Synthesis, characterisation, cytotoxicity and radioprotective effect of novel chiral nitronyl nitroxyl radicals

Xiang-Yang Qin[a], Gui-Rong Ding[b], Xiao-Wu Wang[c], Juan Tan[b], Guo-Zhen Guo[b] and Xiao-Li Sun[a]

[a]Department of Chemistry, [b]Department of Radiation Medicine, Fourth Military Medical University, Xi'an, Shanxi 710032, P.R. China

Nitroxyl compounds have been previously investigated as potential radioprotection drugs. To develop new radioprotectors, two kinds of novel chiral nitronyl nitroxyl radicals: *L*-*tert*-butyl 2-(4, 5-dihydro-4, 4, 5, 5-tetramethyl-3-oxido-1*H*-imidazol-3-ium-1-oxyl-2-yl) pyrrolidine-1-carboxylate (*L*-NNP) and *L*-*tert*-butyl 2-[(4-(4, 5–dihydro-4,4,5,5–tetramethyl–3–oxido-1*H*-imidazol-3-ium-1-oxyl-2-yl)-2-methoxyphenoxy)methyl] pyrrolidine-1-carboxylate (*L*-NNVP) have been synthesised. The cytotoxic and radioprotective effects of these two compounds were then evaluated in rat glioma C6 cells.

Keywords: chiral nitronyl nitroxyl radicals, cytotoxicity, radioprotective effect

In recent years, stable nitronyl nitroxyl radicals, have been extensively studied as a unique and interesting class of antioxidants, for protection against oxidative damage.[1-3] Cellular and *in vivo* pharmacological studies of stable nitroxyls proved that they can be reduced to hydroxylamines or oxidised to oxo-ammonium cations by electron-transfer reactions.[3,4] The redox transformations among the oxidation states of nitroxyl, hydroxylamine, and the oxoammonium cation are shown in Fig. 1.[5,6] Unlike other antioxidants which protect against radiation damage through stoichiometric reactions, nitroxyls can participate in redox reactions and self-replenish, thereby giving a catalytic protective activity.[7] At the same time, nitroxyls readily cross the blood–brain barrier and permeate the cell membrane.[8] These key features indicate the potential of nitroxyls against radiation-induced injury.

So far, Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl) has been shown to be one of the most promising radiation protectors.[9-12] In 2004, a phase II study of Tempol was initiated.[13] However, the results have not yet been reported.

Stable nitroxyls include two types: TEMPO (2,2,6,6-tetramethylpiperidine-*N*-oxyl) and NIT (nitronyl nitroxyl) (Fig.2).[14] At present, NIT radicals for radioprotective effects have not been well characterised. The structure of nitroxyls has an importance influence on their radioprotective activity.[5,15] In addition, biological molecules can discriminate between enantiomers.[16] To search for more effective radiation protectors with minimal cytotoxicity, two kinds of new chiral NIT nitroxyl compounds (*L*-NNP and *L*-NNVP), containing pyrrolidine moieties originating from natural *L*-proline, have been synthesised (Fig.3). Their cytotoxicity and protective effects were evaluated by MTT[17] (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenpyltetrazolium bromide) assay in C6 cells.

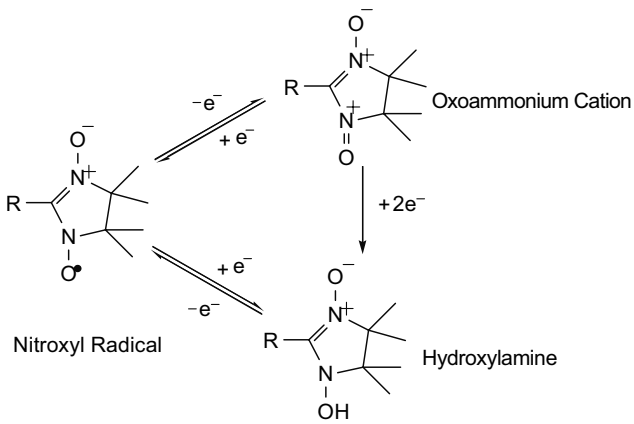


Fig. 1 The redox transformation of the various oxidation states of nitroxyls.

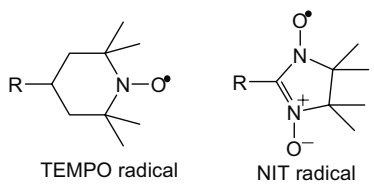


Fig. 2 Two types of nitroxyls.

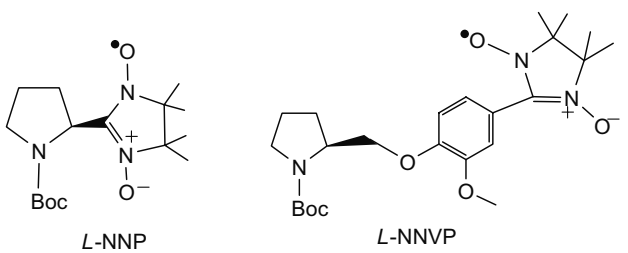


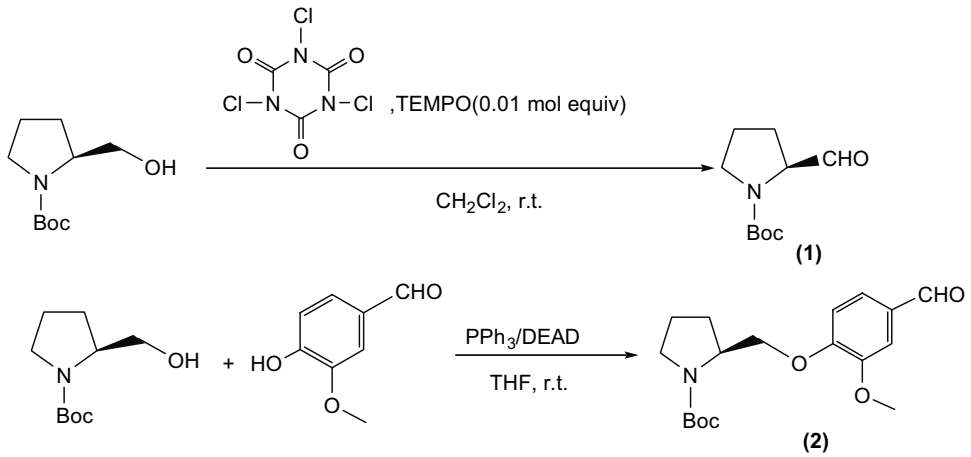
Fig. 3 The structure of chiral nitroxyls (*L*-NNP and *L*-NNVP).

Results and discussion

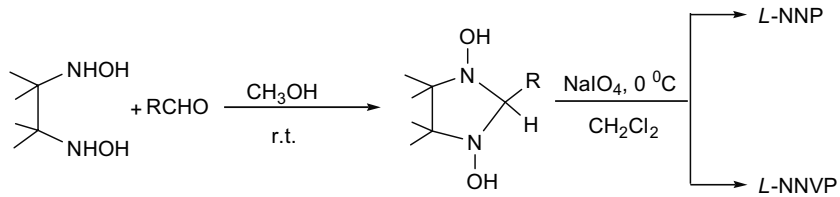
According to Ullman's pioneering work,[18] any aldehyde may give rise to a nitronyl nitroxyl. Therefore, the first step is to obtain chiral aldehydes. As shown in Scheme 1, the precursor of *L*-NNP, aldehyde (**1**) [*L*-*tert*-butyl 2-formylpyrrolidine-1-carboxylate] was obtained by rapid oxidation of *L*-N-Boc-prolinol under mild conditions with trichloroisocyanuric acid in the presence of catalytic TEMPO.[19] The precursor of *L*-NNVP, aldehyde (**2**) {*L*-*tert*-butyl 2-[(4-formyl-2-methoxyphenoxy) methyl] pyrrolidine-1-carboxylate} was obtained by an intermolecular dehydration reaction between *L*-N-Boc- prolinol and vanillin.[20-22] To purify aldehyde (**2**), the side-products triphenylphosphine oxide and diethyl hydrazinedicarboxylate were precipitated by ether and then filtered off. The filtrate was evaporated under reduced pressure and the crude product was purified by chromatography on silica.

The chiral aldehydes as described above were subsequently subjected to condensation with 2,3-bis(hydroxylamino)-2,3-dimethylbutane to generate the corresponding *N, N′*-dihydroxyimidazolidines as stable white solids from methanol solution at room temperature. The target compounds, *L*-NNP and *L*-NNVP, were obtained after $NaIO_4$ was used to oxidise *N, N′*-dihydroxyimidazolidines at 0 °C as shown in

* Correspondent. E-mail: qinxiangyang@fmu.edu.cn



Scheme 1



Scheme 2

Scheme 2. One of the key steps in the synthesis of nitronyl nitroxyl radicals was the oxidation of the *N, N′*-dihydroxyimidazolidines. While PbO_2 was usually used as an oxidant, it is not only time-consuming but also it is difficult to observe the progress of the reaction due to the colour of PbO_2. However, when $NaIO_4$ was used as an oxidant, observation became much easier despite the overoxidation product obtained. Thus, we used aqueous $NaIO_4$ as the best choice of oxidant to give the corresponding chiral nitronyl nitroxyl radicals, provided that the amount of $NaIO_4$ and the time of the oxidation reaction was strictly controlled and the reaction was carried out in an immiscible solvent such as dichloromethane in which the product was soluble.

ESR spectroscopy is the best tool for the study of free radicals, due to its sensitivity and accuracy. As shown in Fig. 4, the ESR spectra of the two types of radicals show five major lines in the ratio 1 : 2 : 3 : 2 : 1, which is expected for coupling of two identical nitrogens.

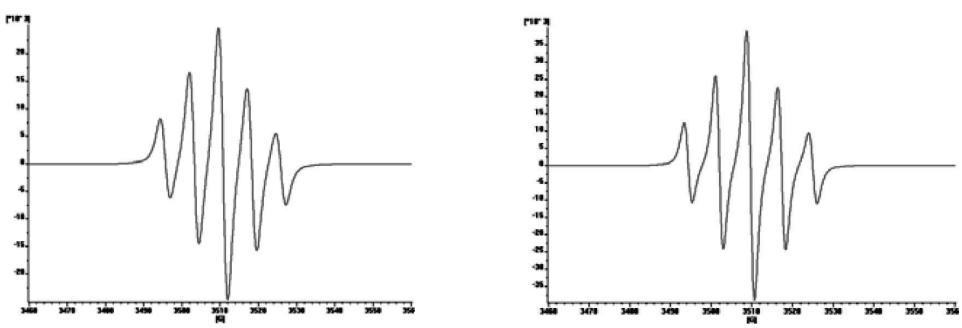
The UV-vis absorption spectra of the two compounds *L*-NNP and *L*-NNVP were determined in dichloromethane at room temperature. They presented absorption in the UV at 320 nm and 360 nm respectively, arising from π–π* transitions of the ONCNO units.[23]

The IR spectra of *L*-NNP and *L*-NNVP are noteworthy in that the characteristic band was observed at 1372 nm and 1358 nm, arising from the N–O stretching frequency.[24]

Experimental

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AV-300 NMR spectrometer (400 MHz) (Bruker, Karlsruhe, Germany), with $CDCl_3$ as solvent and TMS as internal reference. Optical rotation values were measured at 20 °C on a PerkinElmer 343 polarimeter (PerkinElmer Bodenseewerk, Überlingen, Germany). High resolution mass spectroscopy (HRMS) was carried out on a Varian 7.0T ESI-FTICR-MS (Varian. USA) instrument. IR was recorded on a Bruker tensor27 (Bruker optics, German) instrument. Absorption spectra were recorded on a Jasco V-570 UV/vis/NIR (Jasco, Japan) spectrophotometer. The EPR spectra were obtained using a Bruker ESP 300E spectrometer on solutions of radicals dissolved in dichloromethane. Elemental analyses were carried out using a Perkin–Elmer analyser model 240C (PerkinElmer, America).

2,3-bis(hydroxylamino)-2,3-dimethylbutane was prepared by the published method.[25] *N*-Boc-*L*-prolinol was prepared in our laboratory. All other chemical reagents were purchased from the Nanjing Tianzun Zezhong Chemical Limited Company (Nanjing, China). THF was distilled under N_2 from Na/benzophenone and CH_2Cl_2 was distilled from CaH_2. The other chemical reagents were used without further purification.



L-NNP *L*-NNVP

Fig.4 ESR spectra of nitroxyls in dichloromethane.

Synthesis of L -tert-butyl 2-formylpyrrolidine-1-carboxylate

To a mixture of *N*-Boc-*L*-prolinol (1.9 g, 9.5 mmol) and trichloroisocyanuric acid (2.3 g, 10 mmol), CH_2Cl_2 (20 mL) was added, and the mixture was stirred and maintained at 0 °C for 5 min, followed by addition of TEMPO (0.015 g, 0.1 mmol). Then the mixture was warmed to room temperature and stirred for 20 min and filtered on Celite. The precipitate was washed with CH_2Cl_2 (10 mL × 2). The combined organic phases were washed with 15 mL of a saturated solution of Na_2CO_3 and then by 0.1 N HCl (20 mL) and brine (20 mL). The organic phase was dried over Na_2SO_4, and stripped of solvent. The crude product was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (1 : 10) as eluant, giving the product as a colourless liquid (1.68 g, 88.8%). MS(*m/z*): 222.11 (MNa^+); $[\alpha]_D^{20}$ = –102.3 (*c* = 1.1, CH_2Cl_2); IR(KBr)cm^{-1}: 1396, 1363, 2977, 1478 (ν CH_3), 1165, 1123, 912, 858, 772 (ν C–C ring of pyrrolidine), 2711, 2810, 1736 (ν CHO), 1255, 1696 (ν ester bond and amide bond); ^{1}H NMR($CDCl_3$): δ 9.47 (d, 1H, *J* = 2.7 Hz, –CHO), 4.21 (m, 1H, –CH), 3.47 (t, 2H, $–CH_2$), 2.00 (m, 2H, $–CH_2$), 1.87 (m, 2H, $–CH_2$), 1.42 (s, 9H, $–CH_3$); ^{13}C NMR δ($CDCl_3$): 200.4, 155.8, 80.6, 65.0, 46.7, 28.2, 26.7, 23.9; Anal. Calcd for $C_{10}H_{17}NO_3$: C, 60.28; H, 8.60; N, 7.03. Found: C, 60.17; H, 8.66; N, 6.89%.

Synthesis of L-tert-butyl 2-((4-formyl-2-methoxyphenoxy) methyl) pyrrolidine-1-carboxylate

Triphenyl phosphine (1.5 g, 6 mmol), vanillin (0.9 g, 6 mmol) and *N*-Boc-*L*-prolinol (1.33 g, 6.6 mmol) were dissolved in dry THF (45 mL) with vigorous stirring under an atmosphere of nitrogen at 0 °C. A solution of diethyl azodicarboxylate (DEAD) (1 g, 6 mmol) in dry THF (10 mL) was added dropwise over a period of 1 h at 0 °C. The mixture was warmed to room temperature and stirred for 12 h. THF was removed under reduced pressure. Ether was added to the residue to precipitate triphenylphosphine oxide and diethyl hydrazinedicarboxylate, which were then filtered off. The filtrate was evaporated. The crude product was purified by column chromatography on silica gel using hexane/acetone (5 : 1) as eluant, giving a colourless oil product (1.66 g, 82.3%). MS(*m/z*): 358.16(MNa^+); $[\alpha]_D^{20}$ = –67.2 (*c* = 1.1, CH_2Cl_2); IR(KBr)cm^{-1}: 1396, 1366, 2974, 2878 (ν CH_3), 1167, 1136, 1024, 910 (ν C–C ring of pyrrolidine), 1688, 2833, 2723 (ν CHO), 1688, 1342, 1310, 1270 (ν ester bond and amide bond), 1587, 1510, 866, 811, 731 (ν benzene ring); ^{1}H NMR($CDCl_3$): δ 9.80 (s, 1H, –CHO), 7.37 (d, 1H, *J* = 3.0 Hz, –ArH), 7.28 (s, 1H, –ArH), 7.04 (d, 1H, *J* = 6.0 Hz, – ArH), 4.27 (d, 2H, $–CH_2$), 4.04 (m, 1H, –CH), 3.87(s, 3H, $–CH_3$), 3.38 (t, 2H, $–CH_2$), 2.06 (m, 2H, $–CH_2$), 1.83 (m, 2H, $–CH_2$), 1.43 (s, 9H, $–CH_3$); ^{13}C NMR δ($CDCl_3$): 190.98, 154.8, 153.9, 149.8, 129.9, 126.5, 111.8, 109.0, 79.9, 68.7, 60.0, 55.9, 46.9, 28.4, 23.6, 22.6. Anal. Calcd for $C_{18}H_{25}NO_5$: C, 64.46; H, 7.51; N, 4.18. Found: C, 64.29; H, 7.37; N, 4.05%.

Synthesis of L–NNP; General procedure for synthesis of nitronyl nitroxyl radicals

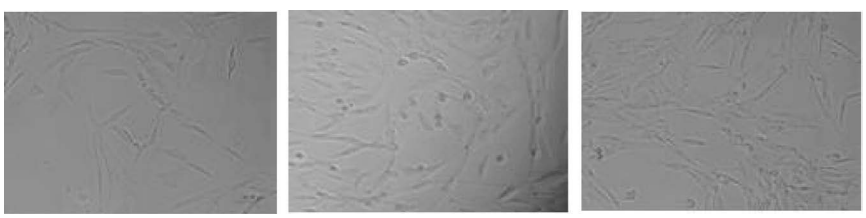
A solution of *L-tert*-butyl 2-formylpyrrolidine-1-carboxylate (0.5 g, 2.5 mmol) and 2,3-bis (hydroxylamino)-2,3-dimethylbutane (0.37 g, 2.5 mmol) in methanol (20 mL) was stirred at room temperature for 6 h (reaction periods of 24 h were used for aldehyde (**2**)). After the reaction was completed, the methanol was removed and the residue was suspended in 40 mL dichlormethane, aqueous $NaIO_4$ (0.53 g, 2.5 mmol in 20 mL) was added dropwise over a period of 5 min at 0 °C, and the mixture was stirred for a further 2 min at 0 °C. The organic layer was dried over anhydrous Na_2SO_4. The deep red solution was then evaporated. The crude product was purified by column chromatography on silica gel using absolute ether/petroleum/acetone (3 : 1 : 0.5) as eluent, giving a deep red solid product (445.3 mg, 54.6%); MS(*m/z*): 349.19 (MNa^+); IR(KBr)cm^{-1}: 1372, 1345, 1156 (ν NO), 1396, 1372, 2882, 2979 (ν CH_3), 1696, 1282, 1251 (ν ester bond and amide bond), 1112, 915, 858, 776 (ν C–C ring of pyrrolidine); UV($\lambda_{max}^{CH_2Cl_2}$): 333 (ONCNO, π→π*, ε = 1.6 × 10^4 $mol^{-1}\cdot cm^{-1}$), 530, 571 (n→π*). EPR (CH_2Cl_2): g factor, 2.0032; a_N (2N), 7.57 G. Anal. Calcd for $C_{16}H_{28}N_3O_4$: C, 58.87; H, 8.65; N, 12.87. Found: C, 58.68; H, 8.51; N, 12.72%. As anticipated, nitronyl nitroxyl radicals showed no NMR signal.

L-NNVP

Dark blue oil product; MS (*m/z*): 485.24 (MNa^+); IR(KBr)cm^{-1}:1358, 1125, 1168 (ν NO), 1391, 2976, 2876 (ν CH_3), 1232, 1026, 909 (ν C–C ring of pyrrolidine), 1692, 1327, 1267 (ν ester bond and amide bond), 1599, 1491, 1530, 1455, 867, 810 (ν benzene ring); UV-vis ($\lambda_{max}^{CH_2Cl_2}$): 289 (benzene ring, π→π*), 342 (ε = 4.4 × 10^3 $mol^{-1}\cdot cm^{-1}$), 368 (ONCNO, π→π*), 560—630 (n→π*). EPR (CH_2Cl_2): g factor, 2.0032; a_N (2N), 7.68 G. Anal. Calcd for $C_{24}H_{36}N_3O_6$: C, 62.32; H, 7.84; N, 9.08. Found: C, 62.19; H, 7.92; N, 9.11%.

Cytotoxicity study

Rat glioma C6 cells were cultured at 37 °C under a humidified atmosphere of 5% CO_2 in DMEM (Dulbecco's Modified Eagle Media) medium supplemented with 10% fetal serum. To study the cytotoxicity of the compounds, cells were dispersed and seeded in 96-well plates with 1 × 10^3 cells/well overnight. Two compounds with different concentrations were then added. PBS (phosphate buffer solution) was used as control. Four replicates were used for each concentration. After 72 h exposure to the compounds, cell viability was determined by the MTT assay.



12Gy-48h-PBS 12Gy-48h- *L*-NNVP 12Gy-48h- *L*-NNP

Fig. 5 The cell morphology.The typical photographs were taken at 48 h after 12 Gy radiation with or without *L*-NNVP (15.63 μg/ml) or *L*-NNP(125 μg/ml) pretreatment. Bar: 40μm.

Table 1 Radioprotective effects of *L*-NNP and *L*-NNVP at the different radiation dosages(*n*=4)

Compd	Drug concentration/μg·mL^{-1}	OD values ($\bar{\chi}$±SD)		
		8Gy	10Gy	12Gy
L-NNVP	125	0.47±0.17	0.74±0.04	0.69±0.05[a]
	62.5	0.54±0.09	0.70±0.04	0.71±0.09[a]
	31.25	0.56±0.005[a]	0.89±0.08[a]	0.70±0.06[a]
	15.63	0.58±0.11[a]	0.75±0.07	0.68±0.05[a]
L-NNP	125	0.49±0.06	0.69±0.17	0.64±0.02[a]
	62.5	0.57±0.07	0.77±0.02[a]	0.61±0.09
	31.25	0.55±0.06	0.84±0.13[a]	0.59±0.07
	15.63	0.74±0.07[a]	0.72±0.07	0.61±0.08
PBS		0.48±0.04	0.64±0.08	0.55±0.08

[a](P<0.05), PBS was used as control (n = 8).

It was found that *L*-NNP and *L*-NNVP at concentration less than 125 μg mL^{-1} and 250 μg mL^{-1} respectively had no effect on cell viability.

Radioprotective effects study

To study the radioprotective effects of *L*-NNP and *L*-NNVP, cells were dispersed and seeded in 96-well plates with 1×10^3 cells/well overnight. Fifteen minutes prior to irradiation, two compounds at four different concentrations (according to the results from compound cytotoxicity) were added to the medium (final concentrations were 15.625, 31.25, 62.5 and 125 μg mL^{-1}). The cells were irradiated with ^{60}Co γ-rays for 8, 10 and 12 Gy respectively. The dose rate used was about 4.64Gy min^{-1}. Cell viability was determined by the MTT assay. PBS was used as control. Four replicates were used for each concentration.

Treatment of cells with *L*-NNP and *L*-NNVP before irradiation at low concentrations (125, 62.5, 31.25 and 15.625μg mL^{-1}) effectively decreased γ-rays irradiation-induced cell death. With lower concentrations (31.25 and 15.625μg mL^{-1}), *L*-NNVP and *L*-NNP exhibited better radioprotective effects for lower dose irradiation (8 and 10 Gy) whereas with higher concentrations (125, 62.5μg mL^{-1}), *L*-NNP and *L*-NNVP exhibited better radioprotective effects for higher dose irradiation (12 Gy). The experimental results are listed in Table 1.

At 24 h and 48 h after irradiation at the different radiation dosages, the cell morphology was observed under an inverted microscope. The micrograph after 48 h at 12 Gy radiation dosages is shown in Fig. 5. As shown in morphology results, in the PBS group, only a few cells survived after radiation. However, the survival rate obviously increased when cells were treated with *L*-NNVP or *L*-NNP before irradiation (Fig. 5). These results suggest that the nitroxyl compounds *L*-NNVP or *L*-NNP provide radioprotective effects in C6 cells.

This work was supported by the National Science Foundation of China (No.20672141; No. 30670492).

Received 9 May 2009; accepted 14 June 2009
Paper 09/10573 doi: 10.3184/030823409X12474221035163
Published online: 10 August 2009

References

1 T. Goffman, D. Cuscela, J. Glass, S. Hahn, C.M. Krishna, G. Lupton and J.B. Mitchell, *Int. J. Radiat. Oncol. Biol. Phys.*, 1992, **22**, 803.
2 S. Goldstein, A. Samuni and A. Russo, *J. Am. Chem. Soc.*, 2003, **125**, 8364.
3 M.C. Krishna, A. Russo, J.B. Mitchell, S. Goldstein, H. Dafni and A.J. Samuni, *J. Biol. Chem.*, 1996, **271**, 26026.
4 M.C. Krishna, D.A. Grahame, A. Samuni, J.B. Mitchell and A. Russo, *Proc. Natl. Acad. Sci*. USA, 1992, **89**, 5537.
5 J.B. Mitchell, A. Russo, P. Kuppusamy and M.C. Krishna, *Ann. N. Y. Acad. Sci.,* 2000, **899**, 28.
6 W. Bi, J.H. Cai, P. Xue, Y.R. Zhang, S.G. Liu, X. Gao, M. Li, Z.B. Wang, M. Baudy-Floc'h, A.S, Green and L.R. Bi, *Bioorg. Med. Chem. Lett.*, 2008, **18**, 1788.
7 M.C. Krishna, A. Samuni, J. Taira, S. Goldstein, J.B. Mitchell and A. Russo, *J. Biol. Chem.*, 1996, **271**, 26018.
8 K. Anzai, M. Ueno, A. Yoshida, M. Furuse, W. Aung, I. Nakanishi, T. Moritake, K. Takashita and N. Ikota, *Free Radical. Biol. Med.,* 2006, **40**, 1170.
9 J.B. Mitchell, W. Degraff, D. Kaufman, M.C. Krishna, A. Samuni, E. Finkelstein, M.S. Ahn, S.M. Hahn, J. Gamson and A. Russo, *Arch. Biochem. Biophys.*, 1991, **289**, 62.
10 S.M. Hahn, L. Wilson, M.C. Krishna, J. Liebmann, W. Degraff, J. Gamson, A. Samuni, D. Venzon and J.B. Mitchell, *Radiat. Res.*, 1992, **132**, 87.
11 S.M. Hahn, Z. Tochner, M.C. Krishna, J. Glass, L. Wilson, A. Samuni, M. Sprague, D. Venzon, E. Glatstein, J.B. Mitchell and A. Russo, *Cancer Res*., 1992, **52**, 1750.
12 S.M. Hahn, F.J. Sullivan, A.M. Deluca, M.C. Krishna, N. Wersto, D. Venzon, A. Russo and J.B. Mitchell, *Free Radical. Biol. Med.,* 1997, **22**, 1211.
13 J.M. Metz, D. Smith, R. Mick, R. Lustig, J.B. Mitchell, M. Cherakuri, E. Glatstein and S.M. Hahn, *Clin. Cancer Res.*, 2004, **10**, 6411.
14 S. Nakatsuji and H. Anzai**,** *J. Mater. Chem.*, 1997, **7**, 2161.
15 B.P. Soule, F. Hyodo, K. Matsumoto, N.L. Simone, J.A. Cook, M.C. Krishna and J.B. Mitchell, *Free Radical. Biol. Med.,* 2007, **42**, 1632.
16 L.D. Barron, *Nature*, 2000, **405**, 895.
17 J.-E. Chung, S.Y. Kim, H.H. Jo, S.J. H. Wang, B. Chae, D.J. Kwon, Y.O. Lew, Y.-T. Lim, J.H. Kim, E.J. Kim, J.-H. Kim, M.-R. Kim, *Biochem. Biophys. Res. Commun.,* 2008, **375**, 420.
18 E.F. Ullman, J.H. Osiecki, D.G.B. Boocock and R. Darcy, *J. Am. Chem. Soc.,* 1972, **94**, 7049.
19 L.D.Luca, G. Giacomelli and A. Porcheddu, *Org. Lett.*, 2001, **3**, 3041.
20 O. Mitsunobu, *Synthesis*, 1981, 1.
21 O. Mitsunobu and M. Yamada, *Bull. Chem. Soc. Jpn,* 1967, **40**, 935.
22 O. Mitsunobu and M. Eguchi, *Bull. Chem. Soc. Jpn,* 1971, **44**, 3427.
23 M. Minguet, D.B. Amabilino, J. Cirujeda, K. Wurst, I. Mata, E. Molins, J.J. Novoa and J. Veciana, *Chem. Eur. J.*, 2000, **6**, 2350.
24 A.K. Hoffmann and A. Henderson, *J. Am. Chem. Soc.*, 1961, **83**, 4671.
25 M. Lamchen and T.W. Mittag, *J. Chem. Soc.(C)*, 1966, 2300.