spontaneously under air to give the desired nitroxide.[4c,8,10] Protection of nitroxides has been reported previously by silylation,[8a] acylation,[8] or alkylation[9] of the related hydroxylamine. Our first attempt, however, ended without success. Phosphoramidite **1** behaved well during solid-phase synthesis and strand deprotection (data not shown). Unfortunately, the amine and not the nitroxide was found as the main product of photolysis. This observation prompted us to investigate the chemical stability of protected nitroxides and their photochemical release in a more systematic way.[6] Finally, we prepared the coumarin-protected deoxyphosphoramidite **2** and used it for the synthesis of a short spin-labeled DNA strand.

Both oligonucleotide assembly and photochemical regeneration of the nitroxide operated well.[6] For spin labeling of RNA, the analogous riboamidite **3** is required. Here we describe an optimized synthesis of this compound. Compared with our previous preparation of amidite **2**, the new method saves time and significantly increases yields. Building block **3** has been incorporated into single and double stranded RNA. High levels of nitroxide recovery allowed us to perform distance measurements with PELDOR spectroscopy directly after irradiation of a palindromic sample without any purification step (Figure 1).

Results and Discussion

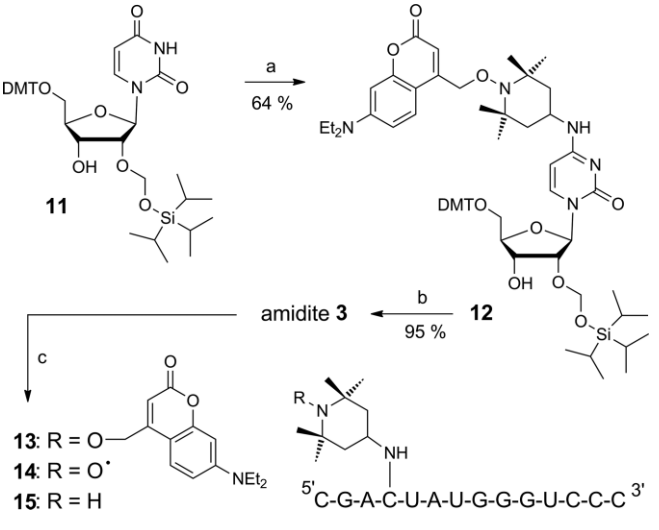
The previous synthesis of phosphoramidite **2** suffered from three low-yielding steps. Commercially available coumarin **4** was oxidized first with SeO_2 to form aldehyde **6**.[11] After tedious purification, the yield was around 40–50 % at best and tended to drop drastically for large-scale preparations. In the new pathway (Scheme 1), condensation of **4** with DMF–DMA leads to the formation of enamine **5** in 82 % yield, which can be converted into aldehyde **6** by oxidation with sodium periodate (80 %). Reduction of **6** and bromination towards **8** was achieved by following published protocols.[6,11] When the purification of intermediates was omitted, 15 g batches of alcohol **7** could be obtained in a rapid procedure from coumarin **4** with 71 % total yield. Coumarin **7** has found other important applications in photochemistry, such as wavelength selective uncaging and two-photon excitation.[7c,12]

The second critical step is alkylation of the hydroxylamine intermediate obtained by reduction of Boc-protected 4-amino-TEMPO with bromide **8**. Whereas the hydrogenation/alkylation method gave only 31 % of product **9**,[6] the new copper-induced procedure increased the yield to 89 %. The reaction type is known from polymer chemistry and involves recombination of the nitroxide with an alkyl radical formed by reduction of compound **8**.[13] After removal of Boc with TMS-I (79 %), the resulting amine **10** reacted with an activated nucleoside obtained from TOM-protected uridine **11**[14] by O-sulfonylation[5] (64 % of **12**, 47 % based on **10**, Scheme 2). Protection of the 3′-hydroxy group is not required under such conditions. Phosphitylation of **12** by using standard procedures gave phosphoramidite **3** in 95 % yield, which was accessible in multigram amounts. Compared with the method published previously for the DNA building block **2**, the total yield starting from coumarin



Scheme 1. Synthesis of the protected TEMPO derivative **10**. *Reagents and conditions*: (a) DMF–DMA, DMF, reflux, 14 h; (b) $NaIO_4$, THF/H_2O, room temp., 1.5 h; (c) $NaBH_4$, THF, r.t., 5 h; (d) see Exp. Sect.; (e) 1. MsCl, Et_3N, CH_2Cl_2, 0 °C, 2 h; 2. LiBr, THF, r.t., 2.5 h;[6] (f) Boc-protected 4-amino-TEMPO, Cu, $Cu(OTf)_2$, bipyridyl ligand, toluene, reflux, 16 h; (g) TMS-I, CH_3CN, 0 °C, 30 min.[6] DMF–DMA = *N*,*N*-dimethylformamide dimethyl acetal. MsCl = methansulfonylchloride. TMS-I = trimethylsilyl iodide.

4 was improved more than 12-fold. To validate the 2′-*O*-TOM amidite **3**, oligoribonucleotide **13** was assembled with a synthesizer from **3** in combination with commercial 2′-*O*-TBDMS-protected phosphoramidites (see the Supporting Information). Although all coupling steps looked very good in the trityl assay, the isolated yield of **13** after deprotection and HPLC purification was somewhat lower than for the analogous DNA oligomer. This observation can be attributed to the lower coupling efficiency of 2′-*O*-TBDMS-protected riboamidites.



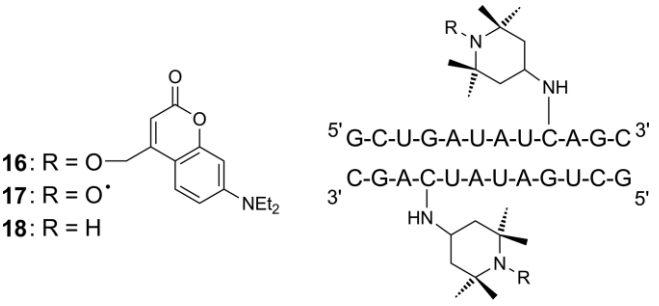
Scheme 2. Synthesis of phosphoramidite **3** and of spin-labeled RNA **13**. *Reagents and conditions*: (a) 1. TPS-Cl, Et_3N, DMAP, CH_2Cl_2, 0 °C → room temp., 20 h; 2. Addition of **10**, DIPEA, DMF, 85 °C, 8 h and r.t., 14 h; (b) *N*,*N*-diisopropylaminocyanoethylphosphoramidic chloride, Et_3N, CH_2Cl_2, r.t., 21 h; (c) see the Supporting Information. TPS-Cl = 2,4,6-triisopropylbenzene-sulfonyl chloride. DMAP = 4-dimethylaminopyridine. DIPEA = diisopropylethylamine.

Photochemical removal of the coumarin group was achieved as reported before by irradiation of RNA **13** at 366 nm for

30 min. The results are similar to those obtained with the analogous DNA.[6] Amine **15** is the dominant product at pH 4.6, whereas at pH 7.4, 55 % of **14** and 45 % of **15** could be observed by HPLC. Best results were achieved at pH 8.5, when 85 % of nitroxide **14** and just 15 % of **15** are formed. The amount of recovered nitroxide (pH 8.5) was also quantified by cw-EPR directly after irradiation at 366 nm for 30 min in a round glass cuvette (Carl Roth 50 × ø 10 mm). The sample was filled into appropriate tubes and the signal obtained was compared to a known reference. More than 80 % yield of recovered spin label (based on the concentration of **13**) was detected (see the Supporting Information, Figure S8), in good agreement with HPLC analysis (see the Supporting Information, Table S1).

The question then arises: are samples of such quality sufficient for the measurement of spin-spin distances by PELDOR spectroscopy? To address this issue, we synthesized the self-complementary RNA strand **16**. Assembly and purification of which worked well, as before. The quality of oligonucleotide **16** was verified by HPLC, UV spectroscopy, and mass spectrometry (see the Supporting Information, Figures S2 and S4). After irradiation of **16** at 366 nm for 30 min as shown above (pH 8.5), cw-EPR again indicated more than 80 % formation of the nitroxide-labeled RNA **17** (see the Supporting Information, Figures S6 and S8), while the reduction product **18** remained a minor constituent of the mixture. Palindrome **17** was annealed to assure duplex formation (see the Supporting Information, Table S2) and used for pulsed EPR experiments without further purification (Scheme 3).

The sample was dissolved in a 20 % d_8-glycerol-buffer mixture and cooled in liquid nitrogen. Calculating the distance distributions from Q-band PELDOR data at 50 K by Tikhonov regularization gave a main distance of 1.85 nm and further distances between 3 and 7 nm (Figure 2 and the Supporting Information, Figure S9). The highest peak at 1.85 nm fits perfectly to the intramolecular distance calculated for an A-type helix with spin labels placed in the major groove. For the modeling, a duplex RNA model was built by using NAB[16] and nitroxide spin labels



Scheme 3. Spin-labeled duplex RNA **17**, bearing a nitroxide on position 9.

were attached with PyMOL.[17] After geometry optimization, the distance between both nitroxides within one duplex RNA was determined (Figure 3). The other peaks with maxima at around 3.5 and 5.7 nm correspond to intermolecular distances of nitroxides in an end-to-end stacked dimer of two palindromic duplex RNAs (see the Supporting Information, Figure S10). The

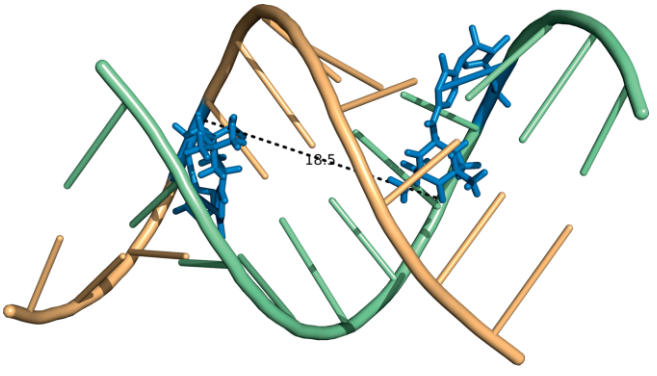


Figure 3. Molecular model of the 12mer-duplex RNA **17** showing a distance of 1.85 nm (depicted as 18.5 Å) between the two spin labels attached to their nucleotide. The structure was generated as an A-form RNA duplex by using NAB.[16] The nitroxide spin labels were built on the corresponding C by using PyMOL.[17] Geometry was optimized by force field calculation implemented in AVOGADRO and YASARA.[18,19]

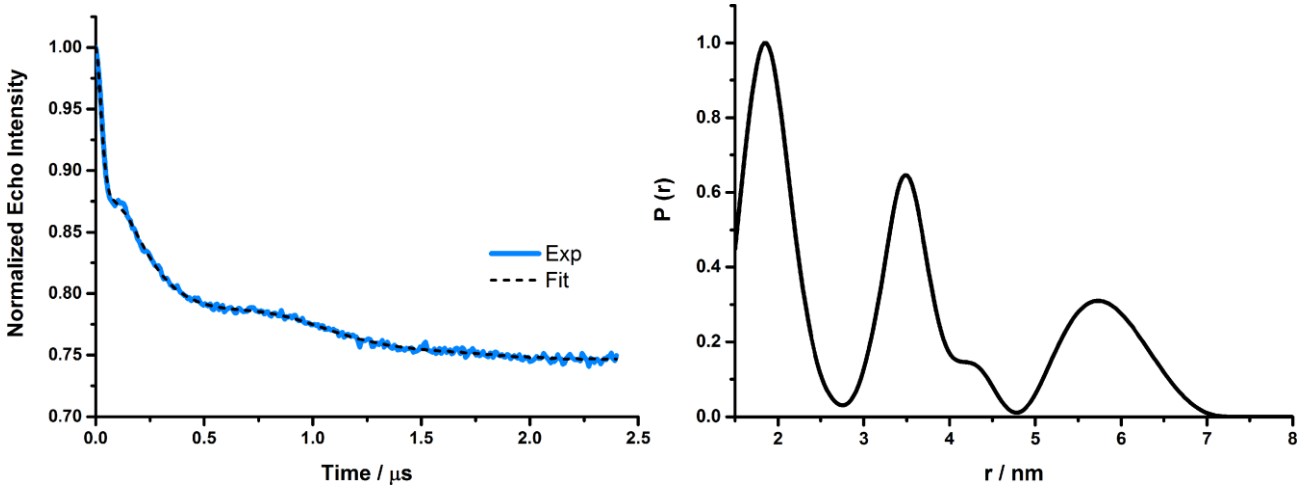


Figure 2. PELDOR measurement of 12mer-duplex-RNA **17** at Q-band frequency. (a) Background-corrected PELDOR trace (in blue) and Tikhonov regularization (in black) by using DEER analysis.[15] (b) Calculated distance distribution with an overall maximum at 1.85 nm. Peaks at around 3.5 and 5.7 nm correspond to intermolecular distances due to stacking of RNA molecules. Data was normalized and a regularization parameter α of 10 was used.

close-by spins between two stacked duplex-RNA dimers will also yield a distance of around 1.85 nm, thereby increasing the amount of this peak in the distance profile. The stacking behavior was already observed in previous RNA studies[1b,1c,2d] and investigations to overcome this, especially for duplex RNA molecules, are ongoing.

Conclusions

Compared with our previous method, the new synthetic pathway towards photolabile precursors of spin-labeled nucleosides saves time and substantially improves the yield. Multigram batches of the corresponding phosphoramidite are easily accessible. Incorporation into RNA strands by using standard protocols works without difficulty. As expected, the coumarin group is not challenged under conditions of 2′-desilylation and TOM removal. The recovery of nitroxides after photolysis of the protecting group and air oxidation is as efficient as in the case of DNA oligonucleotides.

Although the yield of spin labels is not quantitative, samples of good spectroscopic quality are obtained that can be used for PELDOR experiments directly after photolysis without further purification steps. Short spin-labeled RNA strands such as **14** and **17** are also accessible by postsynthetic introduction of TEMPO.[1e,5] Such samples have been successfully used for PELDOR studies.[2d] However, they are still sensitive against thiols. The use of protected spin labels should also prevent nitroxide degradation during standard ligation protocols, thus giving access to long spin-labeled RNA molecules without the requirement for postsynthetic modifications. When using coumarin protecting groups, two-photon excitation is an option to activate spin labels with high local and temporal resolution. For intracellular applications, however, more efficient recovery of the nitroxides would be required when irradiated at physiological pH. Thus, effective and pH-independent deprotection methods for spin labels are the aim of present studies.

Experimental Section

(*E*)-7-(Diethylamino)-4-[2-(dimethylamino)vinyl]-2*H*-chromen-2-one (5): To a solution of coumarin **4** (10.00 g, 43.23 mmol, 1.00 equiv.) in DMF (100 mL) DMF–DMA (11.49 mL, 86.47 mmol, 2.00 equiv.) was added. The reaction mixture was heated to reflux for 14 h. Subsequently conc. $NaHCO_3$ solution and CH_2Cl_2 were added. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were dried with $MgSO_4$ and the solvent was removed under reduced pressure. Purification by silica gel chromatography (CH_2Cl_2/EtOAc, 7:3) yielded the title compound (10.19 g, 82 %) as a brown solid. R_f = 0.30 (CH_2Cl_2/EtOAc, 7:3). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.53 (d, J = 9.1 Hz, 1 H, 5-H), 7.23 (d, J = 13.0 Hz, 1 H, CHC*H*N), 6.55 (dd, J = 9.0, 2.7 Hz, 1 H, 6-H), 6.50 (d, J = 2.7 Hz, 1 H, 8-H), 5.86 (s, 1 H, 3-H), 5.23 (d, J = 13.0 Hz, 1 H, *CH*CHN), 3.41 (q, J = 7.1 Hz, 4 H, CH_2CH_3), 3.00 [s, 6 H, $N(CH_3)_2$], 1.20 (t, J = 7.0 Hz, 6 H, CH_2CH_3) ppm. ^{13}C NMR (125.8 MHz, $CDCl_3$): δ = 163.4, 156.3, 152.3, 150.1, 146.5, 124.8, 108.1, 107.8, 98.1, 93.4, 87.4, 44.6, 12.5 ppm. MS (ESI): m/z = 287.5 [M + H$^+$]. HRMS (MALDI): calcd. for $C_{17}H_{23}N_2O_2$ [M + H$^+$]: 287.17595; found 287.17583.

7-(Diethylamino)-2-oxo-2*H*-chromene-4-carbaldehyde (6): To a solution of enamine **5** (10.00 g, 34.92 mmol, 1.00 equiv.) in THF/H_2O (80 mL 1:1), $NaIO_4$ (22.41 g, 104.76 mmol, 3.00 equiv.) was added. The reaction mixture was stirred for 1.5 h at ambient temperature. The precipitate was filtered off and washed with EtOAc. Half of the solvent was removed under reduced pressure and conc. $NaHCO_3$ solution was added. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were dried with $MgSO_4$ and the solvent was removed under reduced pressure. Purification by silica gel chromatography (EtOAc/*c*-hexane, 1:1) gave title compound **6** (6.84 g, 80 %) as a red solid. R_f = 0.33 (CH_2Cl_2 was used for better TLC-resolution). ^{1}H NMR (250 MHz, $CDCl_3$): δ = 10.04 (s, 1 H, CO*H*), 8.33 (d, J = 9.2 Hz, 1 H, 5-H), 6.66 (dd, J = 9.2, 2.6 Hz, 1 H, 6-H), 6.56 (d, J = 2.6 Hz, 1 H, 8-H), 6.47 (s, 1 H, 3-H), 3.44 (q, J = 7.1 Hz, 4 H, CH_2CH_3), 1.23 (t, J = 7.1 Hz, 6 H, CH_2CH_3) ppm. ^{13}C NMR (63 MHz, $CDCl_3$): δ = 192.4, 161.7, 157.3, 150.7, 143.9, 127.1, 117.7, 109.9, 104.2, 98.2, 45.1, 12.4 ppm. MS (ESI): m/z = 246.4 [M + H$^+$]. HRMS (MALDI): calcd. for $C_{14}H_{16}NO_3$ [M + H$^+$]: 246.11247; found 246.11249.

7-(Diethylamino)-4-(hydroxymethyl)-2*H*-chromen-2-one (7): A solution of aldehyde **6** (6.76 g, 27.58 mmol, 1.00 equiv.) in THF (80 mL) was cooled to 0 °C, treated with $NaBH_4$ (2.09 g, 55.16 mmol, 2.00 equiv.) and stirred for 5 h at ambient temperature. Subsequently, conc. $NaHCO_3$ solution was added and the organic layer was separated. The aqueous layer was extracted with CH_2Cl_2 and the combined organic layers were dried with $MgSO_4$. The solvent was removed under reduced pressure and the residue was purified by silica gel chromatography (EtOAc/*c*-hexane, 1:1). Title compound **7** (6.82 g, quant.) was isolated as a yellow solid. R_f = 0.16 (EtOAc/*c*-hexane, 1:1). ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 7.43 (d, J = 9.0 Hz, 1 H, 5-H), 6.65 (dd, J = 9.0, 2.6 Hz, 1 H 6-H), 6.51 (d, J = 2.6 Hz, 1 H, 8-H), 6.06 (s, 1 H, 3-H), 5.50 (t, J = 5.6 Hz, 1 H, O*H*), 4.66 (dd, J = 5.6, 1.3 Hz, 2 H, CH_2OH), 3.41 (q, J = 7.0 Hz, 4 H, CH_2CH_3), 1.11 (t, J = 7.0 Hz, 6 H, CH_2CH_3) ppm. ^{13}C NMR (125.8 MHz, [D$_6$]DMSO): δ = 161.1, 156.9, 155.6, 150.2, 125.1, 108.5, 105.7, 103.9, 96.8, 59.0, 43.9, 12.3 ppm. MS (ESI): m/z = 248.2 [M + H$^+$]. HRMS (MALDI): calcd. for $C_{14}H_{18}NO_3$ [M + H$^+$]: 248.12812; found 248.12753.

7-(Diethylamino)-4-(hydroxymethyl)-2*H*-chromen-2-one (7), Fast Procedure: To a solution of coumarin **4** (20.00 g, 86.47 mmol, 1.00 equiv.) in DMF (100 mL), DMF–DMA (22.97 mL, 172.94 mmol, 2.00 equiv.) was added. The reaction mixture was heated to reflux for 14 h. Subsequently, conc. $NaHCO_3$ solution and CH_2Cl_2 were added. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were dried with $MgSO_4$ and the solvent was removed under reduced pressure. The residue was resolved in THF/H_2O (150 mL, 1:1), and $NaIO_4$ (55.44 g, 259.41 mmol, 3.00 equiv.) was added. After stirring for 2 h at ambient temperature the precipitate was filtered off and washed with EtOAc. Half of the solvent was removed under reduced pressure and conc. $NaHCO_3$ solution was added. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were dried with $MgSO_4$ and the solvent was removed under reduced pressure. Subsequently, the residue was resolved in THF (120 mL), cooled to 0 °C and $NaBH_4$ (6.54 g, 172.94 mmol, 2.00 equiv.) was added. After stirring for 2 h at ambient temperature, conc. $NaHCO_3$ solution was added. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were dried with $MgSO_4$ and the solvent was removed under reduced pressure. Purification by silica gel chromatography (EtOAc/*c*-hexane, 1:1) gave title compound **7** (15.19 g, 71 % after three steps) as a yellow solid. R_f = 0.16 (EtOAc/*c*-hexane, 1:1). ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 7.42 (d, J = 10.0 Hz, 1 H, 5-H), 6.65 (dd, J = 7.5, 2.5 Hz, 1 H, 6-H), 6.51 (d,

J = 2.5 Hz, 1 H, 8-H), 6.08 (s, 1 H, 3-H), 5.50 (t, *J* = 7.5 Hz, 1 H, O*H*), 4.66 (dd, *J* = 3.5, 1.0 Hz, 2 H, CH_2OH), 3.41 (q, *J* = 7.0 Hz, 4 H, CH_2CH$_3$), 1.11 (t, *J* = 7.0 Hz, 6 H, CH$_2$CH_3) ppm. ^{13}C NMR (125.8 MHz, [D$_6$]DMSO): δ = 161.2, 156.9, 155.6, 150.2, 125.1, 108.5, 105.7, 103.9, 96.8, 59.0, 43.9, 12.3 ppm. MS (ESI): *m*/*z* = 248.2 [M + H$^+$]. HRMS (MALDI): calcd. for $C_{14}H_{18}NO_3$ [M + H$^+$]: 248.12812; found 248.12883.

***tert*-Butyl-1-{[7-(diethylamino)-2-oxo-2*H*-chromen-4-yl]methoxy}-2,2,6,6-tetramethylpiperidin-4-ylcarbamate (9):** *tert*-Butyl-[4-(2,2,6,6-tetramethyl)piperidinyl-*N*-oxyl]carbamate[20] (3.84 g, 14.18 mmol, 1.00 equiv.), bromomethylcoumarin **8**[6] (5.28 g, 17.02 mmol, 1.20 equiv.), copper powder (1.35 g, 21.23 mmol, 1.50 equiv.), Cu(OTf)$_2$ (0.26 g, 0.71 mmol, 0.05 equiv.) and 4,4′-dimethyl-2,2′-bipyridyl (0.39 g, 2.13 mmol, 0.15 equiv.) were suspended in toluene (140 mL). The suspension was degassed, put under argon and heated to reflux for 16 h. The suspension was filtered through silica gel and the residue was eluted with CH_2Cl_2. The solvent was removed under reduced pressure. Purification by silica gel chromatography (CH_2Cl_2/EtOAc, 10:1) gave the title compound **9** (6.30 g, 89 %) as a light-yellow solid. R_f = 0.44 (CH_2Cl_2/EtOAc, 10:1). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.24 (d, *J* = 9.0 Hz, 1 H, 5-H), 6.55 (dd, *J* = 9.0, 2.5 Hz, 1 H, 6-H), 6.51 (d, *J* = 2.5 Hz, 8-H), 6.29 (s, 1 H, 3-H), 4.95 (s, 2 H, OCH_2), 4.28 (br. s, 1 H, N*H*CH), 3.84 (br. s, 1 H, C*H*NH), 3.41 (q, *J* = 7.0 Hz, 4 H, CH_2), 1.86 (d, *J* = 11.5 Hz, 2 H, C*H*H), 1.46 (s, 9 H, *t*Bu), 1.34 (t, *J* = 12.5 Hz, 2 H, CH*H*), 1.28–1.26 (m, 6 H, CH_3), 1.22–1.19 (m, 12 H, CH_3, CH$_2$CH_3) ppm. ^{13}C NMR (125.8 MHz, $CDCl_3$): δ = 162.3, 156.1, 155.2, 151.7, 150.4, 124.4, 108.4, 106.3, 105.9, 97.8, 74.5, 60.4, 46.0, 44.7, 42.0, 32.8, 28.4, 20.9, 12.4 ppm. MS (ESI): *m*/*z* = 503.5 [M + H$^+$]. HRMS (MALDI): calcd. for $C_{28}H_{43}N_3O_5K$ [M + K$^+$]: 540.28343; found 540.28460.

2′-*O*-TOM-5′-*O*-DMT-U° (12): To a solution of 2′-*O*-TOM-5′-*O*-DMT-uridine **11**[14] (0.37 g, 0.50 mmol, 1.00 equiv.) in CH_2Cl_2 (20 mL), Et$_3$N (0.63 mL, 4.50 mmol, 9.00 equiv.) and DMAP (0.009 g, 0.08 mmol, 0.15 equiv.) were added. The reaction mixture was cooled to 0 °C, treated with TPS-Cl (0.20 g, 0.66 mmol, 1.32 equiv.) and stirred for 10 min at 0 °C. The mixture was warmed to ambient temperature and stirred for 20 h. The reaction was quenched with conc. $NaHCO_3$ solution. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were dried with $MgSO_4$ and the solvent was removed under reduced pressure. The residue was dissolved in DMF and DIPEA (0.22 mL, 1.30 mmol, 2.60 equiv.) and 4-[(4-amino-2,2,6,6-tetramethylpiperidin-1-yl-oxy)methyl]-7-(diethylamino)-2*H*-chromen-2-one **10**[6] (0.26 g, 0.65 mmol, 1.30 equiv.) were added. The reaction mixture was stirred for 8 h at 85 °C, cooled to ambient temperature and stirred for 14 h. Subsequently the reaction was quenched with conc. $NaHCO_3$ solution, the organic layer was separated and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were dried with $MgSO_4$ and the solvent was removed under reduced pressure. Purification by silica gel chromatography (EtOAc/*c*-hexane/Et$_3$N, 60:40:1) gave the title compound **12** (0.35 g, 64 %) as a light-yellow foam. R_f = 0.22 (EtOAc/*c*-hexane, 3:2). ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 7.64 (d, *J* = 7.6 Hz, 1 H, N*H*), 7.60 (d, *J* = 7.6 Hz, 1 H, 6-H), 7.43 (d, *J* = 9.1 Hz, 1 H, 6-H), 7.40–7.38 (m, 2 H, Ar-H), 7.32 (t, *J* = 7.6 Hz, 2 H, Ar-H), 7.27–7.24 (m, 5 H, Ar-H), 6.90 (dd, *J* = 8.9, 1.6 Hz, 4 H, Ar-H), 6.68 (dd, *J* = 9.1, 2.4 Hz, 1 H, 6-H), 6.54 (d, *J* = 2.4 Hz, 1 H, 8-H), 6.08 (s, 1 H, 3-H), 5.94 (d, *J* = 4.3 Hz, 1 H, 1′H), 5.54 (d, *J* = 7.5 Hz, 1 H, 5-H), 5.06 (d, *J* = 6.1 Hz, 1 H, 3′OH), 5.01–4.96 (m, 4 H, OCH_2O, NOCH_2), 4.27–4.21 (m, 1 H, C*H*NH), 4.19–4.13 (m, 2 H, 2′H, 3′H), 3.97–3.94 (m, 1 H, 4′H), 3.74 (s, 6 H, OCH_3), 3.43 (q, *J* = 6.8 Hz, 4 H, CH_2CH$_3$), 3.27–3.20 (m, 2 H, 5′H, 5′′H), 1.81–1.79 (m, 2 H, C*H*HCH), 1.43–1.35 (m, 2 H, CH*H*CH), 1.23 (s, 6 H, CH_3), 1.20 (s, 6 H, CH_3), 1.12 (t, *J* = 7.0 Hz, 6 H, CH_3), 0.99–0.93 {m, 21 H, Si[CH(CH_3)$_2$]$_3$} ppm. ^{13}C NMR (125.8 MHz, [D$_6$]DMSO): δ = 163.1, 161.3, 158.6, 156.2, 155.4, 152.8, 150.8, 145.2, 140.5, 135.9, 135.8, 130.2, 128.4, 128.2, 127.2, 125.9, 113.7, 109.2, 105.9, 104.9, 97.3, 95.3, 88.9, 88.2, 86.3, 83.1, 78.6, 74.8, 69.2, 63.6, 60.3, 60.2, 55.5, 45.1, 45.0, 44.5, 41.2, 32.9, 29.1, 21.24, 21.16, 18.24, 18.16, 14.6, 12.8, 11.8 ppm. MS (MALDI): *m*/*z* = 1138.56 [M + Na$^+$]. HRMS (MALDI): calcd. for $C_{63}H_{85}N_5O_{11}SiNa$ [M + Na$^+$]: 1138.59071; found 1138.58911.

Cytidine Phosphoramidite with Protected Spin Label (3): To a solution of 2′-*O*-TOM-5′-*O*-DMT-U° **12** (3.67 g, 3.29 mmol, 1.00 equiv.) in CH_2Cl_2 (60 mL), Et$_3$N (2.31 mL, 16.44 mmol, 5.00 equiv.) and *N*,*N*-diisopropylaminocyanoethylphosphoramidic chloride (1.39 g, 5.89 mmol, 2.00 equiv.) were added. The reaction mixture was stirred for 21 h at ambient temperature. Subsequently, conc. $NaHCO_3$ solution was added and stirred for 5 min at ambient temperature. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2. The combined organic layers were dried with $MgSO_4$ and solvent was removed under reduced pressure. Purification by silica gel chromatography (EtOAc/*c*-hexane/Et$_3$N 70:30:1) gave the title compound **3** (4.10 g, 95 %) as a light-yellow foam. R_f = 0.48, 0.62 (EtOAc/*c*-hexane, 7:3). ^{1}H NMR (500 MHz, [D$_6$]DMSO): δ = 7.68–7.62 (m, 2 H, N*H*, 6-H), 7.43–7.37 (m, 3 H, Ar-H), 7.33–7.24 (m, 7 H, Ar-H), 6.90–6.86 (m, 4 H, Ar-H), 6.68 (dd, *J* = 9.0, 2.0 Hz, 1 H, 6-H), 6.53 (d, *J* = 2.1 Hz, 1 H, 8-H), 6.08 (s, 1 H, 3-H), 5.95–5.93 (m, 1 H, 1′H), 5.55 (d, *J* = 7.4 Hz, 1 H, 5-H), 5.00–4.93 (m, 4 H, OCH_2O, NOCH_2), 4.37–4.18 (m, 3 H, C*H*NH, 2′H, 3′H), 4.10–4.05 (m, 1 H, 4′H), 3.81–3.77 (m, 1 H, POC*H*H), 3.74, 3.73 (s, 6 H, OCH_3), 3.65–3.47 [m, 3 H, NC*H*(CH$_3$)$_2$, POCH*H*], 3.42 (q, *J* = 6.4 Hz, 4 H, CH_2CH$_3$), 3.37–3.32 (m, 1 H, 5′H), 3.25–3.17 (m, 1 H, 5′′H), 2.76–2.73 (m, 1 H, C*H*HCN), 2.65–2.57 (m, 1 H, CH*H*CN), 1.85–1.72 (m, 2 H, C*H*HCH), 1.39 (q, *J* = 12.5 Hz, 2 H, CH*H*CH), 1.22, 1.20 (2 × s, 12 H, CH_3), 1.13–1.01 [m, 18 H, CH$_2$CH_3, NCH(CH_3)$_2$], 0.99–0.93 {m, 21 H, Si[CH(CH_3)$_2$]$_3$} ppm. ^{13}C NMR (125.8 MHz, [D$_6$]DMSO): δ = 162.6, 160.8, 158.2, 155.8, 154.8, 152.3, 150.3, 144.6, 140.2, 135.2, 135.1, 129.7, 127.8, 127.7, 126.8, 125.5, 118.8, 118.7, 113.2, 108.7, 105.4, 104.5, 96.9, 95.1, 88.7, 88.5, 86.1, 86.0, 82.0, 81.8, 77.1, 74.3, 70.5, 63.0, 59.8, 58.7, 58.6, 58.1, 58.0, 55.0, 44.6, 44.5, 44.0, 42.5, 42.4, 40.7, 32.5, 24.22, 24.16, 20.8, 20.7, 19.7, 17.7, 14.1, 12.3, 11.4 ppm. ^{31}P NMR (202.5 MHz, [D$_6$]DMSO): δ = 149.1, 148.8 ppm. MS (MALDI): *m*/*z* = 1338.56 [M + Na$^+$]; calcd. for $C_{72}H_{102}N_7NaO_{12}PSi$ [M + Na$^+$] 1338.70.

Recovery of Spin Labels, EPR Spectroscopy: Buffer systems for deprotection: 10 mM phosphate buffer (NaH_2PO_4/Na_2HPO_4) for pH 4.6, 7.4, and 8.0; 50 mM carbonate buffer ($NaHCO_3$/K_2CO_3) for pH 8.5 and 9.0. Directly after irradiation, 20 µL of the aliquots were filled in quartz EPR tubes of 1 mm inner diameter. Continuous wave (cw) EPR measurements were performed at X-band frequency (9.54 GHz) with a Bruker E500 spectrometer equipped with a TE102 cavity. Experimental parameters: 100 kHz modulation frequency, 0.1 mT modulation amplitude, 0.2 mW microwave power, 40.96 ms time constant, 40.96 ms conversion time, 1024 points, 7 mT sweep width, 20 scans. For PELDOR measurement 20 µL of sample volume with 80 % buffer (pH 8.5)/20 % d_8-glycerol was transferred into 1.6 mm outer diameter quartz EPR tubes (Suprasil, WilmadLabGlass) directly after irradiation and annealing. Pulsed experiments at Q-band frequencies (33.7 GHz) were performed with an ELEXSYS SuperQ-FT accessory unit, a continuous-flow helium cryostat (CF935, Oxford Instruments), a temperature control system (ITC 502, Oxford Instruments), and a Bruker AmpQ 10 W amplifier with a Bruker EN5107D2 cavity at 50 K. Pulse lengths were 32 ns (π/2 and π) for the observer pulses and 20 ns (π) for the pump pulse. The pump pulse frequency was set to the maximum of the echo-detected field swept spectrum and the observer pulses were set 70 MHz lower. For PELDOR experiments, the dead-time free four-

pulse sequence with phase-cycled π/2-pulse was used.[21] Primary experimental data were background-corrected by fitting an exponential decay function for division of the intermolecular contributions. The resulting form factors $F(t)$ were fitted with Tikhonov regularization to obtain distance distributions with the DeerAnalysis2013 software package.[15]

Supporting Information (see footnote on the first page of this article): Additional preparative details and characterization data. Synthesis, analysis, and photochemical deprotection of RNA **13** and **16**. HPLC conditions to determine nitroxide recovery from RNA **13** and **16**. ^{1}H and ^{13}C NMR spectra of all key intermediates and final products, RNA stacking model, CW EPR data, raw PELDOR trace.

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Keywords: DEER spectroscopy · EPR spectroscopy · PELDOR spectroscopy · Photochemistry · Nucleosides · Spin-labeled RNA

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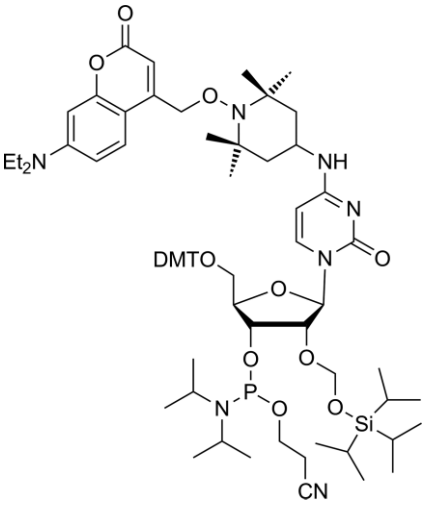
Spin-Labeled RNA



A multigram synthesis of a cytidine phosphoramidite, with a photolabile group on the nitroxide label, is described. No special conditions for RNA-strand assembly are required. After photochemical removal of the coumarin, spontaneous oxidation regenerates the nitroxide in good yield. PELDOR (pulsed electron-electron double resonance) experiments can be run after irradiation without further purification.

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