Vol. 202, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1994 Pages 195-203

SPIN TRAPPING OF NITRIC OXIDE BY NITRONYLNITROXIDES: MEASUREMENT OF THE ACTIVITY OF NO SYNTHASE FROM RAT CEREBELLUM

Yakov Yu. Woldman[2], Valery V. Khramtsov[1*], Igor A. Grigor'ev[3], Igor A. Kiriljuk[3] and Darkhan I. Utepbergenov[4]

[1]Institute of Chemical Kinetics & Combustion,
[2]Institute of Molecular Pathology & Environmental Biochemistry,
[3]Novosibirsk Institute of Organic Chemistry,
[4]Novosibirsk State University, Novosibirsk 630090, Russia

Received May 24, 1994

Nitric oxide (NO) has been shown to be an important mediator in vasodilation, neurotransmission and cellular cytotoxicity. We investigated a new series of nitronylnitroxyl radicals (NNR) as spin traps for NO. It was found these radicals react with NO with rate constants of about 10^4 $M^{-1}c^{-1}$ forming stable iminonitroxides with dramatic changes in EPR spectra. To overcome fast reduction of the radicals (a few seconds in rat cerebella cytosol), NNR with charged trimethylammoniophenyl group (Ib) was incorporated into the inner volume of large unilamellar phosphatidylcholine liposomes. In this case the reduction of the radical Ib in rat cerebella cytosol is slow (ca. 1% per min). The rate of NO production by NO synthase from rat cerebellum measured by NNR, Ib, is in a reasonable agreement with that obtained by spectrophotometric method.

Nitric oxide, NO, is known as messenger molecule with very important functions in the physiology of mammalian cardiovascular, immune and nervous systems (1-3). NO synthases (NOS) constitute an expanding family of unique enzymes responsible for the biosynthesis of nitric oxide in mammalian cells. NO and citrulline are formed through the enzymatic oxidation of L-arginine at the expense of NADPH and molecular oxygen (4,5). The methods of NO detection in biological systems are limited because of its short half-life from less than 1 to several seconds (6,7). These methods are mostly indirect such as

*To whom correspondence should be addressed. Fax: (007)3832-352350.

0006-291X/94 $5.00

those measuring stable metabolites (nitrite, nitrate, L-citrulline), chemical reaction products as methemoglobin, or biological activity (intracellular cyclic GMP, smooth muscle relaxation, inhibition of platelet aggregation). Despite of the fact that NO is a simple radical, it cannot be detected by EPR under normal circumstances, though solid-state spectra have been reported at 77 K (8). Therefore the methods of NO detection in solution by EPR are based on the trapping of nitric oxide with formation of comparatively stable paramagnetic species. Among them there are trapping of NO by oxyhemoglobin, HbFeII, with formation of the nitrosyl derivative $HbFe^{II}$-NO (9); NO trapping by metal-chelator complexes consisting of reduced iron (Fe^{2+}) and dithiocarbamate derivatives (10,11); conversion of nitric oxide into stable nitroxide using 2,5-dimethylhexadiene (12); trapping of NO by nitronylnitroxides with formation of stable iminonitroxides (13,14). The EPR detection of stable nitroxyl (12) or iminonitroxyl (13,14) radicals as the products of the reactions with NO seems to meet a serious limitation due to the reduction of these radicals in biological samples.

In this paper, we propose a new series of nitronylnitroxyl radical (NNR) as spin traps for NO. It was found these radicals react with NO with rate constants of about 10^4 $M^{-1}c^{-1}$ forming stable iminonitroxides (INR) with dramatic changes in EPR spectra. To overcome the fast reduction of NNR and INR in biological samples (a few seconds in rat cerebella cytosol), the NNR with charged trimethylammoniophenyl group (Ib) was incorporated into the inner volume of large unilamellar phosphatidylcholine liposomes. It allows to increase the radical lifetime up to 1 hour and to apply this EPR approach for NO quantitation in biological samples. Indeed, the rates of NO production by NO synthase from rat cerebellum measured by EPR and spectrophotometric method are in a reasonable agreement.

A high efficiency of NO trapping by NNR could be useful for a therapeutic regulation of nitric oxide in case of its overproduction and cytotoxic action in some diseases and disorders, including septic shock and multiorgan failure. The prolongation of neutralization effect of NNR against nitric oxide using their application in liposomes is of principal interest.

MATERIALS AND METHODS

Reagents. NADPH (Reanal, Hungary), superoxide dismutase (iron-containing enzyme, Sigma), hemoglobin (bovine, 2 x cryst., Koch-Light), egg yolk lecithin (standard 10% solution in ethanol,

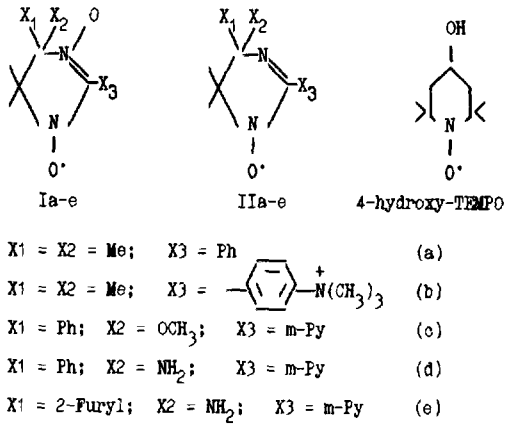


Fig.1. Structures of nitronylnitroxides, Ia-e, iminonitroxides, IIa-e, and ditretalkylnitroxide, 4-hydroxy-TEMPO.

Russia), 3-(4-morpholino)-sydnonimine hydrochloride (SIN-1) (Cassella-Hoechst, Germany), sodium dithionite (Merck, Germany), arginine hydrochloride (Serva), were used.

Spin probes (see fig.1). The radicals Ia and IIa were synthesized following the procedure Ullman and coworkers (15), the radicals Ic-e and IIc-e were synthesized according to ref.(16).

Synthesis of 4,4,5,5-Tetramethyl-2-[4-trimethylammoniophenyl]-2-imidazoline-3-oxide-1-yloxy methyl sulfate (Ib) and 4,4,5,5-Tetramethyl-2-[4-trimethylammoniophenyl]-2-imidazoline-1-yloxy methyl sulfate (IIb). To a stirred solution of 2.0 g (0.0135 mole) of 2,3-bis-hydroxyamino-2,3-dimethylbutane in 40 ml of methanol 3.8 g (0.0138 mole) of 4-trimethylammoniophenyl carboxaldehyde methyl sulfate was added. The mixture was stirred for 5 min to form homogeneous solution and allowed to stand for 48 h at room temperature (25°C). The resulting solution was diluted with 40 ml of methanol and 10 g (0.0418 mole) of lead dioxide was added. The mixture was stirred for 30 min, then precipitate of lead oxides was removed by filtration and washed with methanol, and the filtrate was evaporated in vacuum. The residue was treated with isopropanol and the precipitate formed was isolated by filtration and washed with isopropanol. The isopropanol solutions were used to isolate the iminonitroxide (IIb) (see below). The solid obtained was dissolved in methanol and thoroughly filtered to remove a trace amount of lead oxides and the solution was evaporated in vacuum. The resulting solid was recrystallized from the mixture of methanol and isopropanol, 1:1, for 3 times to give 1.5 g (0.0036 mole, 26%) of nitronyl nitroxide Ib, m.p. 193-203°C (dec.), IR spectrum (KBr, 0.25%, l=1 mm): 725, 1000, 1050, 1220, 1260, 1365, 1390, 1480 cm-1; UV spectrum in H2O, λ_{max}, nm, ($lg\varepsilon$): 570 (2.89), 360 (4.03), 255 (4.04). Elemental analysis: Found C 50.5, H 7.3, N 10.5, S 8.2, $C_{16}H_{28}N_3O_6S$, Calculated C 50.7, H 7.0, N 10.4, S 8.0. Mother solutions and the above mentioned isopropanol solutions were evaporated and the residue were recrystallized from isopropanol to give iminonitroxide (IIb), m.p 164-168°C, IR spectrum (KBr,

0.25%, l=1 mm): 740, 1000, 1050, 1210, 1240, 1365, 1490, 1600 cm-1; UV spectrum in H2O, λ_{max}, nm, (lg ε): 425 (2.60), 300 (3.49), 255 (3.41), 225 (4.06). Elemental analysis: Found C 53.1, H 7.5, N 11.0, S 8.2, $C_{16}H_{28}N_3O_6S$, Calculated C 52.8, H 7.3, N 10.9, S 8.3.

IR and UV spectra of the compounds Ib and IIb were recorded on the spectrometers Specord M20 and Specord UV VIS, respectively.

NO gas was obtained by dropwise addition of $NaNO_2$ (40% in H_2O) to the $FeSO_4$ solution (20%) in HCl (1:1). Gas was purified from higher oxides by passing through NaOH solution.

Bioassay system for NO generation. Rats (Wistar, 150-200 g) were decapitated, cerebellum was removed and homogenized. Homogenate was centrifuged at 4^0C, 18,000g x 20 min. Supernatant was used as a source of NO synthase (17). The protein concentration in the samples was determined by Lowry method (18). Nitric oxide generation by rat cerebella cytosol was initiated by adding the NADPH and arginine in the final concentrations of 0.5 mM to cytosol of about 3-6 mg/ml of protein.

Spectrophotometric measurement of NO generation. Activity of NO synthase as well as NO liberation by SIN-1 was determined by spectrophotometric method (19). Oxyhemoglobin was obtained by adding a few crystals (0.1 mg) of sodium dithionite to methemoglobin solution (0.1 mM), then the solution is freed from excess of reductant by passing through column of G-25 Sephadex. Samples for activity analysis contain 3 μM HbO_2, 2mM $CaCl_2$, 0.5 mM arginine, 0.5 mM NADPH, and appropriate volume of rat cerebella cytosol in the volume of 1 ml total. Absorption difference between 401 and 421 nm was measured vs time, differential extinction coefficient 77200 $M^{-1}cm^{-1}$ being used.

EPR measurement of NO generation. The liberation of NO by SIN-1 as well as NO generation by NO synthase was measured following the EPR signal of both NNR, Ib, and INR, IIb (see fig.2). The radical Ib incorporated into the inner volume of liposomes (see below) was used for NO synthase activity determination. The samples for measurement of the activity of NO synthase are the same as in spectrophotometric studies. EPR spectra were recorded on an ER-200D-SRC (Bruker) spectrometer.

Liposome preparation. Large unilamellar liposomes from egg phospatidylcholine were prepared according to ref.(20). Liposomes with radical Ib were prepared in 0.1 M K-phosphate buffer, pH 7.5, lipid concentration 24 mg/ml, radical concentration 1 mM. The radical, not present in the inner liposomal volume, was removed as follows. The liposomes were centrifuged at 14,000 g, 0^0C for 2 min, supernatant was removed and sedimented liposomes were diluted with 0.07 M K-phosphate buffer, pH 7.5, to the same volume. The procedure was repeated 5 times. As a result the concentration of trapped radical was about 50-100 μM. Efflux of the radicals from the liposomes membrane was tested by measuring of decrease in EPR signal after addition of 50 mM of $K_3Fe(CN)_6$. Reduction of the radicals in the presence of rat cerebella cytosol was tested by the same manner but cytosol instead of ferricyanide was added.

Spectrophotometric stopped-flow experiments were carried out on stop-flow unit for Hitachi-557 apparatus (Hitachi, Japan) in double wavelength mode. Reference wavelength was 750 nm for all cases, the second one was established on the absorption maximum of the radical (556-590nm). Measuring time was 2 s, deadtime is equal to 20 ms. The reaction of NO with the radicals Ia-e was initiated by mixing the 0.1 M K-phosphate buffer solution (pH

7.5) of the radicals and the same buffer saturated with NO. Before mixing both solutions were bubbled with argon, then the solution without the radicals was saturated with NO.

RESULTS AND DISCUSSION

The EPR spectra of the radicals Ia-e consist of a quintet typical for NNR with an intensity ratio 1:2:3:2:1 due to the similar hyperfine interaction constants with N1 and N3 (fig.2a). It was found earlier that treatment of NNR with nitric oxide (13,14,21)

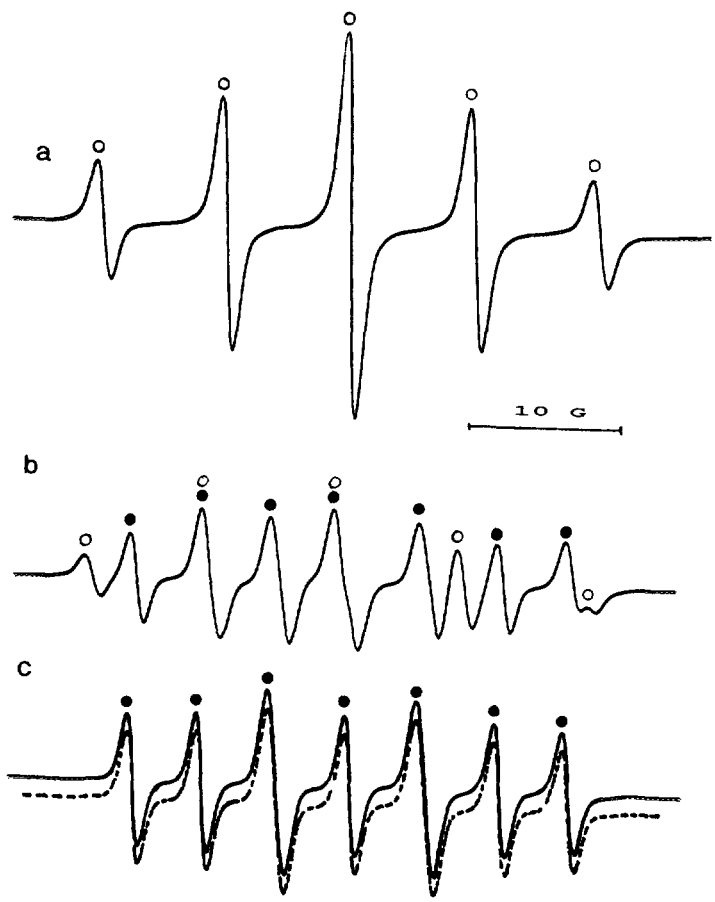
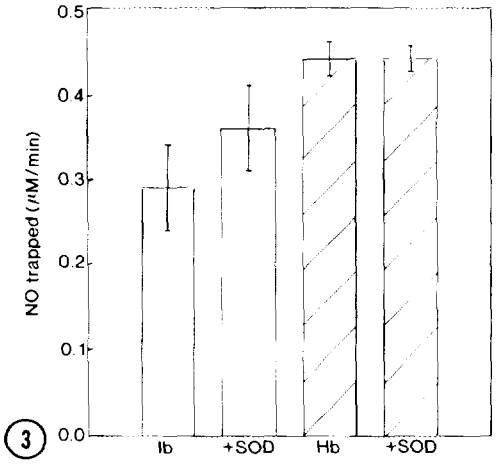


Fig.2. EPR spectra of 100 μM solution of NNR Ib in 0.1 M K-phosphate buffer, pH 7.56, before (a) and after addition of 0.5 mg/ml of sydnonimine SIN-1: 30 min (b) and 50 min (c). The dotted spectrum (c) is EPR signal of 100 μM solution of INR IIb. Hyperfine interaction constants determined by comparison of experimental and simulated spectra are $a_{N1}=a_{N3}=8.1$ G for Ib (a) and $a_{N1}=9.8$ G, $a_{N3}=4.6$ G for IIb (c). Symbols o and • denote the line positions of radicals Ib and IIb, respectively. The spectrometer settings were as follows: microwave power, 10 mW; modulation amplitude, 0.8 G; receiver gain $5x10_3$.

leads to the formation of INR with EPR spectra of seven or nine lines due to the inequivalent nitrogens. Indeed, the time evolution of EPR spectrum of the solution of radical **Ib** under the conditions of NO liberation by sydnonimine SIN-1 shows the appearance of EPR signal of INR, **IIb** (fig.2b). Moreover, the EPR spectrum of **Ib** obtained 50 min after SIN-1 addition coincides with the spectrum of solution of INR, **IIb** (fig.2c), at the same concentration. It supports the stoichiometry 1:1 between **Ib** and **IIb** in according with references (13,14). The measurement of NO liberation by SIN-1 using ratio of normalized peak intensities of **Ib** and **IIb** gives a reasonable agreement with the data obtained by spectrophotometric method at the concentration of **Ib** higher than 0.3 mM (fig.3). However at low concentration of the NNR, **Ia-e**, the efficiency of NO trapping in the presence od oxygen is lower (for example for **Ib** at 100 μM, as in fig.2, it is equal to 0.6).

The stoichiometry between NO and NNR (1:1) at radical concentration higher than 0.3 mM even in the presence of dissolved oxygen is based on the high reactivity of NNR with NO in agreement with (14). To study the kinetics of the reaction of NNR **Ia-e** with NO we have used stopped-flow spectrophotometric method following the absorbance at 570-590 nm ($\varepsilon \approx 10^3$) for NNR. Fig.4 demonstrates the kinetics of decrease of radical concentration in the reaction of **Ib** with NO added in buffer solution. Note that stoichiometry between NO and NNR measured from the plateau level of the kinetics (ca.2:1, fig.4) is in agreement with that observed for the reactions of NNR with NO at high concentration of nitroprusside as donor of nitric oxide (13). This stoichiometry was explained by fast recombination of NO with NO_2 formed in the reaction: NNR + NO → INR + NO_2 (13). Indeed, it was found by the computer simulation of the kinetics that recombination reaction has to be considered to meet an agreement with the experimental data (fig.4). The rate constants for the reaction of NNR **Ia-e** with NO were determined to be $0.59 \times 10^4 M^{-1}s^{-1}$ for **Ia**, $0.6 \times 10^4 M^{-1}s^{-1}$ for **Ib**, $0.64 \times 10^4 M^{-1}s^{-1}$ for **Ic**, $0.82 \times 10^4 M^{-1}s^{-1}$ for **Id**, $1.1 \times 10^4 M^{-1}s^{-1}$ for **Ie**. The obtained rate constant of the reaction of radical **Ia** with NO is in a good agreement with that measured by stopped-flow EPR method (0.515×10^4 $M^{-1}s^{-1}$ (14)). Note that at low rate of NO liberation (up to 3 μM/min of NO) by SIN-1 or by nitroprusside (13) the stoichiometry between NNR and NO is 1:1 (see fig.3). It is due to low concentration of formed NO_2 which does not compete with NNR in the reaction with NO.



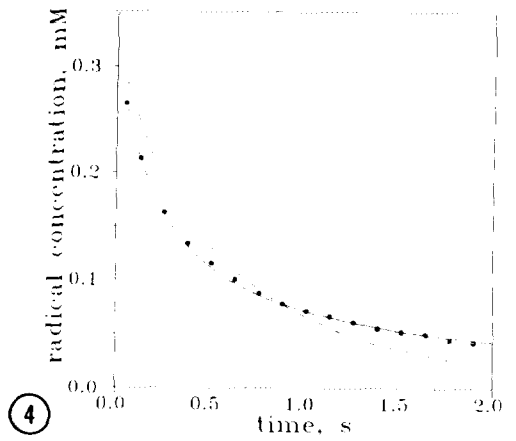


Fig.3. Rates of NO liberation by SIN-1 (0.025 mg/ml) in 0.1 M K-phosphate buffer, pH 7.6 at 22°C measured using radical Ib (□) and oxyhemoglobin (▨). Note that superoxide dismutase (60 U/ml) is affected on the data obtained by EPR due to the simultaneous liberation of superoxide and NO by SIN-1 (23) and the fast reaction of superoxide radical with NO with rate constant of 6.7×10^9 $M^{-1}c^{-1}$ (24).

Fig.4. Kinetics of the radical concentration decrease in the reaction of Ib, 0.32 mM, with NO, 0.6 mM, in 0.1 M K-phosphate buffer at 22°C (●). The solid and dotted curves are non-linear least-squares fits of the data to the numerical solution of the following kinetics schemes. The first one considerates only the reaction **Ib + NO → IIb + NO_2** ($k=0.34 \times 10^4 M^{-1}s^{-1}$, dotted curve); the second one taking into account the recombination of formed NO_2 and NO with rate constant of 6.7×10^9 $M^{-1}c^{-1}$ (24) (solid curve, $k=0.6 \times 10^4 M^{-1}s^{-1}$).

The present data demonstrate the possibility of NO measurement in solution using NNR 1a-e, including on-line detection of NO production. The sensitivity of the approach is 0.03 μM/min of NO production in a flat quartz cell of 0.2 ml. However we met a serious limitation of the method to be applied for the measurement of NO production by NO synthase from rat cerebellum. Namely, the radicals Ia-e were reduced in solutions of rat cerebella cytosol (1-3 mg of protein per ml) in a few seconds with loss of EPR signal. To compare the reduction rates of different types of nitroxides we used model system with thioglycerol as reductant. It was found the rates of the reduction by thioglycerol of NNR and INR to be much higher of that for ditretalkyl nitroxide, hydroxy-TEMPO (see Table 1). This limitation requires an additional defence of NNR against the reduction for their application in biological systems. For that

Table 1. Initial reduction rates of the radicals of different types by thioglycerol in 0.1 M K-phosphate buffer, pH 7.5, at 22°C

Radical, 50 μM	Thioglycerol, mM	Initial rate, nM/s
4-hydroxy-TEMPO	2.5	2.4 ± 0.3
1b	2.5	175 ± 20
2b	0.23	5700 ± 600

purpose we have used charged NNR, **Ib**, incorporated into the inner volume of large unilamellar phospatidylcholine liposomes. It was found low penetration of the liposomes for both NNR, **Ib** (ca.5% per h), and corresponding INR, **IIb** (ca.20% per h). The reduction rates of the radicals in liposomes in rat cerebella cytosol (3 mg of protein per ml) were found to be ca.1% per min for **Ib** and ca.4% per 1 min for **IIb**, which allows to follow their EPR signal during arginine-dependent production of NO. Indeed, it was found the rate of formation of the INR, **IIb**, in rat cerebella cytosol (6 mg/ml of protein) is equal to 0.07 μM/min. Taking into account the efficiency of NO trapping by **Ib** at the concentration used (6% of produced NO as was measured in the experiment with SIN-1 in the same homogenate solution) we found the NO synthase activity equal to 0.28 μM/min per mg of protein. Note that two factors contribute in the NO trapping efficiency of **Ib**: low radical concentration (factor 0.3 in the samples without homogenate for 60 μM of **Ib**) and loss of NO in side reactions in homogenate solution (factor 0.2). The obtained value of NO production in rat cerebella cytosol is in a good agreement with the value of 0.25 μM/min per mg of protein measured by spectrophotometric method.

These results demonstrate a high efficiency of NO trapping by series of new NNR and their application for NO synthase activity measurement by EPR in rat cerebella cytosol. It seems that efficiency of NO trapping by NNR in biological samples can be increased by introduction in their structure negatively charged substitutors as sulfonic acid due to decreasing the rates of radical penetration across liposomal membranes. Note that we described earlier synthesis of different types of NNR (16,22), which allows the introduction of charged substitutors both in 2nd and 4th position of radical heterocycle.

Recently it was found that NNR effectively antagonize NO produced in biological systems such as vascular endothelium (14). The application of hydrophilic NNR incorporated in liposomes seems to be useful for prolongation of their antagonistic action.

ACKNOWLEDGMENTS: We thank Professor L.B.Volodarsky and Professor V.V.Ljakhovich for fruitful discussions.

REFERENCES

1. Moncada,S. Palmer,R.M.J., and Higgs,E.A. (1991) Pharmacol. Rev., 43, 109-142.
2. Nathan,C.(1992) FASEB J. 6, 3051-3064.
3. Bredt,D.S., and Snyde,S.H. (1992) Neuron 8, 3-11.
4. Bush,P.A., Gonzalez,N.E., and Ignarro,L.J. (1992) Biochem. Biophys.Res.Commun. 186, 308-314.
5. Pufahl,R.A., and Marletta,M.A. (1993) Biochem.Biophys.Res. Commun. 186, 308-314.
6. Ignarro,L.J. (1990) Annu.Rev.Pharmacol.Toxicol. 30, 535-560.
7. Taha,Z., Kiechle,F., and Malinski,T. (1992) Biochem.Biophys. Res.Commun., 188, 734-739.
8. Bloom,N.B.D., Raynor,J.B., and Symons,M.C.R., (1972) J.Chem. Soc. A, 3843-3846.
9. Naschimento,L.M., Neto,L.M., and Wajnberg,J. (1991) J.Chem. Phys. 95, 2265-2270.
10. Mordvintcev,P., Mulsh,A., Busse,R., and Vanin,A. (1991) Anal. Biochem. 199, 142-146.
11. Komarov,A., Mattson,D., Jones,M.M., Singh,P.K., and Lai,C.-S. (1993) Biochem.Biophys.Res.Commun. 195, 1191-1198.
12. Gabr,I.M., Rai,U.S., and Symons,M.C.R. (1993) J.Chem.Sos., Chem.Commun., 1099-1100.
13. Joseph,J., Kalyanaraman,B., and Hyde,J.S. (1993) Biochem. Biophys.Res.Commun. 192, 926-934.
14. Akaike,T., Yoshida, M.,Miyamoto, Y., Sato,K., Kohno,M., Sasamoto,K., Miyazaki,K., Ueda,S., and Maeda,H. (1993) Biochemistry 32, 827-832.
15. Ullman,E.F., and Osiecki,J.H. (1970) J.Org.Chem. 35, 3623-3631.
16. Grigor'ev,I., Starichenko, V., Kiriljuk,I., and Volodarsky, L.B. (1989) Izv. AN SSSR, ser.khim. (Russia), 661-667.
17. Mittal,C.K. (1993) Biochem.Biophys.Res.Commun. 193, 126-132.
18. Lowry,O.H., Rosebrough,N.J., Farr,A.L., and Randall,R.G.(1963) J.Biol.Chem. 193, 265-275.
19. Doyle,M.P. and Hoekstra,J.W.(1981) J.Inorg.Biochem.14, 351-358.
20. Szoka,S.Ir., and Papahaijopoulos,D. (1978) Proc.Natl.Acad. Sci. USA, 7, 4194-4198.
21. Nadeau,J.S., and Boocock,D.G.B.(1977) Anal.Chem. 49, 1672-1676
22. Khramtsov,V., Weiner, L. Gogolev, A. Grigor'ev, I. Starichenko V., and Volodarsky,L.B. (1986) Magn.Reson.Chem. 24, 199-207.
23. Darley-Usmar,V.M., Hogg,N., O'Leary,V.J., Wilson,M.T., and Moncada,S. (1992) Free Rad.Res.Commun. 17, 9-20.
24. Huie,R.E., and Padmaja,S.(1993) Free Rad.Res.Commun.18,195-199.