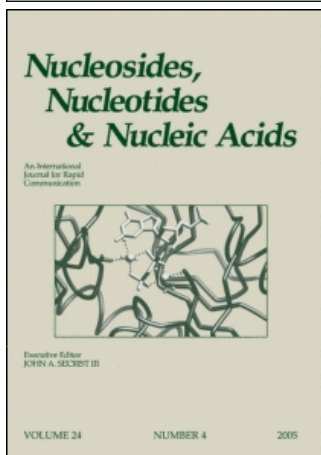


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THE SYNTHESIS OF EPR DIFFERENTIABLE SPINLABELS AND THEIR COUPLING TO URIDINE

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□ *For EPR measurements of RNA, DNA, or proteins, the occurrence of the paramagnetic species is necessary. The aim of this work is to improve the synthesis of two different EPR spinlabels 2,2,6,6-tetramethyl-3,4-dehydro-piperidin-N-oxyl-4-acetylene (TEMPA) **6** and ¹⁵N-labeled TEMPA **6*** and their coupling to uridine. The yield of the synthesis of TEMPA could be increased to 40% and the second nitroxide 2,2,6,6-tetramethyl-3,4-dehydro-piperidin-¹⁵N-oxyl-4-acetylene **6*** could be synthesized with a yield of 11%.*

Keywords RNA structure; sonogashira cross-coupling; side-specific label; nitroxide; EPR

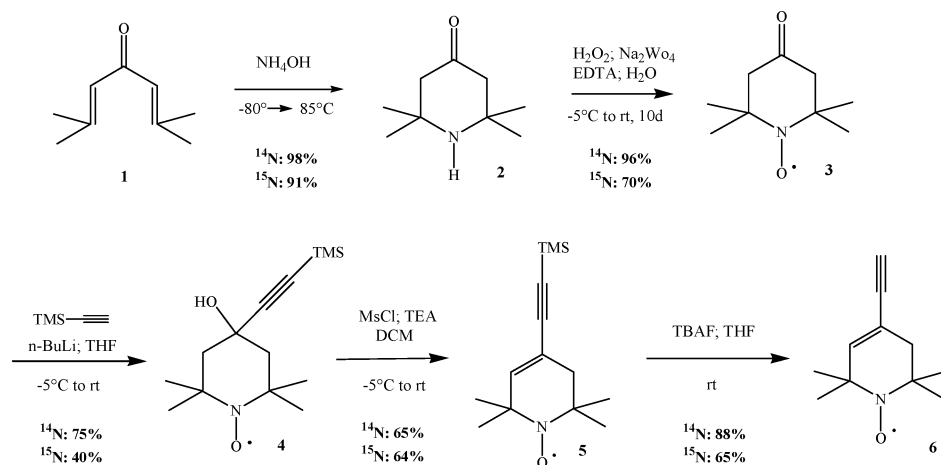
INTRODUCTION

The structure determination of macromolecules, like RNA, DNA, or proteins, is not straightforward but needs a variety of analytic methods. Besides X-ray, NMR, and FRET, electron paramagnetic resonance (EPR) can be applied to elucidate the structure of such systems. Indeed, EPR spectroscopy already proved to be a powerful technique to characterize the local surrounding of paramagnetic centers in proteins.^[1] DNA,^[2] and RNA.^[3]

One requirement to determine the three dimensional structure is the availability of three in EPR distinguishable spinlabels. In most cases the radical 2,2,5,5-tetramethyl-pyrrolin-1-oxyl-3-acetylene (TPA) could be used to obtain paramagnetic species. The use of this spinlabel or similar compounds

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SCHEME 1 Total synthesis of spinlabel TEMPA **6** and **6***.

is described for proteins, DNA, and RNA. Gannett^[4] published a synthesis of the spinlabel **6** 2,2,6,6-tetramethyl-3,4-dehydro-piperidin-*N*-oxyl-4-acetylene (TEMPA) in 2001 and showed its application for DNA labeling. In our group the coupling of nitroxide to RNA could be accomplished and a new spinlabel **6*** had to be synthesized.

Synthesis

To be able to synthesize the ¹⁵N-labeled TEMPA (Scheme 1), the synthesis of both radicals has to start with a ring closure reaction.^[5] Reaction of phorone **1** with ammonia in water yields the six-membered ring **2** with 98% for ¹⁴N- and 91% for ¹⁵N-TEMPA. The oxidation of the nitrogen was performed following the standard procedure with hydrogen peroxide and led to a very good yield of 96% for compound **3** (70% for **3***). For economical reasons and higher reactivity, we used trimethylsilyl instead of triisopropylsilyl-protected acetylene. Product **4** could be obtained as a greenish yellow solid in good yield. The reagents for the elimination step to compounds **5** and **5*** could also be changed from thionylchloride in pyridine to methanesulfonylchloride (MsCl) in triethylamine. The use of these conditions facilitates the purification of 4-acetylene-(trimethylsilyl)-2,2,6,6-tetramethyl-piperid-3,4-en-*N*-oxyl (**5**). The silyl-group was cleaved with TBAF in 88% yield.

The synthesis of the ¹⁵N-labeled compound was performed by the same strategy. Although the structures of both spinlabels are identical, their reactivity is quite different. Thus the reaction time and the temperature of the reaction had to be varied for each step. During the synthesis all com-

TABLE 1 EPR parameters for the three spinlabels

Spinlabel	g -factor	A_{iso} [mT]
TPA	2.006	1.4
^{14}N -TEMPA 6	2.0055	1.5
^{15}N -TEMPA 6*	2.0055	2.1

pounds could be crystallized and analyzed (elemental analysis, ^1H -NMR and ^{13}C -NMR).

***cw*-EPR**

The *continuous wave*-EPR (*cw*-EPR) measurements were carried out with ESP300 *cw* X-Band spectrometer of the Bruker company at the frequency of 9.4267 GHz at 25 K. Both samples had the concentration of 10^{-4} mol/L in toluene and were deoxygenated with argon.

Due to a different geometry the five (TPA) and six-membered rings (TEMPA) possess distinct g -factors (Table 1). The ^{15}N -labeling ($S = 1/2$ instead of $S = 1$ for ^{14}N) affects the hyperfine-coupling (A_{iso}) of the nitroxide **6***, which scales with the gyromagnetic ratio $\gamma^{15}\text{N}/\gamma^{14}\text{N} = 1.4$. The effect is reflected in the EPR-spectra (2 transitions instead of 3), shown in Figure 1.

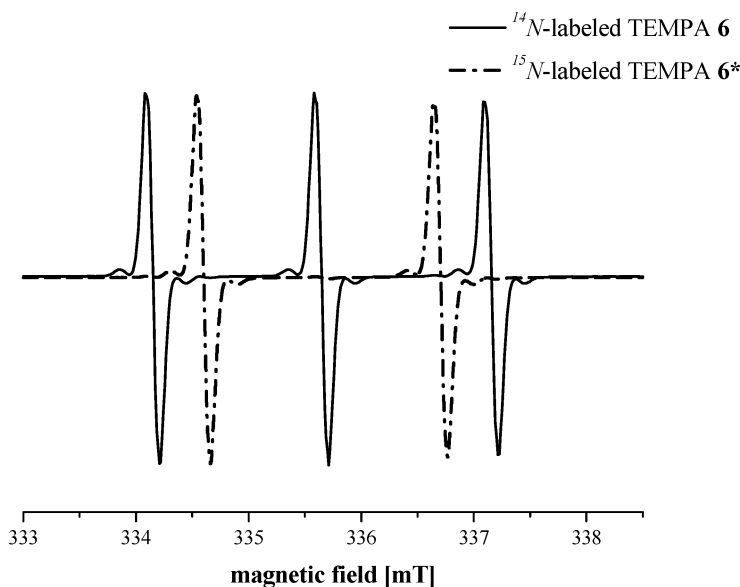
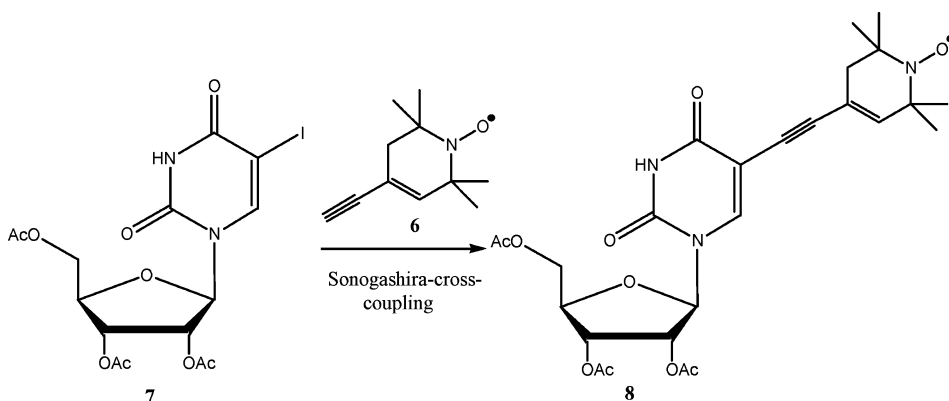


FIGURE 1 *cw*-EPR spectrum of spinlabels **6** and **6***.



SCHEME 2 Sonogashira cross-coupling.

Cross-Coupling

For *pulsed* EPR distance measurements in RNA the synthesized spin-labels will be coupled to the nucleotide base through a Sonogashira cross-coupling during solid-phase synthesis after a method already developed in our laboratory for TPA.^[3]

To optimize the conditions for solid phase, the reaction between both synthesized spinlabels **6** and **6*** and 2',3',5'-tri-*O*-acetyl-5-iodo-uridine **7** was tested in solution (Scheme 2) and the kinetics thereof were followed by HPLC (Figure 2).

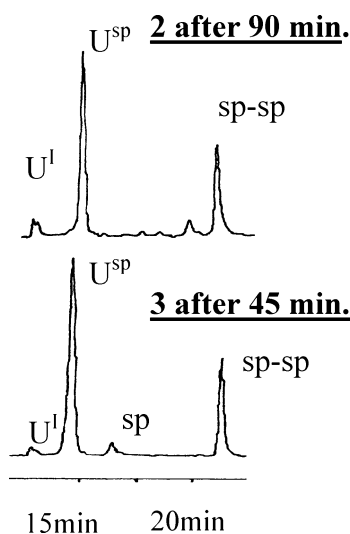


FIGURE 2 Comparison of two HPLC-spectra (sp=spinlabel; U^I =5-Iodo-uridine; U^{sp} =spinlabeled uridine).

TABLE 2 Different test conditions for the cross-coupling with uridine

	Spinlabel	Solvent	Pd	CuI	Yield
1	TPA	DCM	Pd(II)	x	96%
2	TEMPA	DCM	Pd(II)	x	90%
3	TEMPA	THF	Pd(II)	x	97%
4	TEMPA	DMF	Pd(II)	x	28%
5	TEMPA	DCM	Pd(0)	x	69%
6	TEMPA	Piperidine	Pd(0)	/	76% [#]

[#] The hydroxy groups of the sugar are deprotected.

Gradient: 0 min. 10% AcCN + 90% of 0.1% tri-ethylammonium-acetate.

0.1–20 min. 100% AcCN + 0% of 0.1% TEAA.

to 30 min. 100% AcCN + 0% of 0.1% TEAA.

30.1 min. 10% AcCN + 90% of 0.1% TEAA.

The reaction with the spinlabel TPA (Table 2) was taken as reference. Bis(triphenylphosphine)-palladium-dichloride and tetrakis(triphenylphosphine)palladium were used as Pd(II) and Pd(0) respectively. Reactions 1 to 5 are performed with triethylamine as a base.

Yield was calculated by HPLC after a reaction time of 90 min. at room temperature. Reaction 3 in THF with Pd(II) looks like the best condition for the coupling with spinlabel 6.

CONCLUSIONS

After the coupling tests in solution the transfer to the solid phase will be performed and the stability of the labeled RNA will be checked via *T_m*-measurements and CD-spectra.

To optimize the PELDOR parameters for the new nitroxides we are synthesizing different linker systems. On the basis of these results, we will incorporate the three different spin labels into RNA-duplexes to validate the method and then apply it to the determination of more complex 3D-RNA structures.

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