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SPIN TRAPPING USING 2,2-DIMETHYL-2H-IMIDAZOLE-1-OXIDES

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The ability of novel cyclic nitrones, 4-substituted 2,2-dimethyl-2H-imidazole-1-oxides (IMO's) to trap a variety of short-lived free radicals has been investigated using ESR spectroscopy. IMO's scavenge oxygen-, carbon- and sulfur-derived free radicals to give persistent nitroxides. Compared to the spin trap 5,5-dimethyl-pyrroline-1-oxide, a higher lifetime of hydroxyl radical adducts and a higher selectivity related to the trapping of carbon-centered radicals was found. A reaction between IMO's and superoxide was not observed. ESR parameters of 4-carboxyl-2,2-dimethyl-2H-imidazole-1-oxide (CIMO) spin adducts are highly sensitive to the structure of the trapped radical, e.g., different spectra were detected with radicals derived from Na_2SO_3 and $NaHSO_3$. From the data obtained, a successful application of these new spin traps in biological systems can be expected.

KEY WORDS: Free radicals; ESR; Spin trapping; 2H-imidazole-1-oxides (IMO's); Carbon-centered radical; Sulfite radical.

Abbreviations CC, carbon-centered; CDI, 4-Cyano-2,2-dimethyl-2H-imidazole-1-oxide; CIMO, 4-Carboxy-2,2-dimethyl-2H-imidazole-1-oxide; DETAPAC, diethylenetriamine-pentaacetic acid; DMPO, 5,5-dimethylpyrroline-1-oxide; ESR, electron spin resonance; GSH, glutathione; HRP, horseradish peroxidase; IMO, 4-substituted 2,2-dimethyl-2H-imidazole-1-oxide; MEMO, 2,2-dimethyl-4-methoxy-carbonyl-2H-imidazole-1-oxide; m.p., melting point; MW, molecular weight; TMI, 2,2,4-trimethyl-2H-imidazole-1-oxide.

INTRODUCTION

During the last two decades, ESR spectroscopy of spin trapped radicals has become the method of choice for the detection of free radicals formed in biological systems.[1,2,3] The universality of the spin trapping technique has been shown by investigations in model systems,[4,5] cells[6] and organs,[7] as well as by *in vivo* detection of free radicals.[8] However, there are still many problems connected with the application of this technique. The difficulties relate to (i) the physico-chemical properties of the spin traps themselves (e.g., solubility in water, stability, rate constants for trapping of various radicals), (ii) the properties of the spin adducts formed by reaction with biologically relevant radicals (e.g., lifetime, extractability, assignability of the ESR spectra) and (iii) the biological interactions of spin traps and spin adducts

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(e.g., toxicity, pharmacological effects). From this point of view, there is a need to design new spin traps which may overcome problems connected with the commonly used agents. As recently shown by Janzen and coworkers,[9,10] derivatization of spin traps like N-*t*-butyl-α-phenyl nitrone (PBN) is one promising approach in the synthesis of new traps with improved properties. Zhang and coworkers[11,12] used a similar approach, by synthesizing and characterizing derivatives of 5,5-dimethyl-pyrroline-1-oxide (DMPO).

The present paper describes the application of substituted 2H-imidazole-1-oxides (IMO's) as spin trapping compounds; first results with respect to spin trapping by these substances were presented recently.[13] Special emphasis was given here to the reactions of IMO's with sulfur-derived radicals. The hyperfine splitting constants of various types of spin adducts were determined and the lifetime of adducts was compared with that of the corresponding DMPO adducts.

MATERIALS AND METHODS

Chemicals

DMPO (Aldrich) was purified by double distillation. Horseradish peroxidase (HRP, EC 1.11.1.7), xanthine oxidase (EC 1.1.3.22) were from Boehringer Mannheim, cysteine and sodium sulfite were purchased from Merck, Darmstadt. 4-Cyano-2,2-dimethyl-2H-imidazole-1-oxide (CDI) was obtained from the Institute of Organic Chemistry, Novosibirsk, Russia.

All commercial chemicals were of the highest quality available.

Synthesis of 4-Substituted 2,2-Dimethyl-2H-imidazole-1-oxides

4-Carboxy-2,2-dimethyl-2H-imidazole-1-oxide (CIMO) was synthesized in three steps starting from CDI. Briefly: a mixture of CDI (1 g, 7.3 mmol), 50 ml MeOH and 735 μl triethylamine was stirred for 1 h at room temperature. The solution was evaporated *i. vac.* and the residue recrystallized from *n*-hexane/ethylacetate yielding 0.97 g 2,2-dimethyl-4-methoxyiminoyl-2H-imidazole-1-oxide (78.6%), m.p. 119–121°C (120–122°C[14]). This compound (0.97 g, 5.7 mmol) was dissolved in 20 ml MeOH, and 2% aq. HCl was added at 4°C to adjust to pH 1. After 5 minutes, MeOH was evaporated *i. vac.*, and the residue was extracted with $CHCl_3$. Extracts were washed with water, dried over Na_2SO_4, evaporated *i. vac.*, and the residue was crystallized from *n*-hexane/ethylacetate yielding 0.45 g (46%) 2,2-dimethyl-4-methoxy-carbonyl-2H-imidazole-1-oxide (MEMO), m.p. 78–80°C (80–82°C.[14]) The mixture of MEMO (0.2 g, 1.18 mmol), 6 ml EtOH and 1.3 ml 1N NaOH was stirred for 10 min at room temperature, filtered, evaporated *i. vac.* to 1 ml and chromatographed on silica gel plate (Chromatotron®), eluent: HCl_3/MeOH/NH_3 75:22.5:2.5 (v/v), yielding 0.125 g (58.2%) CIMO as the ammonium salt, m.p. 164–167°C (dec.).

2,2,4-Trimethyl-2H-imidazole-1-oxide (TMI) was prepared according to Kirilyuk *et al.*[14] by stirring the mixture of pyruvaldehyde-1-oxim (1.74 g, 20 mmol), 26.6 ml acetone (360 mmol), ammonium acetate (5.4 g, 70 mmol) and 8.0 ml acetic acid (140 mmol) for 72 h at room temperature. After pouring the mixture into 350 ml of water and stirring the solution for 30 min it was extracted using $CHCl_3$ (3 × 100 ml). The $CHCl_3$-extracts were collected, washed with water, dried over

Na_2SO_4, evaporated *i. vac.*, and the residue was crystallized from *n*-hexane under cooling. Yield 0.8 g (31.8%), m.p. 27–29°C (27–29°C.[14])

Analysis of 4-carboxy-2,2-dimethyl-2H-imidazole-l-oxide

$C_6H_{11}N_3O_3$. MS, *m/z* 167 $[M + H]^+$. UV (ethanol) $\lambda_{max} = 293$ nm.

Characterization of Synthesized Compounds

Preparative silica gel chromatographic plates were made by standard procedures. Melting point values were uncorrected and were obtained on a Boetius melting point apparatus. Infrared spectra were recorded on a Specord-75 and UV spectra on a Specord UV/VIS spectrometer (both from Carl Zeiss Jena). Electron spray injection mass spectra were obtained using a Finnigan MAT TSQ 700 spectrometer. ^1H NMR spectra were recorded on a Varian Gemini 200 (200MHz) spectrometer. Measurements of C, H, and N gave results which were within ±0.4% of their theoretical values. Yields have not been optimized.

Generation of Radicals

The generation of the free radicals was performed in an isotonic solution of NaCl; the pH was adjusted to 7.4 (if not stated otherwise). Recording of the ESR spectra was started 1 min after mixing of the components or end of irradiation, respectively.

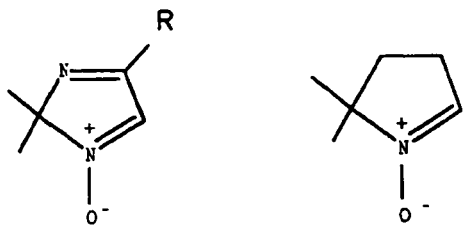
ESR Experiments

ESR experiments were performed on a Bruker ECS 106 X-band spectrometer (equipped with a high sensitivity rectangular-mode cavity ER 4102 ST) operating under the following standard conditions: modulation frequency, 100 kHz; modulation amplitude, 0.1 mT; field set, 348 mT; scan range, 10 mT; microwave power, 20 mW. The samples were placed into a flat quartz cell.

RESULTS

The structures of DMPO and of the novel spin traps used are shown in Figure 1. The main structural difference results from the introduction of the imino group into the 3,4-position of the nitrone cycle. This approach allows the synthesis of various 4-substituted analogs.

Figure 2 demonstrates the ESR spectra of hydroxyl (a) and carbon-centered (b) radical adducts of CIMO. It was found that CIMO (Figure 2 b) has a relatively high reactivity towards carbon-centered (CC) radicals compared to DMPO (Figure 2c). This observation is also confirmed by the spectra shown in Figure 3: When HRP and H_2O_2 were added to a solution of cysteine, DMPO (Figure 3a) trapped mainly ·OH radicals (superimposed on another signal which was not identified) whereas in the presence of CIMO (as well as MEMO and CDI, spectra not shown) CC radicals were trapped in addition to ·OH (Figure 3b). The trapping of free radicals formed by autoxidation of sulfite solutions gave very interesting results. Addition of DMPO to both solutions of sulfite and bisulfite resulted in the formation of the sulfite radical adduct and detection of the corresponding ESR signal (Figure 4a). Using



CIMO: 4 - carboxy - 2,2 - dimethyl - 2H - imidazole - 1 - oxide
R = COOH *(MW=156)*

TMI: 2,2,4 - trimethyl - 2H - imidazole - 1 - oxide
R = CH_3 *(MW=126)*

MEMO: 2,2 - dimethyl - 4 - methoxycarbonyl - 2H - imidazole - 1 - oxide
R = COOMe *(MW=170)*

CDI: 4 - cyano - 2,2 - dimethyl - 2H - imidazole - 1 - oxide
R = CN *(MW=137)*

DMPO: 5,5 - dimethylpyrroline - 1 - oxide
(MW=113)

FIGURE 1 Structure of spin traps

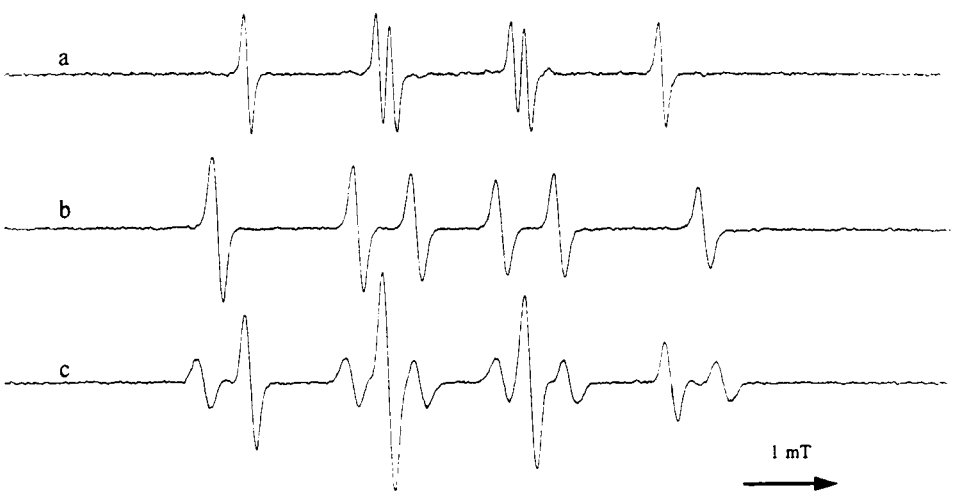


FIGURE 2 ESR spectra of spin adducts of CIMO with hydroxyl (a), carbon-centered radical (b) and DMPO adducts (c) generated by Fenton's reagent in the presence of 10% (v/v) EtOH (experimental conditions according to footnote of Tab. 1, receiver gain, $5 \cdot 10^4$)

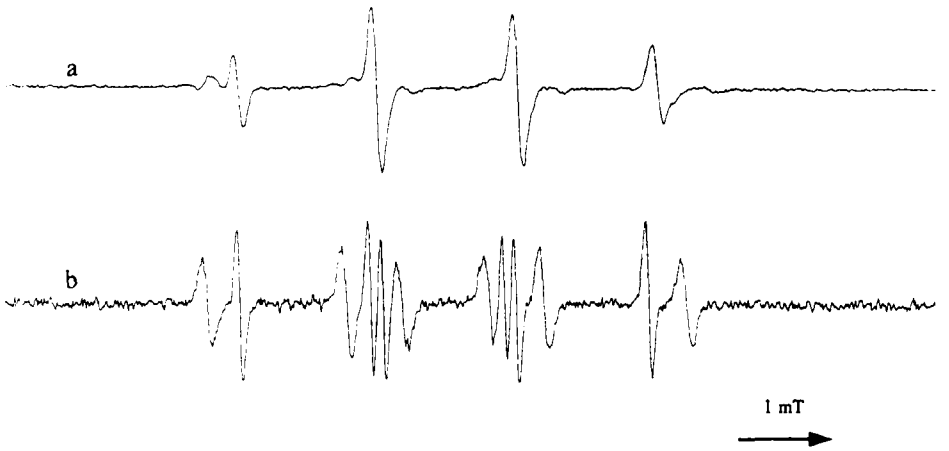


FIGURE 3 ESR spectra of spin adducts generated by addition of horseradish peroxidase/H_2O_2 to a solution of cysteine in the presence of DMPO (a) and CIMO (b) (experimental conditions according to footnote of Table 1, receiver gain, $5 \cdot 10^4$ [a], $2 \cdot 10^5$ [b])

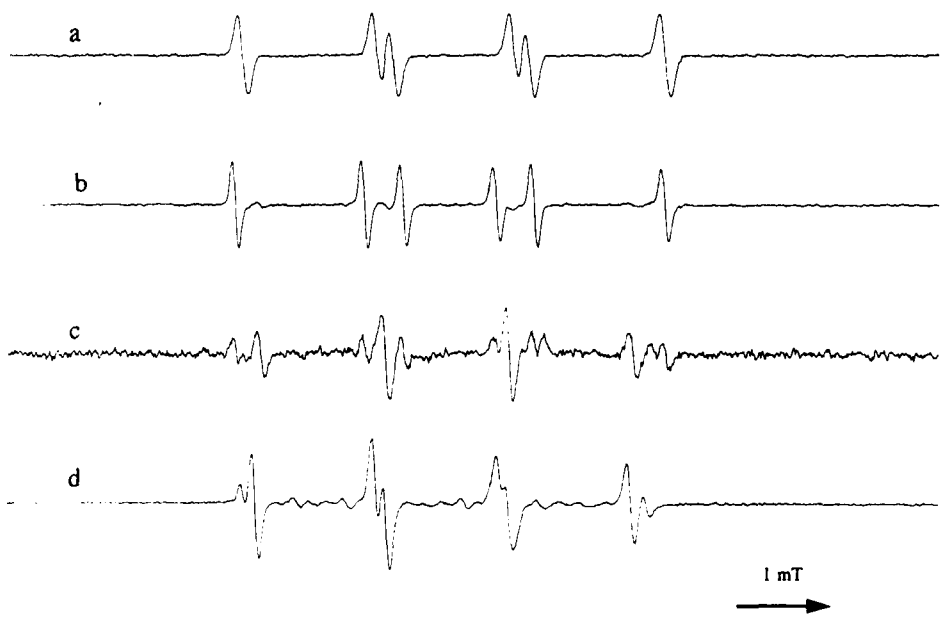


FIGURE 4 ESR spectra of sulfite radical adducts of DMPO (a), CIMO (b) and adducts formed with CIMO in solutions of bisulfite (c) and after UV-photolysis of persulfate (d) (experimental conditions according to footnote of Table 1, receiver gain, $5 \cdot 10^4$ [a, b, d], $2 \cdot 10^5$ [c])

CIMO, different adducts were observed: a sulfite radical adduct in solutions of Na_2SO_3 (Figure 4b), and an adduct assigned to a bisulfite radical in $NaHSO_3$ solutions (Figure 4c). When solutions of persulfate were subjected to UV radiation (experimental conditions according to Table 1) in the presence of CIMO, a signal with

TABLE I

ESR spectra hyperfine splitting constants (given in mT) of adducts of DMPO and various substituted 2H-imidazole-1-oxides with different types of radicals (*, very low intensity; n.i., adduct not identified; §, a_H^{γ})

Generation of radicals†	Adduct	DMPO		CIMO		TMI		MEMO		CDI	
		a_N	a_H^{β}	a_N	a_H^{β}	a_N	a_H^{β}	a_N	a_H^{β}	a_N	a_H^{β}
Fe(II)-H_2O_2 (A)	·OH	1.49	1.50	1.43	1.56	1.42	1.63	1.40	1.50	1.39	1.54
Fe^{2+}/H_2O_2/EtOH(B)	·CH(OH)CH_3	1.59	2.30	1.515	2.13	1.52	2.16	1.50	1.99	1.48	2.03
HX/XO(C)	·OOH	1.17	1.43/ 0.13§	–	–	–	–	–	–	–	–
CYS/HRP/H_2O_2(D)	·OH	1.49	1.50	1.42	1.545	1.415	1.63	1.41	1.49	1.38	1.5
	Cysteinyl	n.i.*		1.515	2.10	–	–	1.49	2.01*	1.46	2.02*
GSH/UV(E)	Glutathionyl (?)	various products of photolytic degradation		1.335	1.57	–	–	1.32	1.52	–	–
				1.43	1.93	1.40	1.80	1.42	2.10*		
Autox. Sulfite(F) (pH ~ 8 . . . 9)	$SO_3^{\cdot -}$	1.46	1.60	1.39	1.80	1.31	1.43	–	–	1.515	1.40
						n.i.					
Autox. Bisulfite(G)	$HSO_3^{\cdot}/SO_3^{\cdot -}$	1.46	1.60	1.33	1.33	–	–	–	–	–	–
(pH ~ 5 . . . 6)		–	–	1.40	1.80	1.39	1.80	–	–	–	–
$S_2O_8^{2-}/UV$(H)	·OH	1.50	1.50	–	–	–	–	1.40	1.50*	–	–
(pH ~ 1)	$SO_4^{\cdot -}$(?)	–	–	1.30	1.40	–	–	–	–	–	–

Model systems for the generation of the radicals were as follows:

†(A) OH radical: 100 μM H_2O_2, 50 μM $FeSO_4$, 10 mM spin trap

(B) CC radicals: Fenton's reagent (see a) in the presence of 10% (v/v) EtOH, 10 mM spin trap

(C) superoxide: 0.4 U/ml xanthine oxidase, 400 μM hypoxanthine, 1 mM DETAPAC, 100 mM spin trap

(D) cysteinyl: 25 mM cysteine, 2000 U/ml HRP, 0.5 mM H_2O_2, 100 mM spin trap

(E) glutathionyl: 25 mM GSH, 100 mM spin trap, 1'UV radiation (λ_{max} = 254 nm)

(F) sulfite: 500 mM Na_2SO_3, 100 mM spin trap

(G) bisulfite: 500 mM $NaHSO_3$, 100 mM spin trap

(H) sulfate (?): 500 mM $(NH_4)_2S_2O_8$, 100 mM spin trap, 1' UV radiation (λ_{max} = 254 nm)

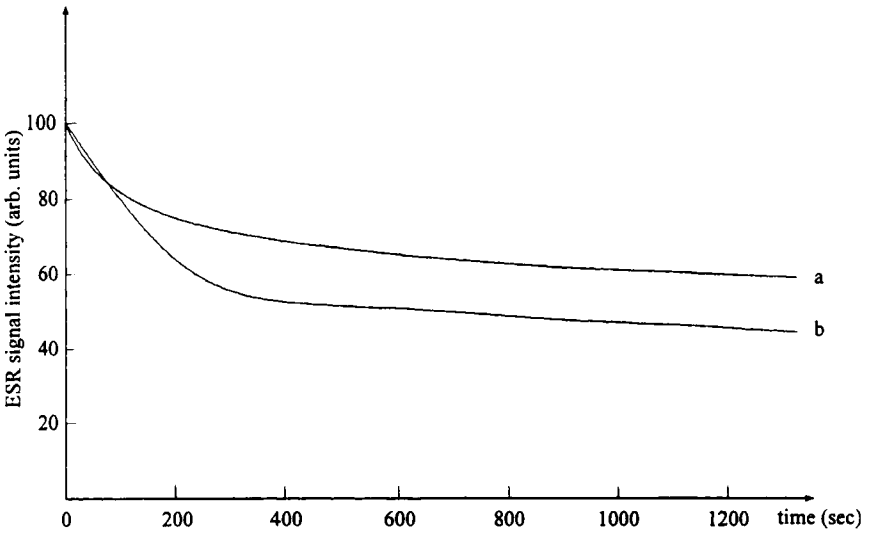


FIGURE 5 Kinetics of ESR signal intensity (normalized time scan) of (a) CIMO-OH and DMPO-OH (b) formed by Fenton's reagent (experimental conditions according to footnote of Table 1, receiver gain, $8 \cdot 10^4$)

the hyperfine splitting $a_N = 1.30$ mT and $a_H^\beta = 1.40$ mT was found (Figure 4 d).

Hyperfine splitting constants of the ESR spectra are summarized in Table 1. It is important to note that the 2H-imidazole-1-oxides tested do not trap superoxide radicals. The lifetime of the OH adducts formed with DMPO and CIMO is compared in Figure 5. In this experiment, a slower decrease as well as a higher plateau of the concentration was detected for CIMO-OH compared to DMPO-OH.

DISCUSSION

It has been found recently that the oxidation of several substituted 2H-imidazole-1-oxides with PbO_2 in MeOH yields 5,5-dimethoxy-3-imidazoline-1-oxyles.[14] The introduction of the imino-group into the 3,4-position of the nitrone ring of pyrroline-N-oxides leads to the 2H-imidazole system, which is expected to show lower reactivity and higher selectivity towards the addition of free radicals and to influence the β-hydrogen hyperfine splitting constant of the spin adduct.

It was reported by Iannone *et al.*[15] that the stability of five-membered ring nitrones is generally higher than the stability of six-membered rings. Comparing various nitroxide radicals in their study, those based on five-membered rings containing a carboxyl group were found to be most stable within cells. Thus, for application in biological systems, a good stability can be expected for IMO's such as CIMO, as well as for the lifetime of their adducts.

In the experiments presented here, the applicability of several 2H-imidazole-1-oxides for the trapping of different radicals was investigated. Spectra of the IMO adducts were assigned to specific radicals, by using DMPO adduct spectral data which were generated in our lab and/or obtained from the literature[2] for comparison. In general, the results confirm that these novel cyclic nitrones can be used

for the detection of free radicals in model systems. It has been shown that various types of radicals, i.e., oxygen-centered, carbon-centered and sulfur-centered species, were trapped with sufficient reactivity. As comparative experiments were performed with DMPO under identical conditions, it can be stated that the reactivity of the 2H-imidazole-1-oxides towards ·OH radicals is lower compared to DMPO. This lower reactivity of IMO's can be compensated by a higher lifetime of the corresponding adducts as shown in Figure 5 (time scan of OH adduct signal intensity in aqueous solutions).

However, there is an important difference compared to DMPO: as expected, a very low reactivity of the IMO's was found with superoxide radicals. Actually, all attempts to detect superoxide with these substances failed. From the point of view that superoxide is the primary species in radical-mediated processes in living systems, this property of the IMO's has to be regarded as a disadvantage. On the other hand, superoxide is in most cases the initiating molecule leading to the generation of more reactive and potentially injurious radicals (i.e., hydroxyl and carbon-centered radicals) which can be detected with IMO's.

A very high selectivity was found for the reaction of 2H-imidazole-1-oxides with carbon-centered radicals. Under identical conditions (generation of α-hydroxyethyl radicals by Fenton's reagent in the presence of 10% [v/v] EtOH), only about 30% of the concentration of DMPO spin adducts were formed with CC radicals whereas CIMO exclusively detects CC radicals in this model system. Moreover, the total concentration of carbon-centered radical adducts was significantly increased. In this context, an interesting effect was also observed concerning the trapping of (cysteinyl) CC radicals generated by reaction of cysteine with HRP/H_2O_2: CC radicals were trapped by CIMO, MEMO and CDI but not by DMPO and TMI. This effect might be due to the fact that among the imidazole traps investigated, TMI has the greatest structural similarity to DMPO with respect to the substitution.

As indicated in the literature,[16] mainly DMPO-OH was produced by UV-irradiation of persulfate. Due to hydrolysis of DMPO-SO_4^-, only traces of this adduct were found (spectra not shown). Under identical conditions, two different signals were detected unambigously with CIMO. The signal with lower intensity seems to correspond to CIMO-OH. Provided that the other signal results from the formation of CIMO-SO_4^-, it can be concluded that this adduct does not undergo hydrolysis as fast as DMPO-SO_4^-. The trapping of sulfite-derived radicals (formed by autoxidation of sulfite and bisulfite solutions, respectively) revealed striking differences between DMPO and CIMO, too: the sulfite radical adduct was detected by DMPO in both sulfite and bisulfite solutions whereas different adducts were found under these conditions with CIMO. The signal with $a_N = a_H^\beta = 1.33$ mT (Figure 4c) is supposed to be derived from the formation of a bisulfite radical; attempts to confirm this assumption by mass spectrometry failed due to the relatively low concentration of the adduct.

Summarizing the data obtained it can be concluded that, in spite of open questions regarding the trapping of certain radicals, the application of substituted 2H-imidazole-N-oxides might be a promising approach for the trapping of free radicals in biological systems. Compared to "classical" spin traps, special advantages can be expected with respect to the trapping of carbon-centered and sulfur-derived radicals.

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