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2007 J. Phys.: Condens. Matter 19 285202

(http://iopscience.iop.org/0953-8984/19/28/285202)

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J. Phys.: Condens. Matter **19** (2007) 285202 (10pp) doi:10.1088/0953-8984/19/28/285202

Synthesis and NMR spectral properties of spin-labelled mRNA 5′ cap analogue: a new tool for biochemical studies of cap binding proteins

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Received 24 October 2006, in final form 12 December 2006
Published 25 June 2007
Online at stacks.iop.org/JPhysCM/19/285202

Abstract

All eukaryotic messenger RNAs (mRNAs) and small nuclear RNAs (snRNAs) comprise a unique chemical structure called a 'cap', i.e. 7-methylguanosine linked by a 5′,5′ triphosphate bridge to the first transcribed nucleoside. Biophysical studies of interactions between the RNA 5′ terminus and proteins that specifically recognize its structure require suitable chemical cap analogues. For the needs of electron spin resonance spectroscopy, a spin-labelled cap analogue, m^7GTP-TEMPO, P^1-(7-methylguanosine-5′) P^3-(2,2,6,6-tetramethyl-1-piperidinyloxy-4) triphosphate, has been synthesized and fully characterized spectroscopically. The structure has been confirmed by one-dimensional (1D) and 2D nuclear magnetic resonance (NMR), electron spin resonance (ESR) and electrospray ionization mass spectrometry (ESI-MS). Despite the presence of a free radical (TEMPO) in the small molecule, complete ^{1}H, ^{13}C, and ^{31}P NMR spectra have been acquired that allowed us to assign all these resonances, including the radical moiety. These are the first well resolved NMR spectra of the TEMPO-containing paramagnetic species, directly obtained and analysed without conversion to an *N*-hydroxylamine derivative.

1. Introduction

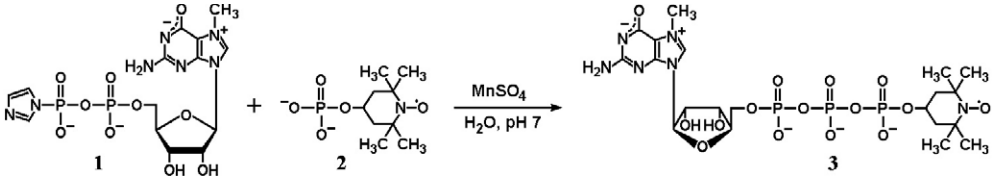
The 5′-terminal structure of eukaryotic mRNA and snRNA consists of 7-methylguanosine connected by a 5′,5′ triphosphate bridge with the mRNA strand [1]. This chemical structure, called a 'cap', plays an important role during several cellular processes involving various cap binding proteins. Among them, the nuclear cap binding complex (CBC)

takes part in pre-RNA splicing [2], polyadenylation of the 3′ terminus [3] and nuclear export [4]; the eukaryotic translation initiation 4E factor (eIF4E) regulates initiation of protein biosynthesis [5], and poly(A)-specific exoribonuclease (PARN) is responsible for cap-dependent mRNA degradation [6–9].

Synthetic analogues of the cap are frequently exercised in biochemical and biophysical research focused on mechanistic bases of intermolecular recognition [10–12], thermodynamics [13–15], conformational changes [16] and kinetics [17] of cap binding by proteins. Analysis of the structure–function relationship upon interaction of these proteins with the mRNA 5′ cap can be performed by means of various physicochemical methods to gain a comprehensive insight into the dynamic nature of recognition of the cap. Solution studies are necessary to complement the structural view provided by x-ray crystallography and multidimensional NMR. Experiments leading to the production of new tools for binding studies in solution are of special importance for proteins of unknown crystal structure. In particular, this is the case with PARN [18], which is a large (147 kDa) dimeric protein [19]. Each monomer of PARN consists of three domains [20]: the exonuclease domain, the RNA recognition motif (RRM), and the R3H domain. A fragmentary structure of PARN without the RRM was published recently [19]. Intriguingly, the results asked more questions than they answered, since specificity of the enzyme to poly(A) was not elucidated, and the 5′ cap binding site was not identified. The latter is expected to be present just in the RRM which is absent in the crystal structure. In this context, fluorescence studies with the use of chemically modified cap analogues would be much needed, as they proved to be very effective in the determination of equilibrium association constants and structural requirements for cap binding in binary and ternary complexes of eIF4E [10] and CBC [12]. Unfortunately, fluorescence measurements suffer from several limitations, e.g. overlapping of both absorption and emission spectra of interacting molecules, weak quenching for Trp → Ala mutants, a long dead-time during stopped-flow experiments, and photobleaching of molecular probes. Hence, we are searching for new possibilities of solution studies that will be a good alternative for optical spectroscopy.

Electron spin resonance (ESR) spectroscopy can provide information similar to that obtained from fluorescence. ESR can be applied both for steady-state studies and for measurements of fast kinetics by means of the stopped-flow technique [21, 22]. Herein, we present a method of chemical synthesis of the first spin-labelled mRNA 5′ cap analogue, referred to in the form of m^7GTP-TEMPO, which contains a free radical as the 2,2,6,6-tetramethylpiperidine-*N*-oxyl residue (TEMPO), joined to 7-methylguanosine 5′,5′ triphosphate via the P^3-phosphoroester bond. This novel cap analogue will serve as a new tool in biochemical and biophysical research on the RNA 5′ cap binding proteins, i.e. different eIF4E isoforms, CBC, and particularly PARN, by means of ESR.

TEMPO is a well-known, commercially available and stable free radical. Due to the ESR activity, various chemical derivatives of TEMPO are widely used for the determination of the relative location of molecules or residues in large molecular systems (see e.g. [23–25]). However, despite the widespread use of this ESR probe, NMR analysis of the TEMPO radical has been lacking. Only recently was the NMR spectrum of TEMPO bound to a cyclodextrine-based rotaxane analysed via comparison with the product of reduction with phenylhydrazine to the *N*-hydroxylamine derivative [26]. The problem is that the NMR spectra of free radical-containing small molecules are hardly detectable due to the strong paramagnetic broadening of resonance signals, so NMR studies of them are often thought to be impossible. Despite these difficulties, we have measured the complete ^{1}H, ^{13}C and ^{31}P NMR spectra of m^7GTP-TEMPO, which led to full assignment of the nuclear magnetic resonances, including all signals of the radical part of the molecule.



Scheme 1. Organic synthesis of the spin-labelled cap analogue, P^1-(7-methylguanosine-5′) P^3-(2,2,6,6-tetramethyl-1-piperidinyloxy-4) triphosphate, referred to as m^7GTP-TEMPO.

2. Materials and methods

*2.1. Chemical synthesis of P^1-(7-methylguanosine-5′) P^3-(2,2,6,6-tetramethyl-1-piperidinyloxy-4) triphosphate (compound **3**, scheme 1)*

5.9 mg of 4-phosphonoxy-2,2,6,6-tetramethyl-1-piperidinyloxy hydrate (compound **2**) purchased from Aldrich, 6.2 mg of 7-methylguanosine 5′-O-(P^2-imidazolide) diphosphate (compound **1**) [27] and 13 mg of manganese sulfate hydrate were dissolved in 0.4 ml of 0.2 M *N*-ethylmorpholine/HCl buffer (pH 7) and magnetically stirred overnight. The mixture was centrifuged, and the main product was isolated from the supernatant by high performance liquid chromatography (HPLC) at the retention time of 4.8 min. HPLC was carried out on a Spectra-Physics SP8800 apparatus equipped with a Supelcosil LC-18-T reverse phase column (25 cm). The mobile phase was a linear gradient of methanol from 0 to 25% (v/v) in 0.05 M NH_4OAc (water solution, pH 5.9) within 15 min; the flow rate was 1.3 ml min^{-1} and the sample was monitored at 260 nm. The collected eluates were lyophilized and gave 1.7 mg of the desired product **3**, m^7GTP-TEMPO, in the form of the ammonium salt, with a yield of 20%.

The mass spectra were recorded on a Waters Micromass Q-TOF spectrometer using negative electrospray ionization, giving the expected molecular peak at $m/z = 690.9$.

2.2. Electron spin resonance (ESR) measurements

The conventional field-swept ESR spectra from aqueous solutions of the spin-labelled cap analogue were obtained with a commercial X-band EPR spectrometer, Model ESP300E from Bruker GmbH (Biospin, Germany), equipped with a standard TE_{102} rectangular resonator. Aliquots of about 7 μl of aqueous samples were transferred into 0.6 mm inner diameter and 0.84 mm outer diameter quartz capillary tubes from VitroCom, NJ, USA (sample height of ∼25 mm) and sealed at both ends with Cha-Seal^{TM} tube sealing compound (Chase Scientific Glass, Inc., Rockwood, TN, USA). ESR spectra were acquired at ambient temperature (21 °C) using the following instrument settings: microwave power 2 mW, modulation amplitude, 1 G_{pp}; modulation frequency, 100 kHz; time constant, 20.5 ms, integration time 40.9 ms; scan time, 49 s; scan width 120 G; receiver gain, 4×10^4. Five scans per EPR trace were accumulated. To quantify the number of spins, the chosen EPR spectra were doubly integrated using Origin 7.5 software from OriginLab Co. As a reference sample, a 25 μM water solution of 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) from Fluka/Aldrich was used.

2.3. Nuclear magnetic resonance (NMR) measurements

For NMR measurements, the spin-labelled mRNA 5′-cap analogue was dissolved in D_2O (isotopic purity D 99.98%, Armar AG, Döttingen, Switzerland) at the concentration of 4 mM. ^{1}H and ^{13}C NMR spectra of m^7GTP-TEMPO were measured at 30 °C on a UnityPlus 500 MHz

spectrometer (Varian, Palo Alto, CA, USA) equipped with a Performa II gradient generator unit, two wave form generators (WFG), high-stability temperature unit, and a 5 mm $^{1}H/^{13}C/^{15}N$ triple-PFG probe head. The ^{1}H NMR reference spectrum of $m^{7}GTP$ was run in the 1/15 M phosphate buffer (pD 7, uncorrected), at a concentration of 17.9 mM, using sodium 3-trimethylsilyl-[2,2,3,3-D_4]-propionate (TSP) as an internal standard in D_2O. ^{31}P-NMR spectra were measured at 25 °C on a UnityInova 400 MHz spectrometer (Varian, Palo Alto, CA, USA) equipped with a Performa II/III gradient generator unit, WFG, high-stability temperature unit, and a 5 mm $^{13}C/^{31}P/^{15}N$ 4NUC auto-switchable PFG probe head. The 10 Hz Lorentzian line-shape broadening function was applied to improve the signal-to-noise ratio in the ^{31}P spectrum, and 34 000 scans were collected.

32 000 and 52 000 data points were collected, and a spectral width of 6 and 32 kHz was used in 1D ^{1}H and ^{31}P experiments, respectively. The 2D total correlation spectroscopy (TOCSY) [28] experiments were performed using a proton spectral width of 6 kHz collecting 2 K data points, with 256 increments (8 scans per increment), and a mixing time of 50 ms. The 2D {^{1}H, ^{13}C} gradient selected heteronuclear single quantum coherence (gHSQC) [29, 30] experiments were performed in proton decoupled mode, with the carbon spectral width of 25 kHz and 256 increments, with 48 scans per increment. The ^{1}H and ^{13}C chemical shifts were determined versus external DSS reference signals, and 1 mM orthophosphoric acid was used as the reference in the ^{31}P spectra.

Acquisition of each 2D spectrum at 25 and 30 °C was preceded and followed by the measurement of the 1D ^{1}H spectrum to check that the sample was stable under these conditions.

2.4. Analysis of ^{31}P nuclear magnetic resonance spectra

Three Lorentz functions were fitted to the ^{31}P spectral data according to the equation

$$Y = A\left(1 + \frac{(x - x_{\max})^2}{\Delta^2}\right)^{-1} + B,$$

by means of non-linear, least-square method using PRISM 3.02 (GraphPad Software, San Diego, CA, USA). The parameters of the fitting curve are as follows: $x_{\max}$, the position of the maximum of the peak; Δ, broadening; A, B, constants. The halfwidth of the ^{31}P NMR signal is given by $2 \cdot \Delta \cdot 161.901$ Hz. The fitted parameters of the signals are given in table 1.

3. Results and discussion

3.1. Chemical synthesis

The spin-labelled cap analogue, P^1-(7-methylguanosine-5′) P^3-(2,2,6,6-tetramethyl-1-piperidinyloxy-4) triphosphate ($m^{7}GTP$-TEMPO, compound **3**, scheme 1) was prepared by a coupling reaction of 7-methylguanosine 5′-diphosphate activated by imidazole (compound **1**) with a commercially available TEMPO derivative (4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy phosphate ester, compound **2**). The intermediate **1** was prepared from guanosine 5′-diphosphate as described previously [27]. The reaction was carried out in aqueous conditions in the presence of manganese(II) salt. Application of the most commonly used method [27, 31, 32] of triphosphate bond formation in anhydrous conditions, i.e. in *N*,*N*-dimethylformamide in the presence of $ZnCl_2$ as a promoter, failed in this case. Therefore, we decided to use an alternative method of coupling in aqueous conditions [27, 33]. The reaction pathway is shown in scheme 1. The yield of coupling of the compound **1** to 4-phosphonoxy-TEMPO was 20%. The final product (**3**) was isolated by preparative HPLC and obtained as an ammonium salt. The analytical HPLC profile of the obtained $m^{7}GTP$-TEMPO is shown

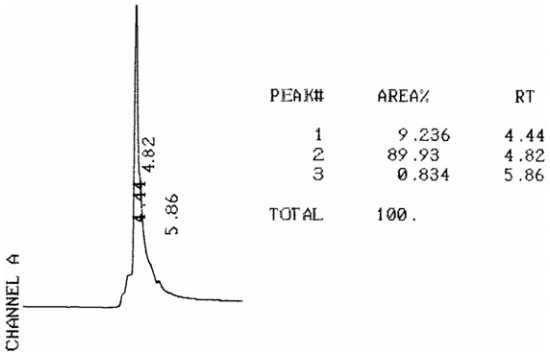


Figure 1. Analytical HPLC profile of the spin-labelled cap analogue, m^7GTP-TEMPO.

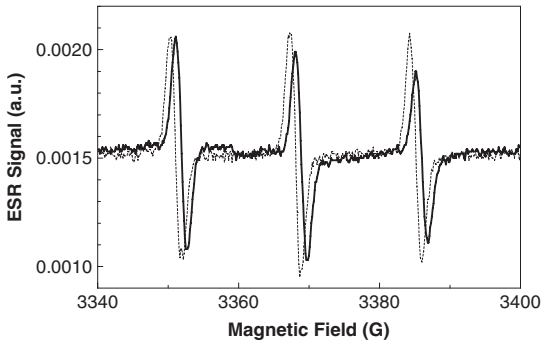


Figure 2. ESR spectra of the spin-labelled cap analogue, m^7GTP-TEMPO (thick solid line), and of the control sample of the unbound nitroxide radical, TEMPOL (thin dotted line), in H_2O.

in figure 1. The purity of this compound (about 90%) is sufficient enough to perform any physicochemical and biophysical experiments.

The synthesis of m^7GTP-TEMPO is the first example of the introduction of a spin label into a nucleotide moiety via phosphoroester linkage.

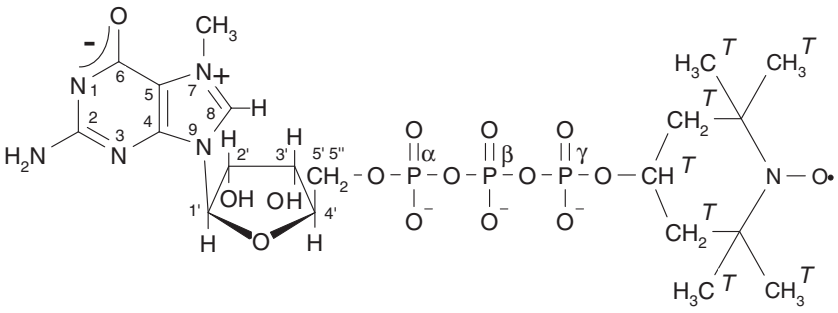
3.2. ESR spectroscopy

Electron spin resonance spectroscopy was used to check whether the nitroxide radical from 4-phosphonoxy-TEMPO had been incorporated into the final product, m^7GTP-TEMPO. As shown in figure 2, the ESR spectrum of m^7GTP-TEMPO reveals a characteristic signal broadening due to a partially hampered rotation of the nitroxide radical covalently bound to the cap analogue. This is in contrast to the control spectrum of the unbound free radical, TEMPOL, shown for comparison.

3.3. NMR spectroscopy

A combination of different types of 1D and 2D NMR spectra allowed us to assign all proton, carbon and phosphorous resonances of the spin-labelled mRNA 5′-cap analogue, m^7GTP-TEMPO (scheme 2, table 1).

In the TOCSY spectrum (figure 3(a)), several separate spin systems can be distinguished. One of them, containing the signals at 6.058, 4.68, 4.528, 4.394, 4.342 and 4.253 ppm, has been



Scheme 2. Structural formula of the spin-labelled mRNA 5′ cap analogue, m^{7}GTP-TEMPO, with identification of all protons, similarly as in table 1.

Table 1. NMR data for m^{7}GTP-TEMPO (scheme 1), m^{7}GTP, and m^{7}GpppG in D_2O.

	$\delta(^1H)$ (ppm)		$\delta(^{13}C)$ (ppm)
Signal	m^{7}GTP-TEMPO	m^{7}GTP [d]	m^{7}GTP-TEMPO[e]
$CH_3{}^{T\ ax}$	1.455[a]		31.9
$CH_3{}^{T\ eq}$	1.483[a]		27.6
$HC\underline{H}{}^{T\ ax}$	1.715[b]		43.9
$\underline{H}CH{}^{T\ eq}$	2.283[b]		43.9
CH^{T}	4.77[c]		70.2
N-CH_3	4.116[a]	4.139	38.5
CH5′	4.253[b]	4.287	67.0
CH5″	4.342[b]	4.375	67.0
CH4′	4.394[b]	4.415	86.6
CH3′	4.528[b]	4.582	n.d.
CH2′	4.68[c]	4.705	77.2
CH1′	6.058[b]	6.075	92.0
	$\delta(^{31}P)$ (ppm)		Halfwidth (Hz)
	m^{7}GTP-TEMPO[f]	m^{7}GpppG [g]	m^{7}GTP-TEMPO[f]	m^{7}GpppG [h]
P^{α}	-11.985 ± 0.007	-12.2	257 ± 4	3
P^{β}	-23.477 ± 0.011	-22.8	225 ± 6	3
P^{γ}	$+0.350 \pm 0.003$	-12.2	130 ± 2	3

[a] Accuracy ±0.001.
[b] Accuracy ±0.006.
[c] Accuracy ±0.01.
[d] Accuracy ±0.005 ppm, data from [41].
[e] Accuracy ±0.4.
[f] Determined as parameters of the Lorentz functions fitted to the spectral data in figure 5.
[g] Accuracy ±0.1 ppm, data from [38].
[h] Reference [41].

assigned to the sugar protons. The proton signals at 1.715 and 2.283 ppm have been assigned to the axial and equatorial protons in CH_2 groups of TEMPO, respectively, on the basis of the proton–carbon correlation spectrum in which both protons are located at the same carbon (figure 3(b)). The difference in their chemical shifts of ∼0.57 ppm is typical for equatorial and axial protons in the chair conformation of cyclohexane-like molecules [34]. These protons are coupled with a constant of about 11 Hz, and form the second spin system with the CH

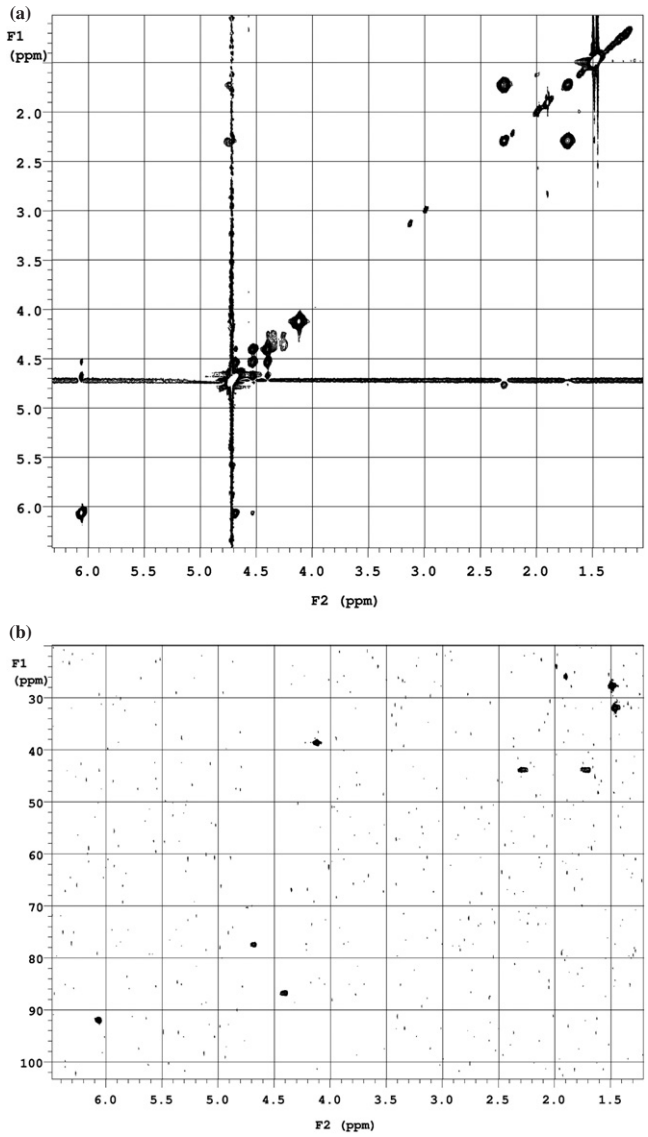


Figure 3. 2D NMR spectra of the spin-labelled cap analogue, m^7GTP-TEMPO, in D_2O at 30 °C. (a) TOCSY spectrum: F1 and F2 denote ^{1}H chemical shifts; (b) {^{1}H, ^{13}C}gHSQC spectrum: F1 and F2 denote ^{13}C and ^{1}H chemical shifts, respectively.

proton of this moiety at 4.77 ppm. The scalar couplings for the remaining protons are not observed in the 1D proton spectra of both spin systems due to the paramagnetic broadening of the signals, with halfwidths of 15–28 Hz (figure 4). The axial and equatorial methyl groups of the TEMPO moiety yield two singlets at 1.455 and 1.483 ppm, with a halfwidth of 8 Hz, while the N7-methyl signal from the base has been found at 4.116 ppm. The latter methyl group is significantly deshielded, since the N7 atom possesses a localized partial positive charge [35]. The relative intensities of these methyl signals confirmed the 1:1 ratio of the cap and TEMPO in the final product. The proton assignment is confirmed by the carbon one (table 1).

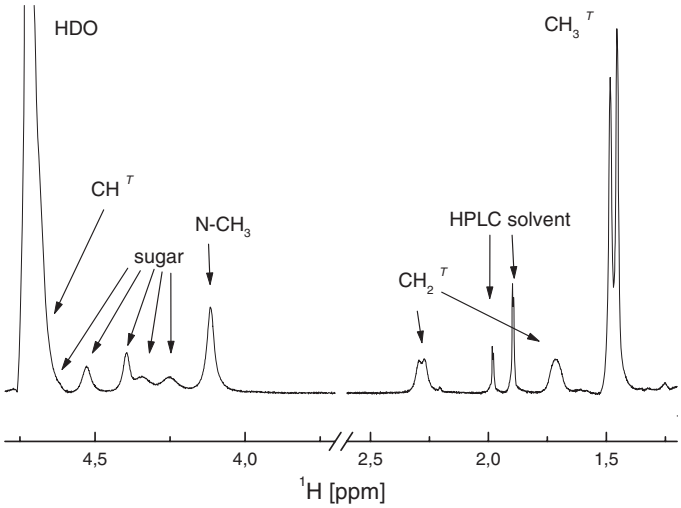


Figure 4. ^{1}H NMR spectrum of m^{7}GTP-TEMPO in D_2O at 30 °C.

In comparison with the spectrum of 7-methylguanosine 5′-triphosphate (m^{7}GTP), ^{1}H chemical shifts of the 7-methylguanosine moiety in the TEMPO-containing product are shifted slightly upfield (table 1). The strongest effect of about −0.05 ppm has been found for the CH3′ sugar proton. Then, the differences of the chemical shifts are about −0.03 ppm for CH5′ and CH5″, about −0.02 ppm for CH2′, N-CH$_3$, CH4′, and < -0.02 ppm for CH1′. These values, together with observation that the C3′ cross peak has not been detected in the gHSQC spectrum, could suggest that a kind of weak interaction between the TEMPO moiety and the 7-methylguanosine is possible. Such interaction would resemble the intramolecular self-stacking of dinucleotide cap analogues which occurs with an equilibrium constant in the range of 1 [32, 36].

Other protons from the m^{7}G base are not observed due to H/D exchange. The signal from H8 is also lacking, though this proton is bound to carbon, due to reversible opening of the imidazole ring of 7-methylguanine nucleotides under the influence of the attack of hydroxide ion on the C8 atom [37]. At neutral pH, the equilibrium is shifted toward the closed structure, yielding deuteration of H8.

A strong paramagnetic broadening that results from the presence of the free radical in the molecule causes the ^{31}P signals to be hardly detectable, requiring more than thirty thousand scans. Nevertheless, three peaks have been identified, at +0.41, −12.02, and −23.16 ppm (figure 5, table 1).

The ^{31}P signals in dinucleotide cap analogues containing different numbers of bridging phosphates are known to be always about −23 ppm for the groups in the middle of the chain and about −12 ppm for the outermost groups that are bound to C5′ of the ribose [32, 38], as shown for P^{1}-(7-methylguanosine 5′) P^{3}-(guanosine 5′) triphosphate (m^{7}GpppG) in table 1. On the basis of these data we can unambiguously assign the above-mentioned ^{31}P chemical shifts to the γ, α and β phosphate group, respectively. Expected doublet and triplet structures of the ^{31}P resonances, resulting from homonuclear scalar coupling with a constant of about 20 Hz [32, 38] are not visible due to the broadening up to 100–200 Hz. Thus, the structure of m^{7}GTP-TEMPO was fully confirmed by NMR.

Line broadening of nuclear magnetic resonances in spin-labelled molecules is distance dependent [39]. In the spectrum of m^{7}GTP-TEMPO, the strongest broadening is observed for

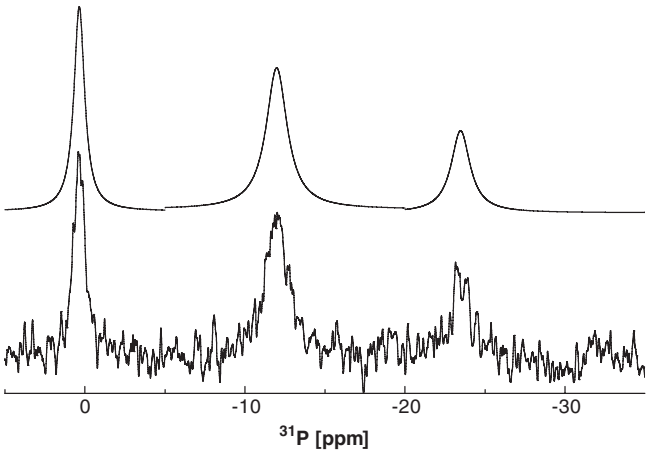


Figure 5. Experimental ^{31}P NMR [161.901 MHz] spectrum of m^{7}GTP-TEMPO, in D_2O at 25 °C (bottom), and the Lorenz functions fitted to the spectral data, with parameters given in table 1 (top).

the β phosphate group (table 1), while the γ one has relatively the sharpest signal. This effect can be attributed to the presence of conformers in which the free radical part of the molecule comes nearer to the 7-methylguanosine moiety due to flexibility of the phosphate chain. Free radical sites have a tendency to weak but stereospecific interactions with tetracoordinated organophosphorus, as was shown by dynamic nuclear polarization studies [40].

3.4. Stability of m^{7}GTP-TEMPO

Stability of the paramagnetic cap analogue has been examined by keeping it in a water solution at 25 °C for six days. The identity of m^{7}GTP-TEMPO has been checked both by means of the analytical RP HPLC and by ESR, at different time intervals. The HPLC profiles did not reveal any particular differences, and the characteristic broadening of the ESR spectra did not change during this time. The doubly integrated ESR spectra did not show evidence of free radical oxidation. These repeated experiments proved that the spin-labelled cap analogue is stable in water and suitable for typical biochemical research.

4. Conclusions

In this work, we have described a method of synthesis of the spin-labelled cap analogue, m^{7}GTP-TEMPO. The synthesis, on a scale of milligrams, allowed us to perform measurements that yielded the complete assignment of ^{1}H, ^{13}C and ^{31}P NMR spectra and confirmed the structure.

This compound has been designed and synthesized as an ESR-active molecular probe for future research involving several mRNA 5′ cap binding proteins, such as PARN, CBC and isoforms of eIF4E of different affinity to the cap. Application of the ESR technique to study the dynamics of formation of their complexes should make it possible to overcome many of technical limitations related to other spectroscopic methods. In this regard, the synthesis of m^{7}GTP-TEMPO opens new possibilities in studying processes of eukaryotic gene expression at the molecular level.

Acknowledgments

We thank Professor Ryszard Stolarski for providing the chemical shifts of m^{7}GTP and helpful discussion, and Professor Michal Dadlez for access to ESI-MS in the Laboratory of Mass

Spectrometry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences. This work was supported by the Polish Ministry of Science and Higher Education (grant No. 2 P04A 033 28).

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