148. Formation of *N*-Hydroxy-amines of Spin Labeled Nucleosides for ^{1}H-NMR. Analysis[1])

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Summary

Amine-oxide radical **1a** was efficiently converted to the corresponding *N*-hydroxy-amine **2a** with sodium dithionite in acetone/water. This reaction was used to develop a procedure for monitoring the NMR. spectra of sodium dithionite reduced amine-oxide radicals and of novel reduced amine-oxide-labeled nucleosides.

The stable amine-oxide free radicals are among the most sensitive probes in the biophysical study of nucleic-acid conformations. Amine-oxide radicals containing nucleic acids can be monitored directly by ESR. within complex biological systems without isolation [1]. Although non-site-specifically spin-labeled polynucleotides have been useful, the ESR. spectra of site-specifically modified polynucleotides should, theoretically, reflect specific conformational transitions more accurately [2]. Therefore, the site of attachment of the spin label must be known, and is most conveniently established on the nucleoside-monomer level [3]. The valuable structural evidence accessible by NMR. spectroscopy, however, cannot be acquired by conventional techniques, since paramagnetic species yield spectra of low resolution [4]. Conversion of amine-oxide radicals to diamagnetic species renders the molecules suitable for subsequent NMR. analysis. Phenylhydrazine reduces most amine-oxide radicals [5] efficiently to the analogous *N*-hydroxy-amines directly in the NMR. sample tube [6]. A technique exploiting both phenylhydrazine and ascorbic acid was used to monitor the NMR. spectrum of a spin labeled transfer RNA [7]. High field resonances were observed after reduction with phenylhydrazine, and ascorbate was used to monitor the low field spectrum. Ascorbate was subsequently shown to convert amine-oxide radicals quantitatively to the corresponding *N*-hydroxy-amines [8]. These methods, however, were not suited for direct application to spin-labeled nucleosides. Phenylhydrazine is known to produce by-products [9] and to contribute resonances in the region of interest

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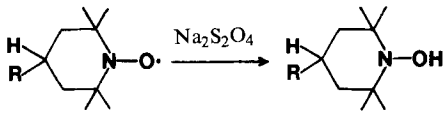
in the case of spin-labeled nucleosides. Furthermore, amine-oxide-radical labeled nucleosides are insufficiently soluble in solvents commonly used for these reducing agents. We observed that sodium dithionite, which was used to convert amine-oxide radicals to diamagnetic species directly in the NMR. sample tube in aqueous solvents [10], also gives well resolved spectra in deuteriated acetone/water in the case of spin-labeled nucleosides, but structurally important exchangeable hydrogen atoms cannot be observed. A procedure was developed allowing the spectra of dithionite reduced amine-oxide radicals and of amine-oxide-radicals labeled nucleosides to be monitored in perdeuteriated dimethylsulfoxide, a commonly used NMR. solvent for nucleosides. It is also demonstrated that the procedure reported here results in the conversion of the amine-oxide moiety to the corresponding *N*-hydroxy-amine.

The representative amine-oxide radical **1a** was efficiently reduced to the analogous *N*-hydroxy-amine **2a** with sodium dithionite in acetone/water 1:1. The mass spectrum showed the molecular-ion peak at *m/z* 173. The *N*-hydroxy-amine **2a** was re-oxidized to starting material with cupric ion in methanol to the extent of 90% (ESR.) in one hour, or to the extent of 80% (ESR.) with no added catalyst in 24 hours. Consequently, the yield of the oxygen sensitive *N*-hydroxy-amine is dependent upon the length of time that it is kept in solution during purification, although it is stable in the crystalline state. An excess of dithionite prevents oxidation of the moisture and air sensitive *N*-hydroxy-amines back to amine-oxide radicals in their preparation for NMR.

Acetone/water is a convenient solvent for dithionite reduction of amine-oxide radicals, since having low solubility in water they are readily soluble in this solvent, particularly the amine-oxide-radical labeled nucleosides. In contrast, reduction in methanol/water 1:1 led to products other than the analogous *N*-hydroxy-amines. These diamagnetic products could not be re-oxidized with cupric



1

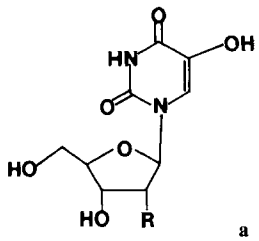
a R = OH
b R = H

2

c R = NH_2
d R = $NHCOCH_2Cl$

3

a $R^1 = R^2 = H$
b $R^1R^2 = O$
c $R^1 = H$; $R^2 = OH$



4

a R = OH
b R = H

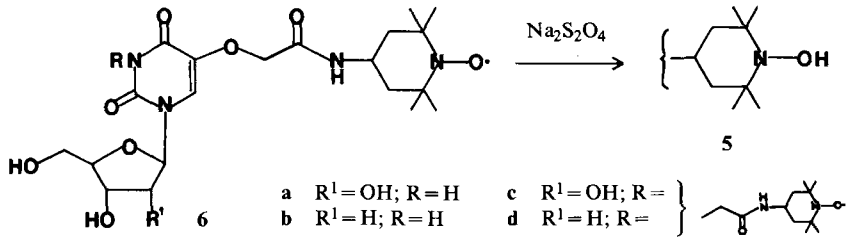
Table. *^{1}H-NMR. Chemical Shifts* (ppm.) in DMSO-d_6

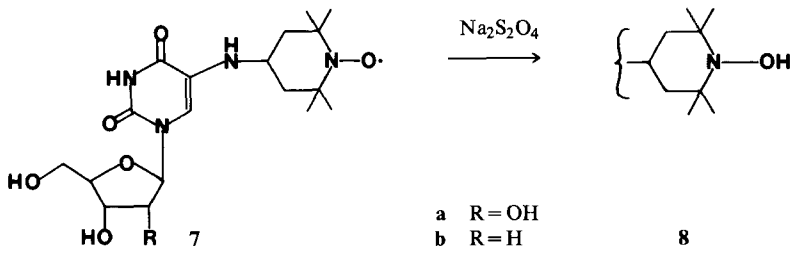
Compound	NH (piperidine)	CH_3	CH_2 (piperidine)	CH_2 (acetamido)	H–C(1')	H–C(6)	NH (uracil)	N–OH
2a		1.30 (*s*)	1.80 (*m*)					
2b		1.38 (*s*)	1.60 (*m*)					8.05 (br. *s*)
2c		1.40 (*s*)	1.80 (*m*)					
2d		1.42 (*s*)	1.80 (*m*)					
3a	0.52 (*m*)	1.06 (*s*)	1.00–1.80 (*m*)					
3b	0.82 (*m*)	1.50 (*s*)	2.66 (*s*)					
3c	0.82 (*m*)	1.08 (*d*)	1.70 (*m*)					
4a[a])					5.80 (*m*)	7.35 (*s*)	11.60 (*s*)	
4b					6.18 (*m*)	7.37 (*s*)	11.60 (*s*)	
6a		1.40 (*s*)	1.80 (*m*)	4.40 (*s*)	5.82 (*m*)	7.80 (*s*)	11.60 (*s*)	8.80 (br. *s*)
6b		1.40 (*s*)	1.77 (*m*)	4.36 (*s*)	6.18 (*m*)	7.70 (*s*)	11.50 (*s*)	9.00 (br. *s*)
6c		1.38 (*s*)	1.80 (*m*)	4.40 (*s*)	5.86 (*m*)	7.96 (*s*)		
6d		1.40 (*s*)	1.76 (*m*)	4.38 (*s*)	6.20 (*m*)	7.80 (*s*)		9.00 (br. *s*)
8a		1.40 (*s*)	1.80 (*m*)		5.87 (*m*)	6.90 (*s*)	11.40 (*s*)	8.90 (br. *s*)
8b		1.38 (*s*)	1.82 (*m*)		6.28 (*m*)	6.92 (*s*)	11.40 (*s*)	8.80 (br. *s*)

[a]) Values of **4a,b** and **6a–d** in part from [3].

ion catalysis in air, even after destruction of residual dithionite (see experimental part). The structures of these products were not investigated.

The preparation for NMR. analysis of amine-oxide radicals and of spin labeled nucleosides consists of first, reduction with sodium dithionite, then drying and subsequent extraction of the residue with DMSO-d_6. The chemical shifts observed for various reduced oxide radicals and for some reduced amine-oxide-radical labeled nucleosides using this procedure are listed in the *Table*. Chemical shift assignments of the *N*-hydroxy-amines **2a–d** and the piperidine analogues





3a–c were verified by comparison of their NMR. spectra with published spectra [6] [11]. The chemical shift assignments of nucleosides **4a, b** were also corroborated with analogous established values [12]. Unambiguous structural information concerning the attachment sites of the spin labels in nucleosides **6a–d** and **8a, b** was obtained from the DMSO-d_6-NMR. spectra, since the resonances of the uracil moiety were well separated from those of the sugar and the spin label moieties, generally occurring from 5–12 δ. This was particularly useful for determining the second alkylation site of the uracil function at N(3) in **5c** and **5d**, since the H–N(3) could be readily monitored *(Table)* [3].

It was noted that acetone may interfere with the dithionite reduction method if no precautionary steps are taken. Namely, spectra monitored immediately after extraction of the reduced material in DMSO-d_6 showed a resonance at 1.22 δ, which after 24 to 48 hours was converted to a resonance at 2.10 δ, corresponding to acetone. However, this interference was readily eliminated by removing the original DMSO-d_6 under vacuum at 40° and then again dissolving the residue in DMSO-d_6.

In summary, extraction of *N*-hydroxy-amines from dithionite reduced amine-oxide radicals with DMSO-d_6 is applicable to the NMR. analysis of uridine nucleosides spin labeled in the 5-position, and provides the basis for determining the attachment sites of amine-oxide radicals in site-specific spin labeling. The piperidine oxide radicals mentioned in this study were efficiently converted to the analogous *N*-hydroxy-amines by sodium dithionite. Whereas phenylhydrazine produces the corresponding *N*-phenoxypiperidines as by-products of oxide-radical reduction [9], no by-products were detected with dithionite reduction in acetone/water. This procedure may have general applicability in the structural determination of other spin-labeled nucleic acid building blocks.

Experimental Part

The secondary amine *N*-oxide precursors and most of the *N*-oxides were purchased commercially *(Aldrich; Eastman)*. Nucleosides **4a, b** and **5a–d** were prepared as previously described [3], and preparation of **7a, b** is to be published. Sodium dithionite was from two sources *(J.T. Baker; Alfa)*. Column chromatography was done with silica gel *(Warner-Chilcott)*, and analytical thin layer chromatography was done on silica gel GF plates *(Eastman)*. Nitrogen dried over drierite was used for the isolation and transfer of the *N*-hydroxy-amines. The 60-MHz-NMR. spectra were recorded with a *Varian* T60 spectrometer, and chemical shifts (δ) are relative to internal TMS. The mass spectra were recorded with a *Hitachi Perkin Elmer* RMU-7 spectrometer. Melting points were determined with a Mel-Temp apparatus and are uncorrected.

General procedure for the preparation of amine-oxide radicals. To 1.0 g of the representative secondary amine **3c** in 8 ml of H_2O were added 10 mg of phosphotungstic acid and 2 ml of 30% hydrogen peroxide. The solution was stirred vigorously at room temperature for 48 h. The orange solution was saturated with NaCl and extracted with ether. The ether extract was dried over K_2CO_3, filtered, and evaporated[3]). The orange residue was chromatographed on a silica gel column (approx. 20 g) in chloroform, or in chloroform containing 1–5% methanol for more polar amine-oxide radicals. The average yield of **1a** was 500 mg (50%).

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[3]) The oxidation procedure is typical of the literature methods for the preparation of amine oxide radicals, but the purification procedure differs from the typical literature employing re-crystallization.

General procedure for synthesis of N-*hydroxy-amines from amine-oxide radicals.* To 500 mg of the representative amine-oxide radical **1a** in 60 ml of acetone/water 1:1 were added 500 mg (1 equiv.) of $Na_2S_2O_4$. The orange solution decolorized instantly upon swirling. After 0.5 h at room temperature the acetone was azeotroped off under diminished pressure. The remaining aqueous solution was extracted with ether, and the ether extract was dried over K_2CO_3. The solution was filtered, evaporated, and the residue crystallized from chloroform/hexane 1:1, yielding 400 mg of **2a** (80%), m.p. 146–151°. One re-crystallization gave white needles, m.p. 160–162° (lit. [8] m.p. 155–158°), Rf 0.53 (methanol/chloroform 13:37). – MS. (*m/z* (rel. intensity)): 173 (16), 172 (3), 158 (100), 140 (43), 102 (99), 74 (31), 71 (39), 57 (51).

Reduction for NMR. in the NMR. sample tube. To 15 mg of amine-oxide radical in 0.2–0.3 ml of acetone-d_6/D_2O 1:1 in an NMR. sample tube were added 1.5 equivalents of $Na_2S_2O_4$. The NMR. spectrum of the corresponding *N*-hydroxy-amine was subsequently monitored.

Reduction of amine-oxide radicals for DMSO-d_6-NMR. spectrometrie. To 15–40 mg of amine-oxide radical containing compounds in 5 ml acetone/H_2O 1:1 were added 1.5 equivalents of $Na_2S_2O_4$. The solution decolorized upon swirling, and was kept at room temperature for 0.5 h. The solvent was evaporated under diminished pressure, and the residue was dried at 50° i.V. oven for 24 h. The residue was extracted with 0.3–0.4 ml DMSO-d_6 and the extract after centrifugation was evaporated i.V. at 40°. The residue was then again dissolved in DMSO-d_6 before aspirating it into an NMR. tube under nitrogen.

General procedure for re-oxidation of N-*hydroxy-amines.* Normally, the reduced material was first adjusted to an apparent pH 4 with dilute H_2SO_4 for 20 min to destroy excess dithionite [13]. The solution was then re-adjusted to pH 8 with dilute NaOH. Extraction with ether, then evaporation of the ether yielded a residue which readily re-oxidized in methanol solution with $Cu(OAc)_2$ catalysis. The *N*-hydroxy amine was re-oxidized by more than 90% (ESR.) after stirring vigorously in air for 1 h. Alternatively, methanol solutions of the *N*-hydroxy-amine re-oxidized overnight in air without an added catalyst to the extent of 80% (ESR.).

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