Supplementary Material

Biomimetic chemistry on the protection of *cis* phospholipid from the thiyl radical isomerization by common antioxidants

Ioannis N. Lykakis,*[a] Carla Ferreri,*[b] and Chryssostomos Chatgilialoglu*[c]

[a] *Department of Chemistry, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece*
[b] *ISOF, Consiglio Nazionale delle Ricerche, Via P. Gobetti 101, 40129, Bologna, Italy*
[c] *Institute of Nanoscience and Nanotechnology, NCSR Demokritos, Agia Paraskevi Attikis 15310, Athens, Greece*
E-mail: lykakis@chem.auth.gr, c.chatgilialoglu@inn.demokritos.gr, cferreri@isof.cnr.it

Dedicated to Prof. Michael Orfanopoulos on the occasion of his 67th birthday and retirement, and for being a splendid teacher, scientist and friend

Table of contents

General methods

GC analysis for the determination of the isomeric ratio of the unsaturated fatty acids was performed after several reaction times. For this reason an Agilent 5860 gas chromatograph equipped with a flame ionization detector and a Rtx-2330 column (90% biscyanopropyl-10% phenylcyanopropyl polysiloxane capillary column; 60 m, 0.25 mm i.d., 0.25-mm film thickness) was used. Temperature started from 190 °C held for 15 min, followed by an increase of 30 °C/min up to 220 °C, held for 15 min, followed by a second increase of 30 °C/min up to 250 °C. Methyl esters of oleic and elaidic acids were identified by comparison with the retention times of authentic samples (*trans* isomers were identified by comparison with authentic samples, which were commercially available). When necessary, GC/MS spectra were recorded on a Hewlett-Packard GC 5890 (series II) coupled to a Hewlett-Packard mass selective detector model 5971A.

General experimental method

POPC was a gift from Chemi SpA. DOPC was purchased from Sigma-Aldrich. LUVET were prepared as earlier described using 2 mM PC. The suspension (1 mL) was then transferred to a 4 mL vial equipped with an open-top screw cap and a Teflon-faced septum, and saturated with N_2O or flushed by N_2. Continuous radiolysis was performed at room temperature (22± 2 °C) on 250 mL samples using a ^{60}Co-Gammacell, with dose rates between 8 and 10 Gy min^{-1}. The exact absorbed radiation dose was determined with the Fricke chemical dosimeter, by taking $G(Fe^{3+})$ = 1.56μmol J^{-1}. Workup and GC analyses of the irradiated reaction mixture were carried out as reported above.

Preparation and Isomerization of DOPC large unilamellar vesicles (LUV)

A. Extrusion methodology

A chloroform solution of DOPC (2 mL; 0.08 mmol of fatty acid content, MW=786.11) was evaporated to a thin film in a test tube under an argon stream and under vacuum for 30 min. Degassed milliQ water (1 mL) was added, and multilamellar vesicles were formed by vortex stirring for 7 min under an argon atmosphere. To obtain large unilamellar vesicle with extrusion technique (LUVET), the lipid emulsion was transferred into a LiposoFast (produced by Avestin, Inc.) and extruded through two polycarbonate membranes with a pore diameter of 100 nm. In each irradiation experiments, an aliquot from the PC suspension was added to a phosphate buffer (PB) solution, affording liposome dispersion with a final lipid concentration of 2.2 mM. 1mL of this final suspension was added into a vial equipped with an open top screw cap and a Teflon-faced septum and saturated with N_2O prior to γ-irradiation. $HOCH_2CH_2SH$ (MSH) (1 mM) and MeOH (40 mM) were consecutively added and the suspension was irradiated. Aliquots of 100 μL were withdrawn and processed at different times, by partitioning between 2/1 chloroform/methanol and brine, extraction and collection of the organic phases dried over anhydrous sodium sulfate, and evaporation of the solvent under vacuum at room temperature. The residue containing the phospholipids was treated with 0.5 M KOH/MeOH, for 10 min at room temperature and then poured into the brine and extracted with n-hexane. The organic layer

containing the corresponding fatty acid methyl esters was examined by GC analysis in comparison with the retention times of authentic samples.

In the presence of hydrophilic inhibitors, such as vitamin C and urate, a suspension of DOPC (1mL) was prepared as described previously and an exact amount of the stock solution containing the inhibitors was added. The final hydrophilic inhibitors concentrations were in the range of 20 μM to 180 μM. The addition volume of the inhibitors solution was not higher than 25 μL, in order the final concentrations of lipids and MSH were kept constants. For this reason, a stock solution of vitamin C and urate in phosphate buffer (pH = 7) were prepared in higher than 2 mM concentration for each one.

B. Methanolic injection

The injection method was used to prepare DOPC liposomes containing defined amounts of the lipophilic vitamin E, or all *trans* retinol acetate, as inhibitors. Stock solutions were prepared freshly and based on the methanol density by weighting in the exact amount of the solvent and immediately preventing further evaporation. Vitamins (antioxidant) were weighted and the solutions were stored in vials at –20 °C for the duration of one set of experiments. 50 μL of chloroform (HPLC) containing various amount of the vitamins were added to 100 μL of a methanolic stock solution with a concentration of 68 mM DOPC. The organic solution was evaporated under vacuum for 20 min and then 150 μL of methanol (HPLC) were added. This solution (150 μL, 45 mM DOPC) were injected with a Hamilton syringe under stirring into 3 ml of phosphate buffer at 40 °C (10 mM) affording a liposome dispersion with a final lipid concentration of 2.2 mM and an overall methanol amount of 4.7% (or 1.2 M). The final hydrophobic inhibitors concentrations were in the range of 5 μM to 120 μM. The suspensions were left under stirring for 15 min at 40 ^{o}C and another 15 min at RT.

The most important factor to control the vesicles size in the final mixture is the concentration of the phospholipids in the initial alcoholic solution injected in the phosphate buffer [30,32]. For this reason, herein a fast stirring and injection rate were kept constants, as well as different initial concentrations of phospholipids in methanolic solution were used. In our experiments MSH was used as the isomerising diffusible agent and the *cis*/*trans* lipids ratio was always compared to that obtained using the DOPC vesicle prepared by extrusion methodology after 40 min irradiation (Table S1, entry 6). After, several experiments the more closed system that gives similar *cis*/*trans* ratio to the one by extrusion methodology with methanol, was that where the lipid concentration in the initial methanolic solution was 40 mM and the final lipid concentration in PB was 2.2 mM (*cis*/*trans* = 33/67). In addition, a specific amount of *i*PrOH was added to the final suspension for both methodologies, but no any significant change in the *cis*-*trans* ratio was observed (Table S1, entries 4 and 7). Figure S1 shows also the disappearance of the oleate residue in DOPC vesicle irradiation, prepared by alcoholic injection methodology. For comparison reason, the *cis*-*trans* ratio of the isomerization process of DOPC vesicles prepared by the extrusion methodology was also presented.

Table S1. Preparation of different sized DOPC liposomes by extrusion methodology and methanolic injection, based on *cis-trans* isomerization ratio of the oleate residue

entries	Liposome suspension	[DOPC] in initial methanolic solution	oleate %	elaidate %
1	DOPC + MeOH (2.2 mM) (245 mM)	150 mM	91	9
2	DOPC + MeOH (2.2 mM) (570 mM)	85 mM	81	19
3	**DOPC + MeOH (2.2 mM) (1180 mM)**	**40 mM**	**33**	**67**
4	DOPC + MeOH + *i*-PrOH (2.2 mM) (1180 mM) (40 mM)	40 mM	29	71
5	DOPC + MeOH (2.4 mM) (570 mM)	-	43	57
6	**DOPC + MeOH (2.4 mM) (1180 mM)**	**-**	**30**	**70**
7	DOPC + *i*-PrOH (2.4 mM) (40 mM)	-	25	75

Entries 1–4: the vesicles were prepared with methanolic injection experiments; entries 5–7; the vesicles were prepared by extrusion methodology.

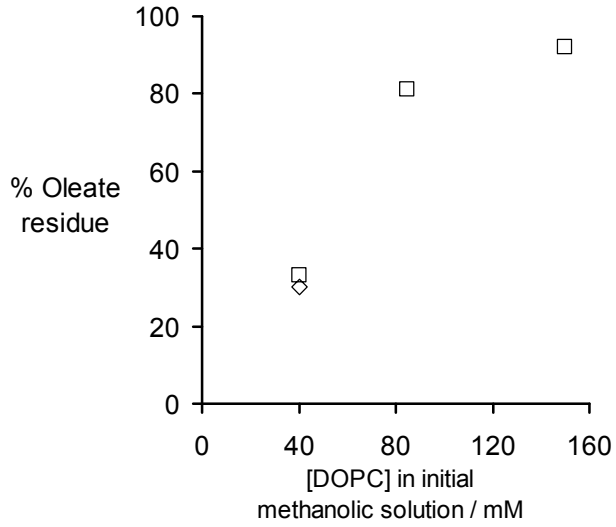

Figure S1. Methyl oleate residue disappearance, under 40 min gamma-radiation (10.2 Gy/min) in the presence 1.2 mM mercaptoethanol in N_2O-saturated flushed phosphate buffer solution at pH=7, based on different concentrations of DOPC in the initial methanolic solution, 40 mM, 85 mM and 150 mM (□), compared with the extrusion methodology (◊).

Table S2

Dose (Gy)	without vit-C	vit-C (0.06 mM)	vit-C (0.12 mM)	vit-C (0.18 mM)
0	100	100	100	100
53	73	98	98	99
105	56	91	96	98
210	41	68	92	96
315	37	52	84	92
420	34	44	68	80

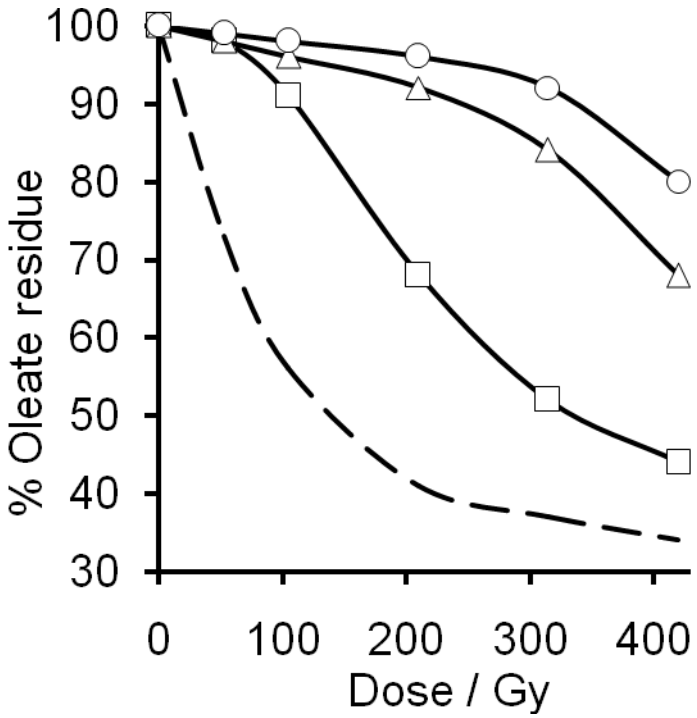

Figure S2. Disappearance of the *cis* isomer (oleate residue) upon γ-radiolysis (dose rate = 10.2 Gy/min) of N_2O-saturated phosphate buffer solution (pH = 7) of DOPC vesicles (2.2 mM) containing 1 mM $HOCH_2CH_2SH$ in the absence (dashed lines) and in the presence of L-ascorbic acid: (□) 60 μM, (△) 120 μM, (○) 180 μM.

Table S3

Dose	without urate	urate (0.06 mM)	urate (0.12 mM)	urate (0.18 mM)
0	100	100	100	100
53	73	81	92	89
105	52	67	85	81
160	44	58	76	72
210	41	53	69	66
315	37	46	58	56
420	34	42	52	50

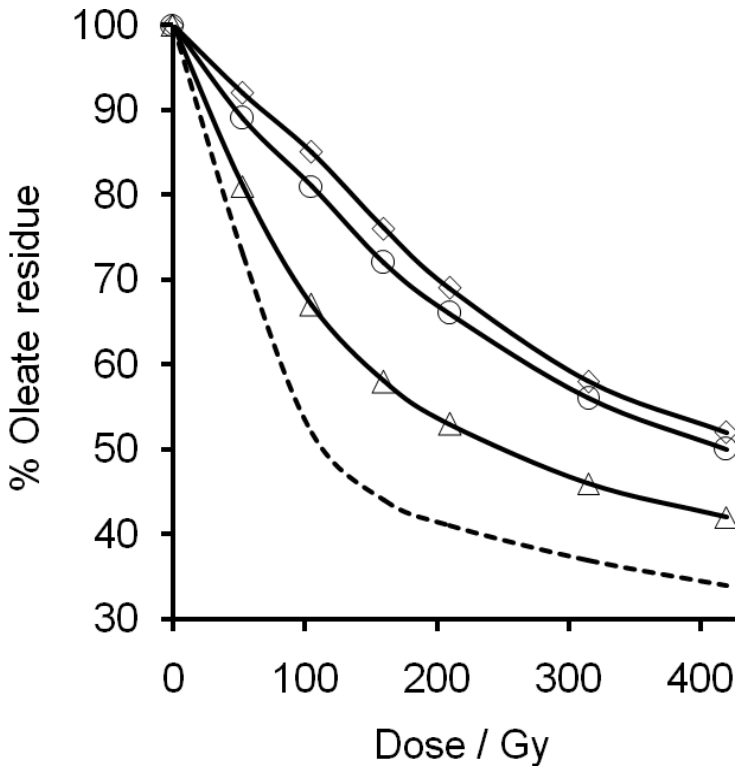

Figure S3. Disappearance of the *cis* isomer (oleate residue) upon γ-radiolysis (dose rate = 10.2 Gy/min) of N_2O-saturated phosphate buffer solution (pH = 7) of DOPC vesicles (2.2 mM) containing 1 mM $HOCH_2CH_2SH$ in the absence (dashed lines) and in the presence of urate: (△) 60 μM, (○) 120 μM, (◊) 180 μM.

Table S4

Dose (Gy)	without vit-A	vit-A (0.005 mM)	vit-A (0.02 mM)	vit-A (0.06 mM)
0	100	100	100	100
105	57	62	72	76
210	43	50	63	68
315	38	46	59	64
420	35	44	56	61

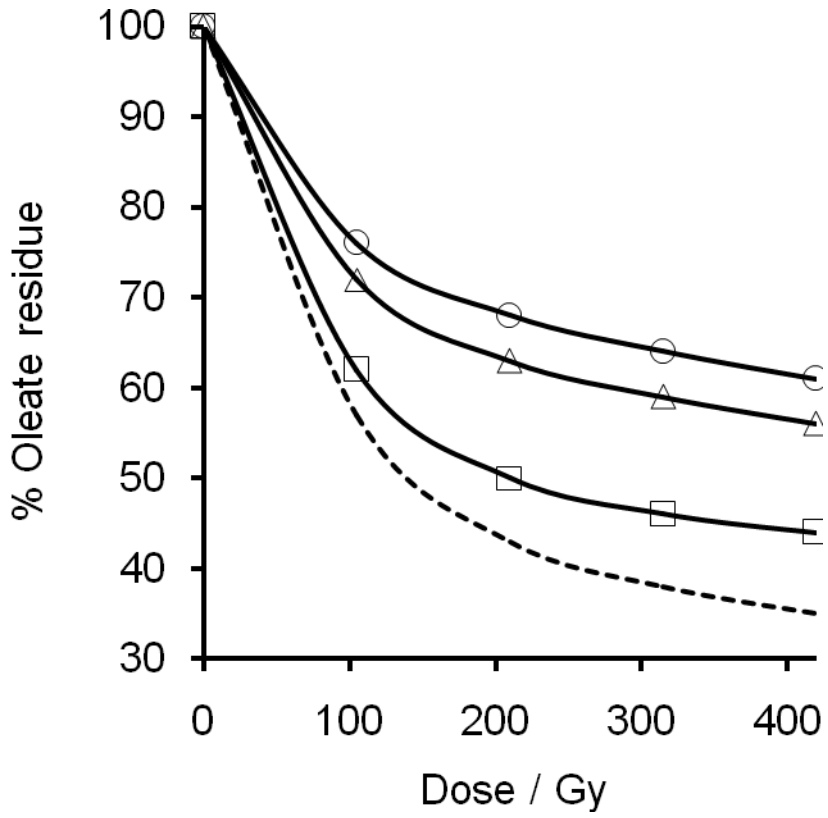

Figure S4. Disappearance of the *cis* isomer (oleate residue) upon γ-radiolysis (dose rate = 10.2 Gy/min) of N_2O-saturated phosphate buffer solution (pH = 7) of DOPC vesicles (2.2 mM) containing 1 mM $HOCH_2CH_2SH$ in the absence (dashed lines) and in the presence of all-*trans* retinol acetate: (□) 5 μM, (△) 20 μM, (○) 60 μM.

Table S5

Dose (Gy)	without vit-E	vit-E (0.02 mM)	vit-E (0.06 mM)
0	100	100	100
105	57	60	61
210	42	46	48
315	36	41	43
420	34	39	42

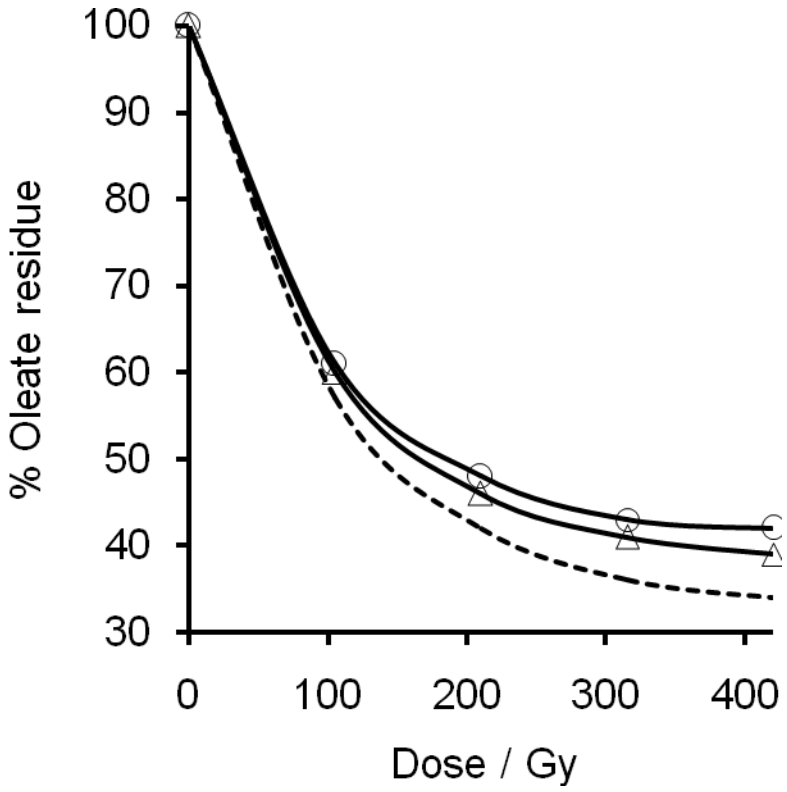

Figure S5. Disappearance of the *cis* isomer (oleate residue) upon γ-radiolysis (dose rate = 10.2 Gy/min) of N_2O-saturated phosphate buffer solution (pH = 7) of DOPC vesicles (2.2 mM) containing 1 mM $HOCH_2CH_2SH$ in the absence (dashed lines) and in the presence of α-tocopherol: (△) 20 μM, (○) 60 μM.

Table S6

	without vit-C				with vit-C (60 μM)			
Dose (Gy)	pH=3	pH=5	pH=7	pH=9	pH=3	pH=5	pH=7	pH=9
0	100	100	100	100	100	100	100	100
53	70	72	73	75	97	98	98	99
105	54	55	56	55	84	92	94	97
210	40	41	41	42	61	67	68	93
315	36	37	37	38	49	51	52	86
420	34	34	35	36	42	44	44	80

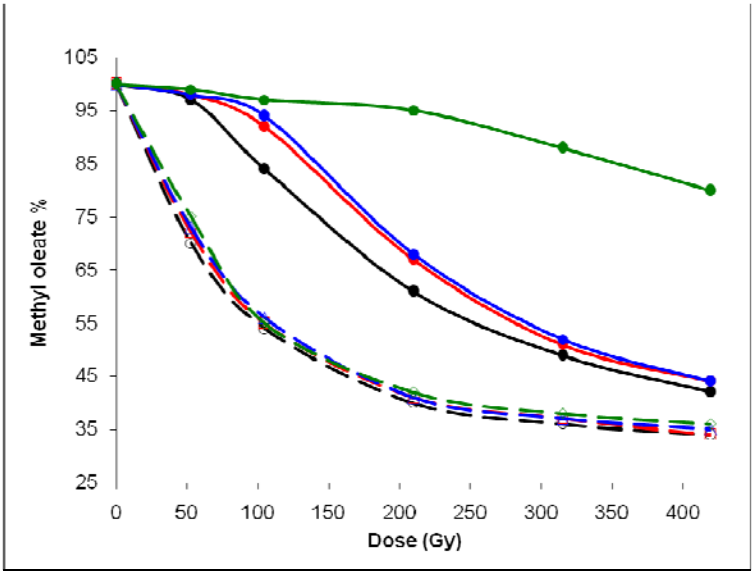

Figure S6. Disappearance of methyl oleate residue in DOPC vesicles (2.2 mM) under Gamma-radiolysis (10.2 Gy/min) with 1 mM mercaptoethanol as the thiyl radicals source, in N_2O-saturated flushed phosphate buffer solution, in the absence (dashed lines) and in the presence of 0.06 mM of vit-C at pH = 9 (●), 7 (●), 5 (●) and 3 (●).

Table S7

	without urate				with urate (60 µM)			
Dose	pH=3	pH=5	pH=7	pH=9	pH=3	pH=5	pH=7	pH=9
0	100	100	100	100	100	100	100	100
53	71	71	71	73	77	87	90	95
105	54	53	55	55	58	74	79	85
210	39	39	40	42	42	53	57	67
315	32	32	33	34	34	44	47	55
420	30	30	31	32	32	38	39	46

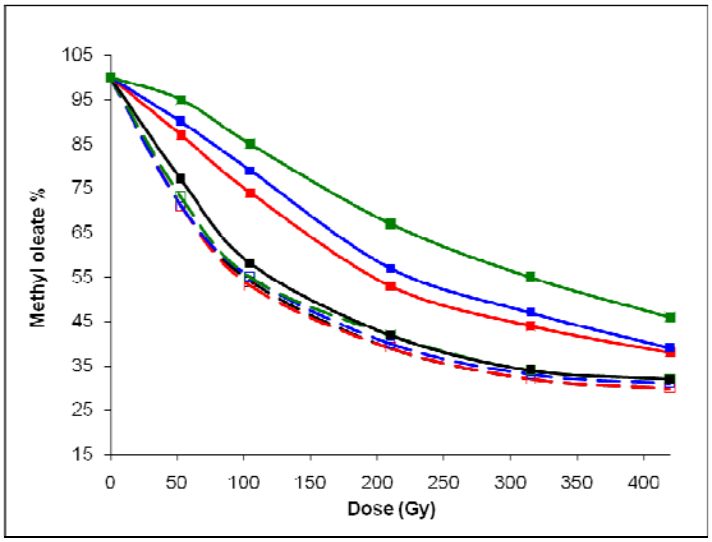

Figure S7. Disappearance of methyl oleate residue in DOPC vesicles (2.2 mM) under Gamma-radiolysis (10.2 Gy/min) with 1 mM mercaptoethanol as the thiyl radicals source, in N_2O-saturated flushed phosphate buffer solution, in the absence (dashed lines) and in the presence of 0.06 mM of urate at pH = 9 (■), 7 (■), 5 (■) and 3 (■).

Table S8

Dose	without vitamines	vit-A+C+E+urate (vivo μM)	vit-A (5 μM)	vit-E (60 μM)	vit-C (60 μM)	Urate (180 μM)
0	100	100	100	100	100	100
105	55	99	62	60	90	82
210	41	88	50	46	68	67
315	36	79	46	41	52	55
420	33	70	44	39	43	49