CHEMISTRY
A European Journal

A Journal of
ChemPubSoc Europe

Accepted Article

Title: A Cytidine Phosphoramidite with Protected Nitroxide Spin Label: Synthesis of a Full-Length TAR RNA and Investigation by in-line Probing and EPR Spectroscopy.

Authors: Timo Weinrich, Eva A. Jaumann, Ute Scheffer, Thomas F. Prisner, and Michael W. Göbel

To be cited as: *Chem. Eur. J.* 10.1002/chem.201800167

Link to VoR: http://dx.doi.org/10.1002/chem.201800167

Supported by
ACES

WILEY-VCH

10.1002/chem.201800167

WILEY-VCH

A Cytidine Phosphoramidite with Protected Nitroxide Spin Label: Synthesis of a Full-Length TAR RNA and Investigation by in-line Probing and EPR Spectroscopy.

Timo Weinrich,[a] Eva A. Jaumann,[b] Ute Scheffer,[a] Thomas F. Prisner,[b] and Michael W. Göbel*[a]

Abstract: EPR studies on RNA are complicated by three major obstacles related to the chemical nature of nitroxide spin labels: Decomposition while oligonucleotides are chemically synthesized, further decay during enzymatic strand ligation and undetected changes in conformational equilibria by the steric demand of the label. Here we present possible solutions for all three problems: A 2-nitrobenzyloxymethyl protective group for nitroxides, stable under all conditions of chemical RNA synthesis that can be removed photochemically. By careful selection of ligation sites and splint oligonucleotides high yields are achieved in the assembly of a full-length HIV-1 TAR RNA labeled with two protected nitroxides. PELDOR measurements of spin labeled TAR in the absence and presence of arginine amide indicate an arrest of interhelical motions upon ligand binding. Finally, even minor changes in sample conformation due to the presence of spin labels are detected with high sensitivity by in-line probing.

Introduction

The analysis of RNA structure and dynamics by EPR spectroscopy [1-6] requires labeling of the samples with paramagnetic species. This is normally achieved by covalent attachment of persistent nitroxide radicals [7-34] although noncovalent labeling has become an interesting alternative.[35,36] Due to the limited stability of nitroxides, postsynthetic attachment has some advantages. Alkylation of 2'-amines,[7-11,20] of 4-thiouridine,[14,30-32] and of thio-phosphates [13,15-19,32] are typical approaches. Other methods include Sonogashira reactions,[21-24] substitutions at convertible nucleotides [26,27] and 1,3-dipolar cycloadditions.[29] Most elegant in terms of synthesis is the direct introduction by DNA [37-48] and RNA [12] phosphoramidites already containing the nitroxide moiety. However, decomposition of nitroxides may occur during oligonucleotide assembly or strand ligation unless specific precautions are taken.[41,42] For spectroscopic reasons, rigid linker structures are desirable. Cytidine analog **1** [21,37] for example is well suited for pulsed electron-electron double resonance experiments (PELDOR or DEER, Figure 1). The nitroxide part is introduced postsynthetically by Sonogashira coupling.[21] Sigurdsson's nitroxides **2** [41] and **3**,[12] even more rigid than **1**, can be incorporated into DNA or RNA as phosphoramidites when modified synthetic conditions are used to limit decomposition of the radical.

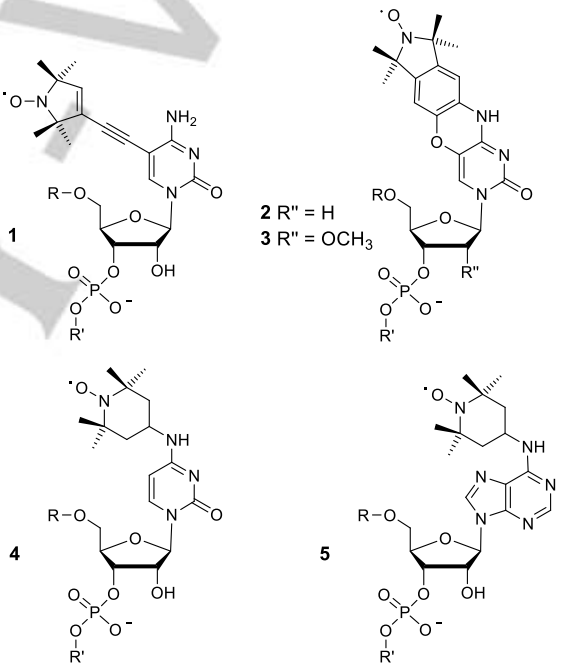


Figure 1. Some nitroxide labeled ribonucleotides used for EPR studies of RNA.

PELDOR experiments [49,50] on oligonucleotides labeled with **2** or **3** not only detect the distance between the nitroxides but also their relative orientation.[51] Starting from convertible nucleotides, Höbartner has introduced a method for the postsynthetic generation of nitroxides **4** and **5**.[26] She also suggested a ligation technique based on deoxyribozymes thus avoiding nitroxide reduction by thiols.[27] Addition of thiols normally is required to keep ligase proteins active. Although spin labels **4** and **5** are less rigid when compared to **2** or **3** and also cause some duplex destabilization, successful PELDOR studies have been published.[5,26] Furthermore, if some decomposition is accepted, deoxynucleoside analogs of nitroxides **4** and **5** can be incorporated into DNA strands by specifically adapted phosphoramidite chemistry.[40,43,46]

[a] T. Weinrich, Dr. U. Scheffer, Prof. M. W. Göbel
Institute for Organic Chemistry and Chemical Biology
Goethe University Frankfurt
Max-von-Laue-Str. 7, D-60438 Frankfurt am Main, Germany.
E-mail: M.Goebel@chemie.uni-frankfurt.de

[b] E. A. Jaumann, Prof. T. F. Prisner
Institute for Physical and Theoretical Chemistry
Goethe University Frankfurt
Max-von-Laue-Str. 7, D-60438 Frankfurt am Main, Germany.

Supporting information for this article is given via a link at the end of the document.

To provide a more general solution to the problem of nitroxide instability we have developed a protection strategy that is based on the alkylation of hydroxylamines with light sensitive groups (Figure 2). A phosphoramidite building block related to **6** could be incorporated into DNA using standard conditions without destruction of the protected label. After photolysis of the coumarin group, the nitroxide radical was formed by spontaneous air oxidation.[52] More recently, we have optimized the synthesis and adapted it to ribonucleotides. Using phosphoramidite **6** we prepared a short palindromic RNA labeled with two protected hydroxylamines. Irradiation of the sample under air then recovered the nitroxides. No further purification was required for subsequent PELDOR analysis.[53] However, the conversion of protected hydroxylamines into nitroxides depends on pH. In neutral or slightly acidic media N-O cleavage is the dominating process leading to amines instead of nitroxides. At pH 8.5 the radical prevails but still 15 % of the material is permanently reduced.[53] Our strategy for minimizing the loss of nitroxides was then to separate the light sensitive group and the N-O bond by a short additional spacer. First trials with 2-nitrobenzyloxycarbonyl failed. This group is cleaved off during nucleobase deprotection by ammonia. We therefore synthesized amidite **7**. The acetal linker should be resistant against nucleophiles but not against strong acids. However, 2-nitrobenzyloxymethyl (NBOM) has been already used to protect 2'-hydroxy groups in RNA chemistry.[54,55] It withstands the conditions of solid phase synthesis and can be removed without causing photochemical damage to the RNA strands.[55,56]

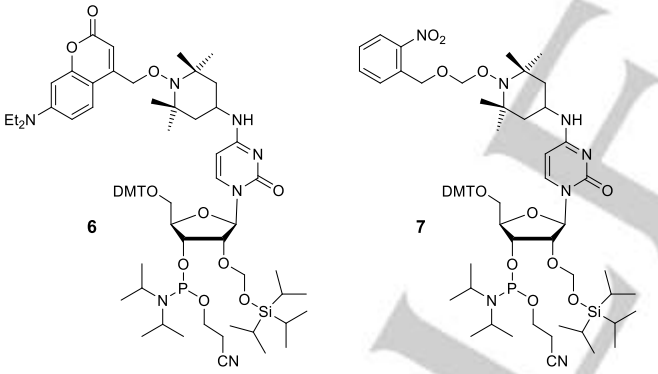
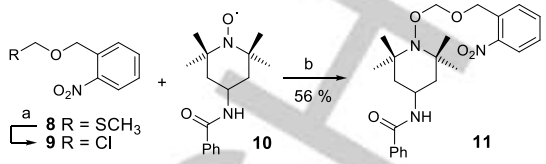


Figure 2. Cytidine phosphoramidites with a protected TEMPO spin label.

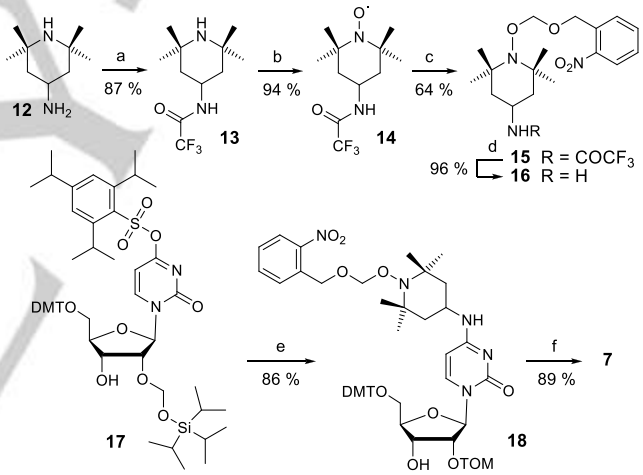
Results and Discussion

To test the stability of the NBOM protected hydroxylamine, TEMPO derivative **10** was coupled with **9** in a copper mediated reduction.[57,58] In this process, radicals obtained from **9** recombine with the nitroxide forming the *O*-alkylated product **11**. Compound **9** is accessible from 2-nitrobenzyl alcohol by Pummerer rearrangement (**8**) and chlorination (Scheme 1).[59,60] Subsequently compound **11** was challenged by reagents typical for oligonucleotide synthesis and ligation: ammonia, iodine, triethylamine trihydrofluoride, dithiothreitol and trifluoroacetic acid. Compound **11** proved to be sufficiently stable (see page S6).



Scheme 1. Preparation of model compound **11**. a) **8**, SO_2Cl_2, 1 h, rt; b) **9**, **10**, Cu, $Cu(OTf)_2$, 4,4´-dimethyl-2,2´-bipyridyl, toluene, 20 h, reflux.

Trifluoroacetylation of **12** to give **13**, followed by oxidation (**14**) and reductive alkylation led to intermediate **15**. After removal of trifluoroacetyl with KOH, amine **16** reacted with the *O*-activated uridine derivative **17** [27,61] in the presence of an unprotected 3'-hydroxy group. Phosphitylation of product **17** then led to multi-gram amounts of amidite **7** in good yield (Scheme 2). The 2'-TOM protection has been chosen to eliminate the risk of 2',3'-silyl shifts and to enable the synthesis of longer RNA strands in future work.



Scheme 2. Synthesis of phosphoramidite **7**. a) Trifluoroacetic anhydride, pyridine; b) Na_2WO_4, H_2O_2; c) **9**, Cu, $Cu(OTf)_2$, 4,4´-dimethyl-2,2´-bipyridyl, toluene, 20 h, reflux; d) KOH, MeOH; e) **16**, diisopropylethylamine, DMF, 80 °C f) Et_3N, *N,N*-diisopropylaminocyanoethylphosphoramidic chloride.

The self complementary RNA oligonucleotides **19** and **22** were prepared from phosphoramidite **7** and commercial 2'-O-TBS protected amidites by standard solid phase synthesis (Figure 3). According to the trityl assay, compound **7** reacted well and normal yields of RNA could be isolated by HPLC. The integrity of the protected TEMPO groups was verified by mass spectrometry. Photochemical removal at 365 nm in aqueous buffer (at pH 4.2, 7.4, 8.0, 8.5) was complete after 20 min. At any pH value tested, only traces of the reduction products **21** and **24** could be detected (see page S10). However, a strong new peak appeared in each case in addition to the nitroxides **20** and **23**. This peak did not occur when **20** was synthesized from

amidite **6**.[53] The products are slowly converted into **20** and **23** at pH 7.0, faster at pH 8.5 and disappeared almost completely upon heating to 90 °C for 60 min (see page S11). We assign them to the hemiacetals **19a** and **22a**.

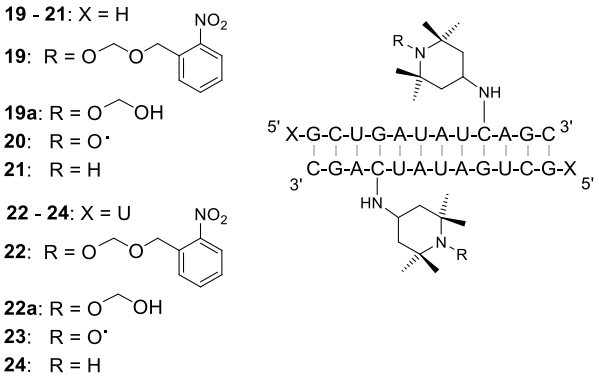


Figure 3. Spin labeled RNA palindromes **20** and **23** bearing a nitroxide on position 9 or 10.

For PELDOR experiments, samples of RNAs **19** or **22** were irradiated at pH 7.4, annealed to form the palindromic duplexes **20** or **23** and used directly for EPR spectroscopy without any further purification (see page S12). The labeling efficiency of **20** was 95 % according to HPLC and 90 % as determined by EPR. Consistent with our previous finding, both experiments showed a main distance of 1.8 nm.[53] In case of RNA **20**, end-to-end stacking of the duplexes leads to a small population of longer distances.[53] To preclude this effect, an extra uridine residue was attached to RNA **22**. The PELDOR data after photochemical deprotection (**23**) now shows a single and well defined distance (Figure 4).

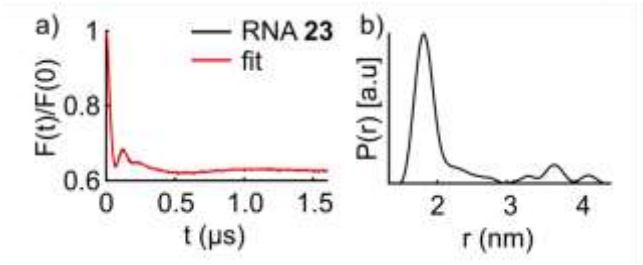


Figure 4. PELDOR measurement of RNA **23** (black). a) PELDOR time trace after background correction (original data see page S20) and fit with Tikhonov regularization (red) by using DeerAnalysis.[62] b) PELDOR distance distribution with a dominant intramolecular value ~1.8 nm.

To demonstrate the stability of the protective group under conditions of enzymatic RNA ligation, we assembled a 59mer full-length TAR RNA (Figure 5) from three RNA fragments (Figure 6). The non-labeled analog **25** was also prepared for comparison. Covalent coupling of fragments by ligase proteins or by deoxyribozymes [27] is a successful strategy to prepare samples of long RNAs which can be studied by NMR [63] or EPR [31,64] spectroscopy or by a combination of both.[30]

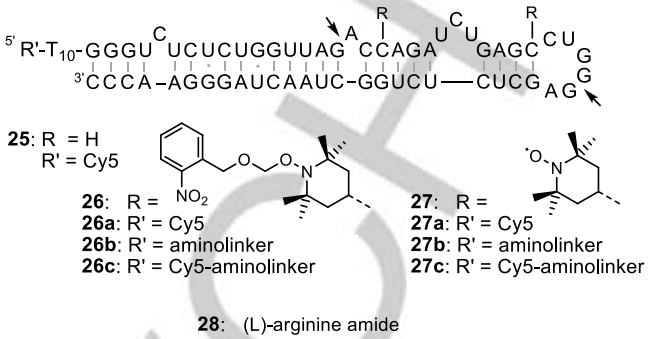


Figure 5. Structure of TAR oligonucleotides **25** – **27**. Spin labels are in positions 19 and 29. Arrows point to ligation sites. The fluorescent dye enables in-line probing by a DNA sequencer.

After chemical synthesis of RNA fragment **30a** from phosphoramidite **7** and successful ligation (see below), the 59mer RNA **26b** was photochemically deprotected to yield the twofold spin labeled TAR RNA **27b**, finally studied by EPR in the absence and presence of arginine amide **28**.

RNA **29**: 5'Cy5-T_{10}-GGGUCUCUCUGGUUAG3'

RNA **29a**: 5'$_{aminolinker}$-T_{10}-GGGUCUCUCUGGUUAG3'

RNA **30**: 5'pACCAGAUCUGAGCCUGG3'

RNA **30a**: 5'pAC**C**AGAUCUGAG**C**CUGG3'

RNA **31**: 5'pGAGCUCUCUGGCUAACUAGGGAACCC3'

Splint DNA **32**: 3'GAGAGACCAATCTGGTCTAG5'

Splint DNA **33**: 3'ACTCGGACCCTCGAGAGACC5'

Splint DNA **34**: 3'GATTGATCCCTTGGG5'

Figure 6. Sequences of RNA and of splint oligonucleotides used to assemble RNAs **25** - **27**. p stands for a 5' phosphate. Positions of the protected TEMPO label are shown in bold letters.

For ligation of RNA **25** we used T4 RNA ligase 2 [64] in a buffer containing dithiothreitol, the dye-labeled RNA **29** and the two 5' phosphorylated fragments **30** and **31** (molar ratio 1:1:1). Ligation sites between two purines were chosen for optimized stacking of the fragments (see arrows in Figure 5). To guide the ligation and to prevent unfavorable backfolding of RNA, we also added DNA splint oligonucleotides (see page S17). Best results were obtained in the presence of three splints **32**, **33**, and **34** (1.5 equiv. in each case) leading to about 80 % ligation yield of **25** that was purified further by gel electrophoresis. When RNA **29**, the TEMPO modified fragment **30a** (1.5 eq.), and **31** were ligated by the three-splint approach, up to 80 % analytical yield of the full-length RNA **26a** could be observed. The steric demand of the protected TEMPO moieties seems not to impede

the reaction. Dye labeling helps to optimize the ligation protocol. However, it interferes with photochemical deprotection. For PELDOR experiments we therefore chose RNA **26b** lacking the fluorescent dye. It was assembled in the three-splint ligation by replacing RNA fragment **29** by **29a**. Material used for PELDOR was purified by gel electrophoresis (ligation scale: 40 nmol; 27-46 % isolated yield) and RP-HPLC. Light induced removal of nitrobenzyl then occurred without problems. However, when hemiacetal cleavage was enforced by heating to 90 °C for 60 min, product **27b** partially hydrolyzed. This problem could be minimized by heating to 70 °C for 5 h and to 90 °C for only 10 min (pH 5.5-6.0). To check the conformational integrity of the RNA sample by in-line probing,[65,66] an aliquot of **27b** was converted into dye-labeled **27c** by reaction of the amino linker with an active ester of Cy5.

U23 and C24 of the UCU bulge in the ligand-free TAR RNA are known from NMR studies to form a continuous stack with their neighbors.[67] The two additional bases in one half of the duplex cause a bend (~50 °) between the upper and lower helical axes [67-69] and allow for large inter-helical motions.[70] Upon binding of arginine amide or Tat-derived peptides, the unpaired bulge nucleotides are expelled from the stack and an almost straight and rigid helix results,[68-71] where U23 is brought into close proximity to A27.[72] Consistent with these findings, the PELDOR experiment with ligand-free RNA **27b** shows a broad distance distribution around 2.7 ± 0.4 nm, reflecting a dynamic structure (Figure 7a). In the presence of arginine amide, however, the structure becomes more ordered and shows a narrow distance distribution of 2.3 ± 0.2 nm (Figure 7b). The corresponding data taken from the NMR structure of the TAR-arginine complex (1ARJ) [72] agrees well with the PELDOR result.

The influence of the spin labels on local conformation has been studied by in-line probing,[65] a method leading to partial RNA cleavage specifically in sites of enhanced conformational mobility. When applied to the Cy5 labeled RNA **25**,[66] the single nucleotide bulges at C5, A17 and the hexaloop (C30-A35) show the expected number of signals (Figure 8a). However, four peaks are visible for the bulge U23-U25. The additional peak is caused by the well known lability of the base pair A22/U40.[67,73] Figure 8b shows in-line probing of RNA **26c** containing two protected spin labels on C19 and C29. One of the former single nucleotide bulges now opens up to a three nucleotide bulge A17, C18, and C19 (black arrows in Figure 8b). Note that the spacing between C18 and C19 is twice as usual due to the reduced mobility of fragments containing the spin label. Furthermore, four extra peaks appear between U25 and C29 (red arrows) giving direct evidence for a destabilization of the upper stem region. Much less structural perturbation is seen in the deprotected RNA **27c** (Figure 8c). The pattern is very similar to that of the unmodified RNA. Only the base pair C18/G44 seems to be slightly destabilized.

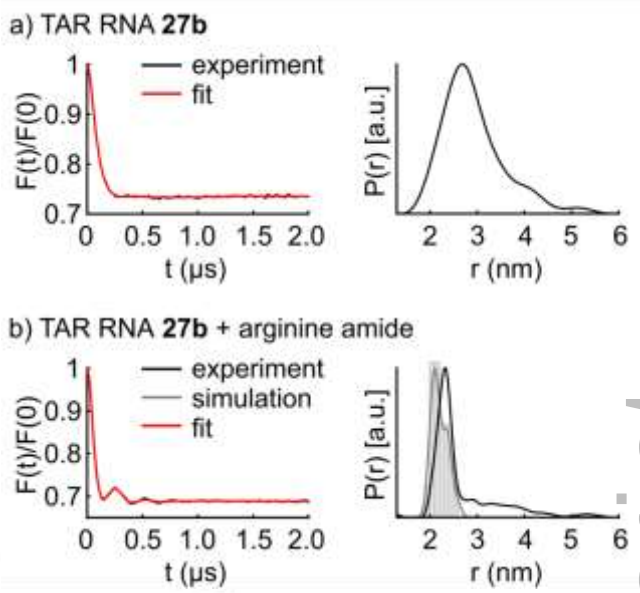


Figure 7. PELDOR measurements of TAR RNA **27b** a) without and b) with arginine amide. PELDOR time traces after background correction (original data see page S21) and fit with Tikhonov regularization (red) by using DeerAnalysis.[62] a) TAR RNA **27b** without any ligand bound shows a broad distance distribution of 2.7 ± 0.4 nm. b) In presence of arginine amide, the distance distribution of TAR RNA **27b** becomes narrow and the mean distance slightly shorter with 2.3 ± 0.2 nm. The distance compilation based on the arginine-bound TAR RNA NMR structure (1ARJ) [72] is shown in grey.

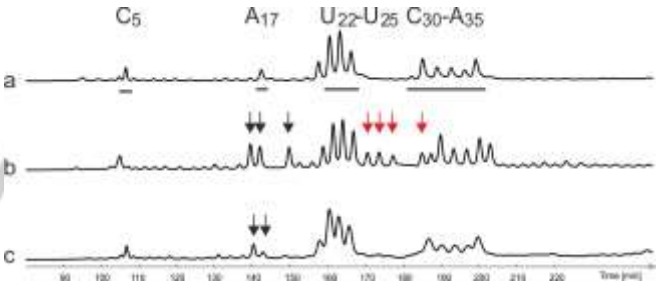


Figure 8. Secondary structure analysis by in-line probing. This technique is based on Mg^{2+} induced partial hydrolysis of 5' dye-labeled RNA in positions of unrestricted conformational mobility such as single strands, loops and bulges. The resulting fragments are separated and visualized by gel electrophoresis in a DNA sequencer (see page S19). a) Unmodified TAR RNA **25**. b) RNA **26c** modified by protected spin labels on C19 and C29. c) Deprotected RNA **27c**.

Conclusions

Phosphoramidite **7** is accessible in good yield and in gram quantities. In oligonucleotide solid phase synthesis, it behaves like a normal 2'-O-TOM protected building block. No special reaction conditions are required because the NBOM group is stable during all steps of chain assembly, RNA deprotection and enzymatic ligation. In contrast to coumarin groups we have used earlier in amidite **6**, photochemical removal of NBOM does not lead to N-O cleavage, not even at acidic pH. Short RNA samples such as **20** and **23** are ready for PELDOR experiments after

irradiation in the presence of air and after annealing. A minor disadvantage is the stability of the hemiacetal intermediate. The need to heat up the sample for liberating the nitroxide might become a critical step when long RNA targets (e.g. 300mers) are envisioned.

TAR, the 59mer *trans*-activation response element of HIV-1, belongs to the best characterized RNAs known today. TAR and related model oligonucleotides have been studied by a plethora of biochemical and spectroscopic techniques including NMR,[67,70-72,74] cw-EPR [9] and PELDOR.[8,75] It is not too complex and forms a stable stem-loop structure. Therefore, TAR is a good test case for evaluating our approach of combining EPR with methods of secondary structure mapping. Fragment coupling with T4 RNA ligase 2 [64] was effective in producing spin labeled TAR samples of high purity in yields of 0.25-0.41 mg per batch. The results of PELDOR experiments fully agree with the known structure and dynamics of TAR and its complex with arginine amide. It should be noted, however, that the TEMPO label present in amidite **7** is inferior to more rigid building blocks such as **1** or **3**. Furthermore, the TEMPO label is known to decrease the melting point of RNA helices.[26] While thermal denaturation studies demonstrate the global impact of modifications on RNA secondary structure, the local effects are invisible. Local perturbations induced by spin labels, on the other hand, can have misleading effects on EPR data interpretation. Such local disturbance can be visualized by in-line probing.[65] The presence of protected spin labels in RNA **26c**, for example, opens up the A17 bulge and destabilizes the upper stem. After removal of NBOM, these effects largely disappear. Such controls are highly recommendable for EPR studies on more complex RNAs when delicate conformational equilibria may be severely disturbed by the introduction of spin labels. Furthermore, electrophoretic analysis of dye-labeled RNAs such as **27c** is a sensitive method to detect even minor levels of hydrolytic degradation. Having established our strategy of nitroxide protection, fragment ligation and in-line probing, it seems worthwhile to extend it on advanced spin labels applied to challenging cases of RNA protein interactions. Apart from pure EPR studies, long range distances derived from PELDOR experiments can provide valuable complementary data for structure determination by NMR.[30,31] Spin labeled RNA samples are also required for NMR studies using paramagnetic relaxation enhancement.[76] Although, compared to PELDOR, 5-10fold larger sample quantities are required, upscaling of the methods described here would not be unfeasible.

Acknowledgements

Financial support by the Deutsche Forschungsgemeinschaft (DFG) (collaborative research center 902) and the International Max Planck Research School (IMPRS) of the MPI of Biophysics in Frankfurt is gratefully acknowledged.

Keywords: enzymatic ligation of RNA • PELDOR spectroscopy • photolabile protection • secondary structure mapping • TEMPO

[1] A. J. Fielding, M. G. Concilio, G. Heaven, M. A. Hollas, *Molecules* **2014**, *19*, 16998-17025.
[2] B. Endeward, A. Marko, V. P. Denysenkov, S. T. Sigurdsson, T. F. Prisner, *Methods in Enzymology* **2015**, *564*, 403-425.
[3] T. F. Prisner, A. Marko, S. T. Sigurdsson, *J. Magn. Res.* **2015**, *252*, 187-198.
[4] S. A. Shelke, S. T. Sigurdsson, *Eur. J. Org. Chem.* **2012**, 2291-2301.
[5] K. Halbmair, J. Seikowski, I. Tkach, C. Höbartner, D. Sezer, M. Bennati, *Chem. Sci.* **2016**, *7*, 3172-3180.
[6] C. Gmeiner, G. Dorn, F. H. T. Allain, G. Jeschke, M. Yulikov, *Phys. Chem. Chem. Phys*. **2017**, *19*, 28360-28380.
[7] M. K. Bowman, A. G. Maryasov, N. Kim, V. J. DeRose, *Appl. Magn. Reson.* **2004**, *26*, 23-39.
[8] N.-K. Kim, A. Murali, V. J. DeRose, *Chem. Biol.* **2004**, *11*, 939-948.
[9] T. E. Edwards, T. M. Okonogi, B. H. Robinson, S. T. Sigurdsson, *J. Am. Chem. Soc.* **2001**, *123*, 1527-1528.
[10] T. E. Edwards, S. T. Sigurdsson, *Nat. Protoc.* **2007**, *2*, 1954-1962.
[11] S. Saha, A. P. Jagtap, S. T. Sigurdsson, *Chem. Commun*. **2015**, *51*, 13142-13145.
[12] C. Höbartner, G. Sicoli, F. Wachowius, D. B. Gophane, S. T. Sigurdsson, *J. Org. Chem.* **2012**, *77*, 7749-7754.
[13] P. Z. Qin, S. E. Butcher, J. Feigon, W. L. Hubbell, *Biochemistry* **2001**, *40*, 6929-6936.
[14] P. Z. Qin, K. Hideg, J. Feigon, W. L. Hubbell, *Biochemistry* **2003**, *42*, 6772-6783.
[15] Q. Cai, A. K. Kusnetzow, W. L. Hubbell, I. S. Haworth, G. P. C. Gacho, N. Van Eps, K. Hideg, E. J. Chambers, P. Z. Qin, *Nucl. Acids Res*. **2006**, *34*, 4722-4730.
[16] Q. Cai, A. K. Kusnetzow, K. Hideg, E. A. Price, I. S. Haworth, P. Z. Qin, *Biophys. J.* **2007**, *93*, 2110-2117.
[17] G. P. G. Grant, N. Boyd, D. Herschlag, P. Z. Qin, *J. Am. Chem. Soc.* **2009**, *131*, 3136-3137.
[18] X. Zhang, C.-S. Tung, G. Z. Sowa, M. M. Hatmal, I. S. Haworth, P. Z. Qin, *J. Am. Chem. Soc.* **2012**, *134*, 2644-2652.
[19] P. Nguyen, X. Shi, S. T. Sigurdsson, D. Herschlag, P. Z. Qin, *ChemBioChem* **2013**, *14*, 1720-1723.
[20] O. Schiemann, A. Weber, T. E. Edwards, T. F. Prisner, S. T. Sigurdsson, *J. Am. Chem. Soc*. **2003**, *125*, 3434-3435.
[21] O. Schiemann, N. Piton, J. Plackmeyer, B. E. Bode, T. F. Prisner, J. W. Engels, *Nat. Protoc.* **2007**, *2*, 904-923.
[22] N. Piton, Y. Mu, G. Stock, T. F. Prisner, O. Schiemann, J. W. Engels, *Nucl. Acids Res*. **2007**, *35*, 3128-3143.
[23] I. Krstić, O. Frolow, D. Sezer, B. Endeward, J. E. Weigand, B. Suess, J. W. Engels, T. F. Prisner, *J. Am. Chem. Soc*. **2010**, *132*, 1454-1455.
[24] I. Krstić, R. Hänsel, O. Romainczyk, J. W. Engels, V. Dötsch, T. F. Prisner, *Angew. Chem. Int. Ed.* **2011**, *50*, 5070-5074.
[25] C. M. Grytz, A. Marko, P. Cekan, S. T. Sigurdsson, T. F. Prisner, *Phys. Chem. Chem. Phys.* **2016**, *18*, 2993-3002.
[26] G. Sicoli,F. Wachowius, M. Bennati, C. Höbartner, *Angew. Chem. Int. Ed.* **2010**, *49*, 6443-6447.
[27] L. Büttner, J. Seikowski, K. Wawrzyniak, A. L. Ochmann, C. Höbartner, *Bioorg. Med. Chem*. **2013**, *21*, 6171-6180.
[28] I. Tkach, S. Pornsuwan, C. Höbartner, F. Wachowius, S. T. Sigurdsson, T. Y. Baranova, U. Diederichsen, G. Sicoli, M. Bennati, *Phys. Chem. Chem. Phys*. **2013**, *15*, 3433-3437.
[29] M. Kerzhner, D. Abdullin, J. Wiecek, H. Matsuoka, G. Hagelueken, O. Schiemann, M. Famulok, *Chem. Eur. J.* **2016**, *22*, 12113-12121.
[30] O. Duss, E. Michel, M. Yulikov, M. Schubert, G. Jeschke, F. H.-T. Allain, *Nature* **2014**, *509*, 588-592.
[31] O. Duss, M. Yulikov, G. Jeschke, F. H.-T. Allain, *Nat. Commun*. **2014**, *5*, 3669-3677.
[32] J. M. Esquiaqui, E. M. Sherman, J.-D. Ye, G. E. Fanucci, *Biochemistry* **2016**, *55*, 4295-4305.
[33] E. S. Babaylova, A. V. Ivanov, A. A Malygin, M. A. Vorobjeva, A. G. Venyaminova, Y. F. Polienko, I. A. Kirilyuk, O. A. Krumkacheva, M. V.

Fedin, G. G. Karpova, E. G. Bagryanskaya, *Org. Biomol. Chem*. **2014**, *12*, 3129-3136.

[34] E. S. Babaylova, A. A. Malygin, A. A. Lomzov, D. V. Pyshnyi, M. Yulikov, G. Jeschke, O. A. Krumkacheva, M. V. Fedin, G. G. Karpova, E. G. Bagryanskaya, *Nucl. Acids Res*. **2016**, *44*, 7935-7943.

[35] S. A. Shelke, G. B. Sandholt, S. T. Sigurdsson, *Org. Biomol. Chem.* **2014**, *12*, 7366-7374.

[36] N. R. Kamble, M. Gränz, T. F. Prisner, S. T. Sigurdsson, *Chem. Commun*. **2016**, *52*, 14442-14445.

[37] A. Spaltenstein, B. H. Robinson, P. B. Hopkins, *J. Am. Chem. Soc.* **1988**, *110*, 1299-1301.

[38] R. A. Persichetti, R. R. Sinden, J.-L. Duh, A. M. Bobst, *Synth. Commun*. **1991**, *21*, 1013-1019.

[39] T. R. Miller, S. C. Alley, A. W. Reese, M. S. Solomon, W. V. McCallister, C. Mailer, B. H. Robinson, P. B. Hopkins, *J. Am. Chem. Soc.* **1995**, *117*, 9377-9378.

[40] C. Giordano, F. Fratini, D. Attanasio, L. Cellai, *Synthesis* **2001**, 565-572.

[41] N. Barhate, P. Cekan, A. P. Massey, S. T. Sigurdsson, *Angew. Chem. Int. Ed.* **2007**, *46*, 2655-2658.

[42] P. Cekan, A. L. Smith, N. Barhate, B. H. Robinson, S. T. Sigurdsson, *Nucleic Acids Res*. **2008**, *36*, 5946-5954.

[43] P. Cekan, S. T. Sigurdsson, *J. Am. Chem. Soc*. **2009**, *131*, 18054-18056.

[44] D. P. Gophane, S. T. Sigurdsson, *Chem. Commun.* **2013**, *49*, 999-1001.

[45] D. P. Gophane, B. Endeward, T. F. Prisner, S. T. Sigurdsson, *Chem. Eur. J*. **2014**, *20*, 15913-15919.

[46] D. B. Gophane, S. T. Sigurdsson, *Beilstein J. Org. Chem.* **2015**, *11*, 219-227.

[47] M. Azarkh, V. Singh, O. Okle, I. T. Seemann, D. R. Dietrich, J. S. Hartig, M. Drescher, *Nat. Protoc*. **2013**, *8*, 131-147.

[48] V. Singh, M. Azarkh, T. E. Exner, J. S. Hartig, M. Drescher, *Angew. Chem. Int. Ed*. **2009**, *48*, 9728-3730.

[49] G. Jeschke, *Annu. Rev. Phys. Chem*. **2012**, *63*, 419-446.

[50] O. Schiemann, T. F. Prisner, *Quart. Rev. Biophys*. **2007**, *40*, 1-53.

[51] O. Schiemann, P. Cekan, D. Margraf, T. F. Prisner, S. T. Sigurdsson, *Angew. Chem. Int. Ed*. **2009**, *48*, 3292-3295.

[52] I. Seven, T. Weinrich, M. Gränz, C. Grünewald, S. Brüß, I. Krstić, T. F. Prisner, A. Heckel, M. W. Göbel, *Eur. J. Org. Chem.* **2014**, 4037-4043.

[53] T. Weinrich, M. Gränz, C. Grünewald, T. F. Prisner, M. W. Göbel, *Eur. J. Org. Chem.* **2017**, 491-496.

[54] M. E. Schwartz, R. R. Breaker, G. T. Asteriadis, J. S. deBear, G. R. Gough, *Bioorg. Med. Chem. Lett*., **1992**, *2*, 1019-1024.

[55] S. Pitsch, *Helv. Chim. Acta*, **1997**, *80*, 2286-2314.

[56] C. Brieke, F. Rohrbach, A. Gottschalk, G. Mayer, A. Heckel, *Angew. Chem. Int. Ed.* **2012**, *51*, 8446-8476.

[57] M. Kamigaito, T. Ando, M. Sawamoto, *Chem. Rev*. **2001**, *101*, 3689-3745.

[58] K. Molawi, A. Studer, *Chem. Commun*. **2007**, 5173-5175.

[59] P. K. Yong, A. Banerjee, *Org. Lett*. **2005**, *7*, 2485-2487.

[60] T. Benneche, P. Strande, K. Undheim, *Synthesis* **1983**, 762-763.

[61] S. Pitsch, P. A. Weiss, L. Jenny, A. Stutz, X. Wu, *Helv. Chim. Acta* **2001**, *84*, 3773-3795.

[62] G. Jeschke, V. Chechik, P. Ionita, A. Godt, H. Zimmermann, J. Banham, C. R. Timmel, D. Hilger, H. Jung. *Appl. Magn. Reson.* **2006**, *30*, 473-498.

[63] O. Duss, C. Maris, C. von Schroetter, F. H.-T. Allain, *Nucl.Acids Res*. **2010**, *38*, e188.

[64] I. Lebars, B. Vileno, S. Bourbigot, P. Turek, P. Wolff, B. Kieffer, *Nucl. Acids Res*. **2014**, *42*, e117.

[65] E. E. Regulski, R. R. Breaker *Methods Mol. Biol*. **2008**, *419*, 53-67.

[66] B. Strauss, A. Nierth, M. Singer, A. Jäschke, *Nucl. Acids Res*. **2012**, *40*, 861-870.

[67] F. Aboul-ela, J. Karn, G. Varani, *Nucleic Acids Res.* **1996**, *24*, 3974-3981.

[68] F. A. Riordan, A. Bhattacharyya, S. McAteer, D. M. J. Lilley, *J. Mol. Biol*. **1992**, *226*, 305-310.

[69] M. Zacharias, P. J. Hagerman, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 6052-6056.

[70] H. M. Al-Hashimi, Y. Gosser, A. Gorin, W. Hu, A. Majumdar D. J. Patel, *J. Mol. Biol*. **2002**, *315*, 95-102.

[71] S. W. Pitt, A. Majumdar, A. Serganov, D. J. Patel, H. M. Al-Hashimi, *J. Mol. Biol*. **2004**, *338*, 7-16.

[72] F. Aboul-ela, J. Karn, G. Varani, *J. Mol. Biol*. **1995**, *253*, 313-332.

[73] R. A. Colvin, M. A. Garcia-Blanco, *J. Virol*. **1992**, *66*, 930-935.

[74] C. H. Wunderlich, R. G. Huber, R. Spitzer, K. R. Liedl, K. Kloiber, C. Kreutz, *ACS Chem. Biol*. **2013**, *8*, 2697-2706.

[75] Y. Sun, P. P. Borbat, V. M. Grigoryants, W. K. Myers, J. H. Freed, C. P. Scholes, *Biophys. J.* **2015**, *108*, 893-902.

[76] K. A. Schnorr, D. B. Gophane, C. Helmling, E. Cetiner, K. Pasemann, B. Fürtig, A. Wacker, N. S. Qureshi, M. Gränz, D. Barthelmes, H. R. A. Jonker, E. Stirnal, S. T. Sigurdsson, H. Schwalbe, *J. Biomol. NMR* **2017**, *68*, 53–63.

10.1002/chem.201800167

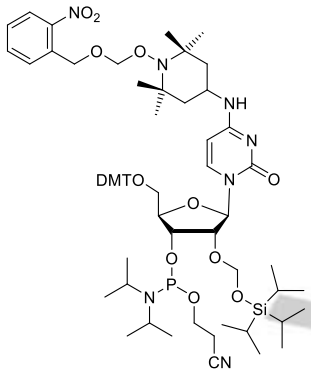
WILEY-VCH

Entry for the Table of Contents (Please choose one layout)

Layout 1:

FULL PAPER

Nitroxides are important spin labels for EPR studies of biopolymers. However, sample preparation can be severely restricted by their limited chemical stability. The 2-nitrobenzyloxymethyl group protects the TEMPO label against all critical conditions of chemical RNA synthesis and enzymatic ligation. After photo-chemical removal, the nitroxide radical is recovered by spontaneous air oxidation. Assembly of a spin labeled 59mer RNA is shown as example.



*Timo Weinrich, Eva A. Jaumann, Dr. Ute Scheffer, Prof. Dr. Thomas F. Prisner, Prof. Dr. Michael W. Göbel**

Page No. – Page No.

A Cytidine Phosphoramidite with Protected Nitroxide Spin Label: Synthesis of a Full-Length TAR RNA and Investigation by in-line Probing and EPR Spectroscopy.

Layout 2:

FULL PAPER

((Insert TOC Graphic here; max. width: 11.5 cm; max. height: 2.5 cm))

*Author(s), Corresponding Author(s)**

Page No. – Page No.

Title

Text for Table of Contents