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Synthesis and study of the cancer cell growth inhibitory properties of α-, γ-tocopheryl and γ-tocotrienyl 2-phenylselenyl succinates

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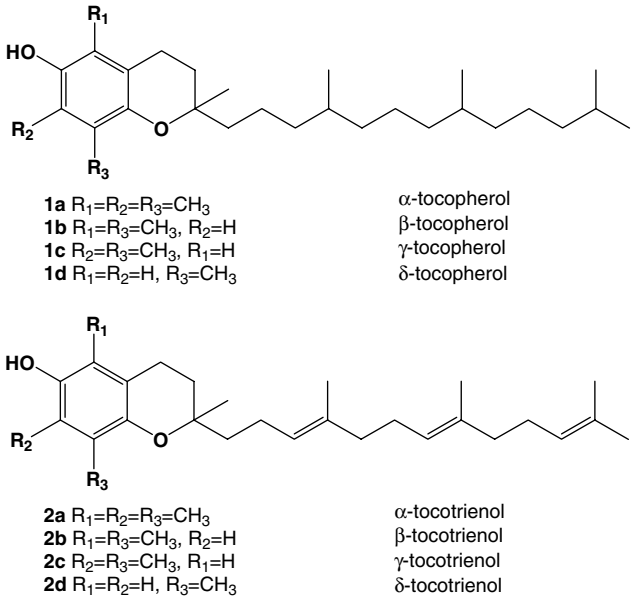
Abstract—Vitamin E succinate selenium-conjugated molecules were synthesized and their apoptogenic properties were evaluated. 4-Methyl-2-phenylselenyl succinate (**4**) was prepared by the reaction of sodium benzeneselenolate with 2-bromosuccinic anhydrite in methanol solution. The methyl ester was converted to the acid (**5**) by hydrolysis with aqueous hydrochloric acid. Reaction of the 2-phenylselenyl succinic anhydrite (**6**) with α-tocopherol (**1a**), γ-tocopherol (**1c**), and γ-tocotrienol (**2c**) in acidic conditions gave the respective esters. The free radical scavenging properties of α-tocopheryl-2-phenylselenyl succinate (**7**), γ-tocopheryl-2-phenylselenyl succinate (**8**), and γ-tocotrienyl-2-phenylselenyl succinate (**9**) were evaluated in comparison with those of α-tocopheryl succinate (**10**), γ-tocopheryl succinate (**11**), and γ-tocotrienyl succinate (**12**), respectively, and the free tocopherols and γ-tocotrienol. Compounds **7**–**9** induced a statistically significant decrease in prostate cancer cell viability compared to **10**–**12**, respectively, or **5**, exhibiting features of apoptotic cell death and associated with caspase-3 activation. These data show that structural modifications of vitamin E components by **5** enhance their apoptogenic properties in cancer cells.

1. Introduction

Selenium and vitamin E have attracted great attention after the decision of the National Institutes of Health to sponsor the selenium and vitamin E chemoprevention trial (SELECT) for the prevention of prostate cancer.[1] The term vitamin E refers to one or more structurally related phenolic compounds called tocopherols (compounds **1a**–**d**) and tocotrienols (compounds **2a**–**d**) (Scheme 1). Vitamin E components have three main distinct moieties, described as (i) the functional domain, responsible for the antioxidant activity and therefore, vitamin E properties, epitomized by the hydroxyl group in α-tocopherol (**1a**), (ii) the signaling domain, comprised of the aromatic rings (phenol- and chromanol-) and activated by the monoesterification of dicarboxylic acids with the phenol oxygen,[2,3] and (iii) the hydrophobic domain, responsible for docking the agents in circulating lipoproteins and biological membranes.[4] In addition, the structure of the aliphatic chain may play a role in the apoptotic properties of vitamin E isoforms, modifying membrane docking and lipid solubility.[5]



Scheme 1. Components of vitamin E.

Keywords: Selenium; Vitamin E; Apoptosis; Succinate.
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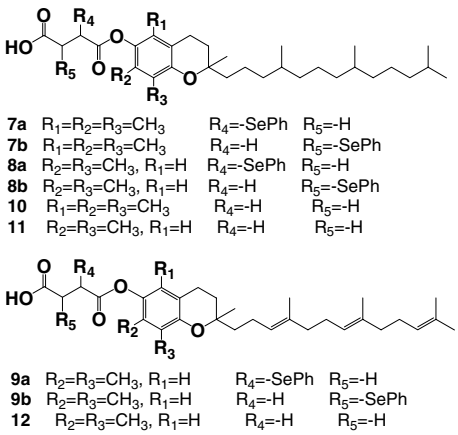
Although there have been many studies about the biological and health effects of α-tocopherol, much attention has recently been arouse interest in by the effects of other vitamin E components, such as γ-tocopherol (**1c**) and γ-tocotrienol (**2c**).[6–10] γ-Tocopherol traps peroxonitrite and is more effective than α-tocopherol in protecting lipids against such peroxidation.[11] γ-Tocotrienol induces apoptosis in human breast cancer cells, whereas α-tocopherol does not.[8,12]

Vitamin E derivatives, such as α-tocopheryl succinate (**10**), which are deprived of their radical inhibitory activity by esterification of the phenolic oxygen with a dicarboxylic acid, modulate the growth of a variety of malignant cell lines by suppressing DNA synthesis and inducing apoptosis more effectively than α-tocopherol alone.[2,13–19] Structure–activity studies revealed the key role of succinyl moiety to promote pro-apoptotic cascades of events through synergistic pathways.[20] The α-tocopheryl moiety has been shown to be involved in protein phosphatase 2A (PP2A) activation, leading to the inactivation of protein kinase C (PKC) and the dephosphorylation of the anti-apoptotic mitochondrial protein bcl-2. The charged succinyl moiety caused destabilization of both lysosomal and mitochondrial membranes leading to further α-tocopheryl-induced cytochrome c release and thus, augmentation of the apoptotic signal. These observations further suggest that hydrolysis of the esteric bond within vitamin E monoesters might augment rather than reduce their anticancer activity through the generation of biologically active moieties.

There has been a growing interest in the synthesis of organoselenium compounds with respect to enzymology and bioorganic chemistry because these compounds are much less toxic compared to inorganic selenium species. Several of these organoselenium compounds, including selenomethionine and aromatic selenium molecules, have been found to inhibit both tumorigenesis in a variety of animal models[21–24] and human tumor cell growth in vitro.[25] Cancer cell death mechanisms have been associated with structural characteristics of organoselenium molecules.[26]

Furthermore, multiple epidemiological observations indicate that α-tocopherol together with selenium has a synergistic effect expressed as cancer chemopreventive activity.[27–29] It has been demonstrated that the impact of selenium deficiency on cancer risk is also more profound at low serum α-tocopherol concentrations.[30] Furthermore, γ-tocopherol seems to be essential in order for α-tocopherol and selenium synergism to be expressed. In addition, methylselenilic acid, a selenium metabolite, and α-tocopheryl succinate have shown synergism in apoptosis induction in human prostate cancer cells through the activation of distinctive initiator and effector caspases.[31]

In order to obtain further insight into the synergism between selenium and vitamin E, and to enhance the pro-apoptotic properties of vitamin E, we have developed a new strategy introducing organoselenium and succinate moieties into the functional domains of vitamin E compounds (Scheme 2). The introduction of succinate moiety targets to induce apoptosis of the cancer cells, whereas at the same time tocopherols and tocotrienols are expected to serve as vehicles transferring selenium to the sites of action of vitamin E succinate. Three novel selenium molecules have been synthesized, namely: α-tocopheryl-2-phenylselenyl succinate (**7**), γ-tocopheryl-2-phenylselenyl succinate (**8**), and γ-tocotrienyl-2-phenylselenyl succinate (**9**). To our knowledge, **7** is the first selenium containing derivative of tocopherol succinate **10**, whereas **8** and **9** are the first selenium containing derivatives of γ-tocopherol and γ-tocotrienol. Cell proliferation and growth assays on androgen unresponsive prostate cancer cells treated with **7**–**9** exhibit statistically significant lower IC_{50} values compared to succinates **10**–**12**, respectively. Morphologic characteristics of apoptotic cell death are visualized in cells treated with **7**–**12**. Cell death is mediated by activation of the caspase apoptotic cascade. Prostate cancer cells are resistant to 2-phenylselenyl succinic acid (**5**), suggesting that the increased pro-apoptotic activity is not totally attributed to the selenium but rather to the structural changes imposed onto the functional and signaling domains of the **7**–**9** monoesters.



7a $R_1=R_2=R_3=CH_3$ R_4=-SePh R_5=-H
7b $R_1=R_2=R_3=CH_3$ R_4=-H R_5=-SePh
8a $R_2=R_3=CH_3$, R_1=H R_4=-SePh R_5=-H
8b $R_2=R_3=CH_3$, R_1=H R_4=-H R_5=-SePh
10 $R_1=R_2=R_3=CH_3$ R_4=-H R_5=-H
11 $R_2=R_3=CH_3$, R_1=H R_4=-H R_5=-H

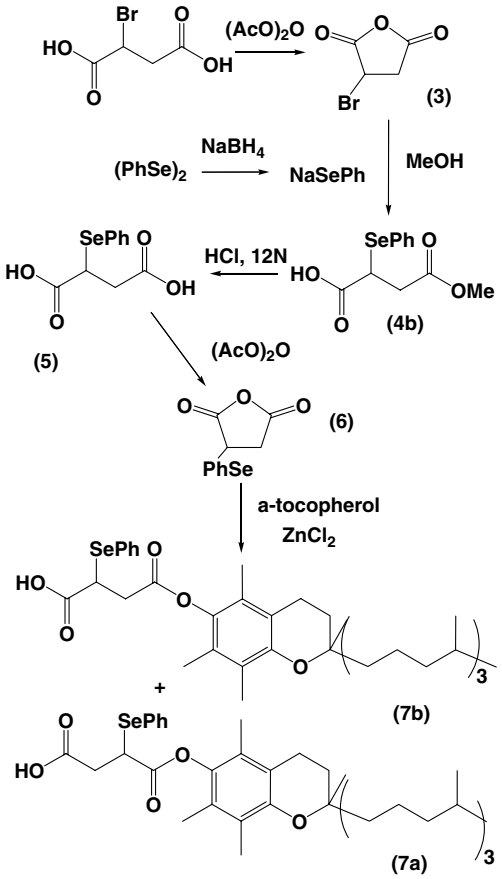
9a $R_2=R_3=CH_3$, R_1=H R_4=-SePh R_5=-H
9b $R_2=R_3=CH_3$, R_1=H R_4=-H R_5=-SePh
12 $R_2=R_3=CH_3$, R_1=H R_4=-H R_5=-H

Scheme 2. Structures of compounds **7**–**12**.

2. Results and discussion

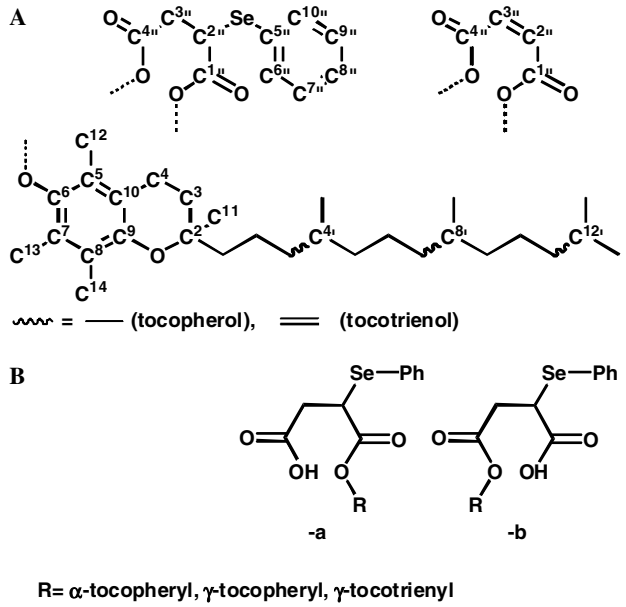
2.1. Synthesis and stability

The synthesis of **7** and its structure is shown in Scheme 3. The first step in the synthesis of selenium molecules involves the synthesis of **5** by the reaction of 2-bromosuccinic anhydrite (**3**) with the sodium salt of phenylselenol in methanol and subsequent hydrolysis of the formed 4-methyl-2-phenylselenyl succinate (**4**) with concentrated hydrochloric acid. Acidic catalysts were used for the synthesis of esters because of the facile elimination of the phenylselenyl group at high pHs. Specifically, **7**–**9** were prepared by the reaction of 2-phenylselenyl succinic anhydrite (**6**) with **1a**,**c**, and **2c**, respectively, in the presence of zinc chloride (Scheme 3). Each product of these reactions consisted of two isomers, –**a** and –**b**, where –**a** has been esterified at C1 and –**b** at the



Scheme 3. Synthesis of compounds **3**–**7**.

C4 carbon atom of the phenylselenyl succinate moiety (Schemes 2–4), as evident by ^{77}Se and ^{1}H NMR spectra. The above selenium compounds are stable in solid state and in organic solvent solutions for several months.



Scheme 4. (A) Numbering of the carbon atoms of the selenium molecules. (B) Geometrical isomers of **7**–**9** monoesters.

However, addition of a base causes decomposition of the compounds and formation of phenylselenol salt and unsaturated carboxylic acids according to reaction 1. Phenylselenol is further oxidized to diphenyldiselenide.

$$\mathbf{5} \xrightarrow{\text{NaOH or } Et_3N} \text{fumaric acid} + \text{phenylselenol}. \quad (1)$$

This reaction is slow at room temperature (less than 5% of **5** is decomposed in one day as evident by ^{1}H NMR in aqueous solution at pH 9) and accelerates at higher temperatures. In the culture media (pH 7.2–7.4), both **5** and **7** are stable for more than three weeks as evident by TLC.

2.2. NMR characterization

Complete assignment of the peaks observed in the NMR spectra was done by analyzing the chemical shifts (Table 1), the integrals as well as the 2D {^{1}H} COSY, {^{1}H} NOESY, {^{1}H, ^{13}C} HMQC, and {^{1}H, ^{13}C} HMBC spectra. The numbering of the carbon atoms, and consequently the hydrogen atoms is shown in Scheme 4. The sensitive 2D {^{1}H, ^{13}C} HMBC NMR was used for determining the long-range (two- and three-bond) connectivity and for identification of the **4** and the **7**–**9** isomers. The following pulse sequence proposed by Bax and Summers was applied.[32]

$$\begin{array}{lccccccc} ^{1}\mathrm{H} & 90^{o}_{x} - \Delta_1 - & & -\Delta_2 - & & -t_{1/2} - 180^{o}_{x} - t_{1/2} - & & -\mathrm{Acq}(t_2) \\ ^{13}\mathrm{C} & & 90^{o}_{\psi} & & 90^{o}_{\psi} & & 90^{o}_{x} & \end{array}$$

The duration of Δ_2 was set to 50 or 70 ms for the detection of two-bond (^{1}H–^{12}C–^{13}C) couplings and to 150 ms for the detection of three- and four-bond couplings, respectively. A part of the HMBC spectrum containing the ^{13}C peaks of C$^{1''}$ and C$^{4''}$, and the ^{1}H peaks of the C$^{2''}$–H for both isomers of **7**, with Δ_2 equal to 70 ms, is shown in Figure 1. The spectrum shows the two-bond connectivity between the C$^{2''}$-H protons and the carbonyl groups [^{1}H–C–^{13}C(O)OR, R = H or γ-tocopheryl–] in proximity, making the identification of the two isomers indisputable.

^{77}Se NMR was also utilized for the identification of the **7**–**9** isomers. The ^{77}Se chemical shifts of the **7a**–**9a** isomers [–C$^{2''}$(SePh)(H)C$^{1''}$(O)OR, R = α-tocopheryl-, γ-tocopheryl-, and γ-tocotrienyl-] showed to be sensitive to the type of substituents on the chroman group. In particular, replacement of α-tocopheryl-, with γ-tocopheryl- or γ-tocotrienyl- resulted in a downfield shift of the selenium peak from 635.7 to 640.4 and 640.8 ppm, respectively. On the other hand, the ^{77}Se chemical shifts of the **7b**, **8b**, and **9b** isomers [-C$^{2''}$(SePh)(H)C$^{3''}$C$^{4''}$(O)OR, R = α-tocopheryl-, γ-tocopheryl-, and γ-tocotrienyl-] are unaffected from R (652.1, 652.5, and 652.4 ppm, respectively).

2.3. Radical scavenging activity

The new molecules were evaluated for their radical scavenging activity via the ability to react with the DPPH· radical.[33,34] The second-order rate constants (k_2) for

Table 1. ^{77}Se and selected ^{13}C and ^{1}H NMR chemical shifts and coupling constants of selenium compounds

Compound (solvent)	Se (ppm)	$C^{1''}$ (ppm)	$C^{2''}$, $H_x^{2''}$ (ppm)(J_{xa}, J_{xb}(Hz))	$C^{3''}$, $H_a^{3''}$, $H_b^{3''}$ (ppm)(J_{ab}(Hz))	$C^{4''}$(ppm)
5 (CD_3CN)	634.80	173.2	37.0, 3.90 (10.00, 5.00)	36.6, 2.78 (7.50)	172.2
4 ($CDCl_3$)	646.95	178.40	37.21, 3.97 (10.29, 5.71)	36.95, 3.04, 2.81 (17.14)	171.50
6 ($CDCl_3$)	668.52	173.70	37.41, 4.39 (10.00, 3.00)	36.26, 3.53, 2.94 (19.2)	172.73
7b ($CDCl_3$)	652.1	170.16	37.12, 4.02 (10.23, 5.11)	36.89, 3.12, 3.02 (17.73)	177.93
7a ($CDCl_3$)	635.7	176.93	36.27, 4.15 (9.89, 4.09)	37.52, 3.05, 2.85 (17.01)	171.53
8b ($CDCl_3$)	652.5	170.49	37.25, 4.09 (10.00, 5.00)	37.09, 3.12, 2.96 (18.75)	178.74
8a ($CDCl_3$)	640.4	176.93	36.63, 4.16 (10.00, 5.00)	37.37, 3.09, 2.94 (18.13)	171.80
9b ($CDCl_3$)	652.4		4.09 (10.36, 5.38)	3.15, 2.89 (18.38)	
9a ($CDCl_3$)	640.8		4.16 (10.00, 4.62)	3.18, 2.81 (17.90)	

The numbering is according to Scheme 4.

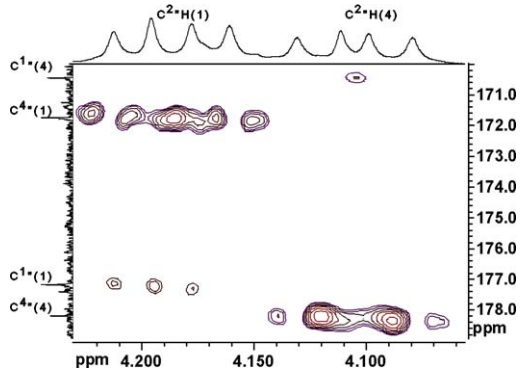


Figure 1. Part of the 2D {^{1}H, ^{13}C} HMBC spectrum of **8** solution in $CDCl_3$ (100 mM) at room temperature. Δ_2 = 70 ms. The numbers **1** and **4** in parentheses represent the isomers **8a** and **8b**, respectively.

the free radical scavenging activity (DPPH·) are listed in Table 2.

Free tocopherols and tocotrienol were much more effective radical scavengers than the esterified ones. The addition of phenylselenyl group on the succinate esters of vitamin E components did not increase the radical scavenging activity, whereas **4** and **5** were inactive. The fact that esterification of tocopherols does not completely cease the antioxidant activity is attributed to the single electron-transferring (SET) mechanism.[35]

Table 2. The second-order rate constants (k_2) of the reaction of DPPH· (20.0 μM) with the radical inhibitors (60.0–300 μM) in methanol at 25 °C

Compound	DPPH· scavenger activity k_2 ($\times 10^5$ μM s^{-1})[a]
None	
5	0.083 (0.002)
4	0.094 (0.003)
1a	56 (0.8)
10	3.4 (0.2)
7	3.5 (0.2)
1c	30 (4)
11	5.3 (0.2)
8	3.1 (0.1)
2c	33 (1)
12	4.1 (0.1)
9	3.0 (0.1)

Standard deviations are shown in parentheses.
Concentrations were calculated from the absorption at 515 nm.
[a] Calculated from the slope of the plot pseudo-first-order rate constant k_{obsd} versus concentration of antioxidant.

In this mechanism, the electron is transferred quicky from DPPH· to the chromanol oxygen followed by the decomposition of the ester bond. The differences in radical scavenging reaction rates between the esterified and the free tocopherols are attributed to the inertness of the C–O ester bond compared to the more labile O–H bond.

2.4. In vitro cancer cell growth inhibitory and apoptotic properties

Compounds under investigation were used in cell viability assays with: (i) crystal violet and (ii) trypan blue on androgen unresponsive prostate cancer cell lines DU-145 and PC-3. Effects on cancer cell survival as demonstrated by the IC_{50} values determined by crystal violet assay and their correlations with structural modifications are shown in Table 3 and presented in Fig. 2. To disclose differences between cell growth inhibitory properties, IC_{50} values subjected to one-sample Student's *t* test comparative statistical analysis for the two cell lines between succinate and phenylselenyl succinate monoesters are summarized in Tables 4A and 4B. An equimolar combination of **5** and **10** was not more effective than **10** alone in either cell line, suggesting that the two compounds have no biochemical interactions modifying their cell growth inhibitory properties in the culture media (data not shown).

There has been mounting evidence that structural characteristics of both vitamin E derivatives and selenium compounds play a significant role in the mechanisms of cell death.[20,26] To explore whether structural modifications within phenylselenyl succinate monoesters augment apoptosis rather than necrosis, cells subjected to trypan blue exclusion test were further assessed for apoptotic characteristics. An advantage of the trypan blue exclusion assay is that besides quantifying cell death it allows for assessment of apoptosis.[36] Cancer cell growth inhibitory properties of compounds **4**, **5**, and **7**–**12** as expressed by their IC_{50} concentrations were determined in both crystal violet proliferation and trypan blue exclusion assays (Fig. 3I and II). Both assays revealed a statistically significant higher cancer cell growth inhibitory activity in phenylselenyl succinate monoesters compared to their succinate counterparts in DU-145 but not in PC-3 cells. Such a cell line specific effect could be attributed to the ability of structural modifications in these selenium compounds to specifically modulate pathways unique to the DU-145 cell line.

Table 3. Structure–function correlations of vitamin E components and newly synthesized phenylselenyl succinate and succinate monoesters

Compound	PC-3		DU-145	
	IC_{50} [mM] (±SD)	Apoptotic features	IC_{50} [mM] (±SD)	Apoptotic features
5	0.137 (0.001)	–	0.121 (0.007)	–
4	0.125 (0.003)	–	0.115 (0.005)	–
1a	0.072 (0.002)	–	0.078 (0.001)	–
1c	0.063 (0.001)	±	0.068 (0.001)	±
2c	0.051 (0.009)	±	0.058 (0.001)	±
10	0.064 (0.002)	+	0.072 (0.001)	+
11	0.045 (0.001)	+	0.056 (0.001)	+
12	0.039 (0.001)	+	0.046 (0.002)	+
7	0.052 (0.002)	+	0.061 (0.001)	+
8	0.039 (0.001)	+	0.047 (0.001)	+
9	0.036 (0.002)	+	0.042 (0.001)	+

Effect on cell survival is expressed by IC_{50} values (±SD) over 72 h of incubation and determined by crystal violet proliferation assay.
Apoptotic cell death is assessed by the visualization of apoptotic features (i.e., pyknotic nuclei, apoptotic bodies, and apoptotic rings) in cells stained with DAPI.
±: indicates condensed chromatin as a feature of early apoptosis.

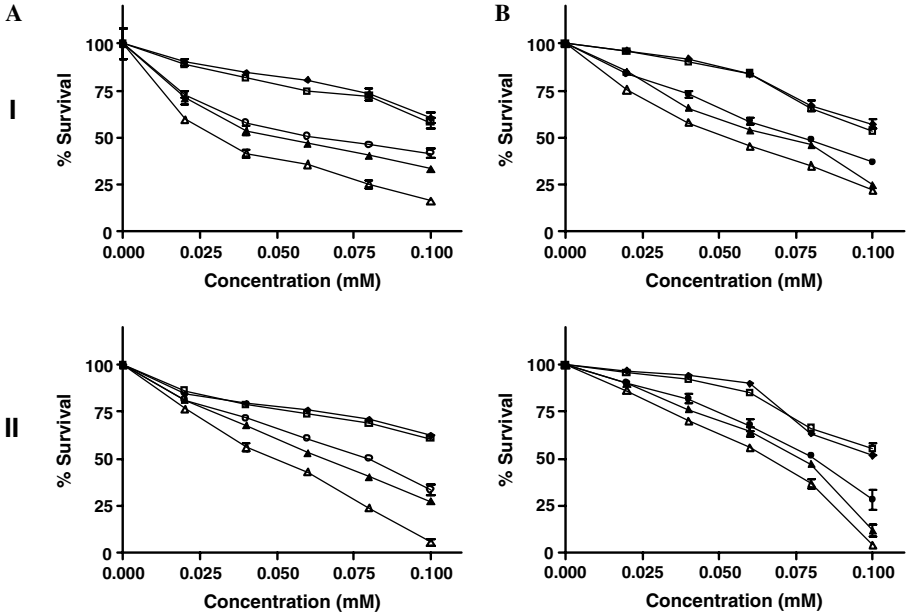


Figure 2. Effect of free vitamin E components **1a**,**1c** and **2c** and newly synthesized organoselenium compounds **4**, **5** on the viability of prostate cancer cells. Results on PC-3 and DU-145 cell lines are represented in panels A and B, respectively. Increasing concentrations of **5** (filled diamond), **4** (open squares), **1a** (circles), **1c** (filled triangles), and **2c** (open triangles) were tested against untreated controls. Cells were plated in triplicate and allowed to attach and reach confluence. Subsequently, test compounds were added at concentrations of 0.00–0.10 mM and incubated for over 72 h. Cell viability was determined by: (I) staining with crystal violet and measuring the OD_{620} of the cell lysates and (II) subjecting the cells to trypan blue exclusion assay. Survival is estimated as a percentage of viable cells in untreated controls. Points represent the mean of triplicate points of two experiments ±SD.

Apoptotic cell death was further confirmed by 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) staining of nuclei that revealed features of apoptosis, such as apoptotic bodies, apoptotic rings, and condensed chromatin, after treatment of cells with **7**–**12** monoesters, whereas homogeneously stained nuclei were disclosed in cells treated with free **1a**,**c**, **2c**, **4**, and **5** (Fig. 4).

It has already been shown in several cell lines that α-tocopheryl succinate induces apoptosis through the activation of the caspase cascade,[15,37,38] while structure–function studies of vitamin E derivatives attribute the generation and transduction of apoptotic signals to the chroman domain. The presence of the active form of caspase-3, as a key executioner molecule, is a sign of ongoing and irreversible apoptosis. Assessment of the enzymatic activity of caspase-3 is considered as a reliable method for capturing the apoptotic process.[39,40] It would thus be of interest to assess if the substitution of succinate with phenylselenyl succinate moiety within tocopheryl/tocotrienyl monoesters elicits specific apoptotic cascades through the activation of caspases. To study this hypothesis, the proteolytic activity of effector caspase-3 was evaluated in cells treated with **7**–**12** monoesters at IC_{50} concentrations (Fig. 3III). While the structural modifications due to phenylselenyl group on the

Table 4A. p Values between compounds **1a**,**c**, **2c**, **4**, **5**, and **7**–**12** for cell growth inhibitory properties of PC-3 cells

PC-3 p/p	**5**	**4**	**1a**	**1c**	**2c**	**10**	**11**	**12**	**7**	**8**	**9**
5	—	0.119	0.015	0.011	0.048	0.011	0.007	0.007	0.009	0.007	0.007
4	—	—	0.018	0.013	0.020	0.014	0.008	0.007	0.010	0.007	0.007
1a	—	—	—	0.105	0.045	0.097	—	—	0.037	—	—
1c	—	—	—	—	0.069	—	0.036	—	—	0.026	—
2c	—	—	—	—	—	—	0.052	—	—	—	0.046
10	—	—	—	—	—	—	0.035	0.026	0.068	—	—
11	—	—	—	—	—	—	—	0.099	—	0.100	—
12	—	—	—	—	—	—	—	—	—	—	0.211
7	—	—	—	—	—	—	—	—	—	0.050	0.045
8	—	—	—	—	—	—	—	—	—	—	0.205
9	—	—	—	—	—	—	—	—	—	—	—

Statistical analysis is performed with one-sample t test for 95% confidence for IC_{50} values from three experiments with crystal violet staining in triplicate points.

Table 4B. p Values between compounds **1a**,**c**, **2c**, **4**, **5**, and **7**–**12** for cell growth inhibitory properties of DU-145 cells

DU-145 p/p	**5**	**4**	**1a**	**1c**	**2c**	**10**	**11**	**12**	**7**	**8**	**9**
5	—	0.332	0.004	0.003	0.003	0.004	0.003	0.014	0.003	0.002	0.002
4	—	—	0.005	0.004	0.003	0.005	0.003	0.015	0.004	0.002	0.002
1a	—	—	—	0.019	0.010	0.035	—	—	0.012	—	—
1c	—	—	—	—	0.021	—	0.016	—	—	0.006	—
2c	—	—	—	—	—	—	—	0.015	—	—	0.008
10	—	—	—	—	—	—	0.012	0.007	0.017	—	—
11	—	—	—	—	—	—	—	0.019	—	0.013	—
12	—	—	—	—	—	—	—	—	—	—	0.030
7	—	—	—	—	—	—	—	—	—	0.008	0.007
8	—	—	—	—	—	—	—	—	—	—	0.030
9	—	—	—	—	—	—	—	—	—	—	—

Statistical analysis is performed with one-sample t test for 95% confidence for IC_{50} values from three experiments with crystal violet staining in triplicate points.
Numbers in bold refer to compounds; numbers inside the table refer to p values. Statistically significant difference is for $p < 0.05$.

functional domains of succinate monoesters do not affect significantly their radical scavenging activity (Table 2), **7**–**9** have a significantly higher pro-apoptotic effect associated with the induction of the effector caspase apoptotic cascade (Table 3 and Figs. 3III, 5II). Interestingly, even at IC_{50} concentrations, moderate differences are disclosed between the succinate (**10**–**12**) and phenylselenyl succinate (**7**–**9**) monoesters of each vitamin E derivative. These observations raise the notion that such structural modifications on the succinate moiety induce modulations of different apoptotic pathways which synergistically augment apoptotic signals through the effector caspase cascade.

The efficacy of phenylselenyl succinate monoesters to induce apoptosis was further quantified by analyzing data from: (a) trypan blue exclusion and (b) caspase-3 proteolytic enzymatic activity assays in cells treated with equimolar concentrations of each compound (Fig. 5). Both assays revealed a statistically significant augmentation in pro-apoptotic activity of phenylselenyl succinate monoesters over their succinate counterparts ($0.01 < p \leqslant 0.05$) in DU-145 but not in PC-3 cell lines. Statistically significant differences were further disclosed between phenylselenyl succinate monoesters and phenylselenyl succinic acid in both assays ($p < 0.005$), further confirming the implication of the effector caspase cascade. This effect is partially attributed to the silencing of radical inhibitory activity of vitamin E in these esters.

In fact, prostate cancer cells were resistant to **5**, suggesting that the increased pro-apoptotic activity may not be attributed to selenium but rather to the structural changes imposed onto the functional domain with the additional benzene ring. These modifications introduce additional lipophilicity onto the functional domain of these esters.

Although the hydrophobic aliphatic chain does not seem to play a key role[5] in the induction of apoptosis, it is a necessary structural element for vitamin E compounds to exert the activity of the signaling and functional domains. This is further supported by the small differences in apoptotic activity disclosed between **8** and **9**. In this study, the derivatives of the γ-members of the vitamin E family (**8**–**9** and **11**–**12**) exerted higher pro-apoptotic activity than those of the α-counterparts (**7** and **10**). Furthermore, features of early apoptosis such as condensed chromatin were visualized in cells treated with **1c**. This is verified by the post hoc analysis of data, which yields statistically significant differences between γ-analogues and the other compounds. In addition, this is in agreement with previously reported data[2] where γ-tocopherol was the only member of that vitamin E family that induced a cell line specific apoptotic effect, showing the key role of chromanol structures in regulating apoptotic pathways.

The introduction of the aromatic selenium into the esterified tocopherols and tocotrienols further enhanced their apoptogenicity. Monoesters **7**–**12** had lower IC_{50} values

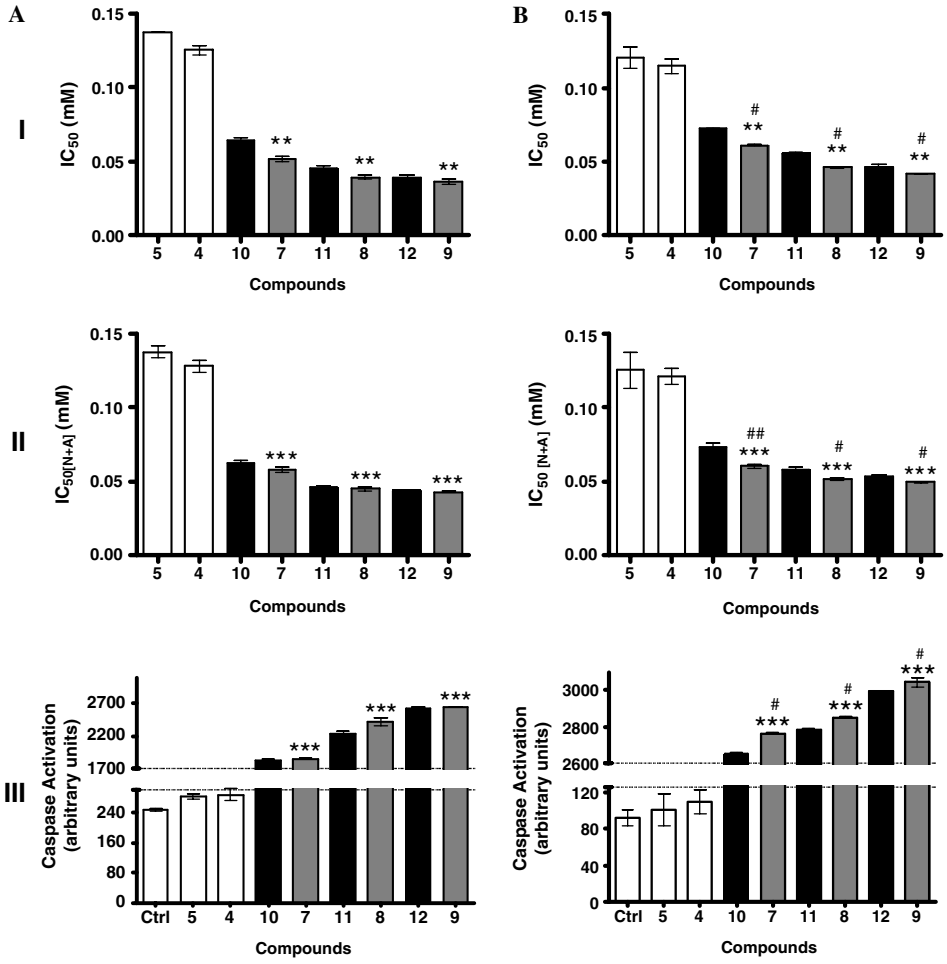


Figure 3. Effects of newly synthesized compounds **4**, **5**, and **7**–**12** on prostate cancer cells' viability (I and II) and caspase-3 enzymatic activity (III). Results on PC-3 and DU-145 prostate cancer cells are represented in panels A and B, respectively. Values represent means of triplicate points of two experiments ±SD. (I) Growth inhibitory properties of **4**, **5**, and **7**–**12** expressed by their IC_{50} values in viability assays as determined by staining with crystal violet and measuring the OD_{620} of the cell lysates. Compounds **7**–**9** monoesters are assessed against **5** (∗) and **10**–**12** monoesters (#). (II) Growth inhibitory properties of **4**, **5**, and **7**–**12** expressed by their IC_{50} values in viability assays as determined by the trypan blue exclusion test. **7**–**9** monoesters are assessed against **5** (∗) and **10**–**12** monoesters (#). (III) Caspase-3 enzymatic activity. Compounds **4**, **5**, and **7**–**12** induce activation of caspases in relation to their effect on cell viability. Cells were treated with the indicated compound at concentrations equal to IC_{50}, as determined in viability assays (Table 3), over 72 h. Cell lysates were subjected to assessment of caspase-3 activity, using the caspase-3-specific fluorogenic substrate z-DEVD-AFC as described in Section 4.4.4. Data show that **7**–**12** induced cell death associated with increase in caspase-3 activity. Arbitrary units of proteolytic activity elicited by IC_{50} concentrations of **7**–**9** are assessed against **5** (∗) and **10**–**12** monoesters (#). Single symbol indicates $0.01 < p \leqslant 0.05$. Double symbol indicates $0.005 < p \leqslant 0.01$. Triple symbol indicates $p < 0.005$. Comparisons of **7**–**9** monoesters with **5** yield $p < 0.0001$. Significance of differences in caspase activation correlates with significance of differences in cell viability expressed by IC_{50}. All bars are represented with ±SD.

and augmented caspase-3 proteolytic activity than those of their free counterparts. Selenium containing monoesters **7**–**9** had lower IC_{50} and augmented caspase-3 proteolytic activity than **10**–**12**, respectively, in DU-145 but not in PC-3 prostate cancer cells (Fig. 3). Enzymatic cleavage and activation of distinctive initiator or effector caspases previously reported to be responsible for the synergy between α-tocopheryl succinate and the selenium metabolite methyselenic acid in apoptosis induction[31] could be implicated in cell death mediated by **7**–**9**. Mechanistic studies with phenylselenyl succinate monoesters and succinate derivatives are currently undertaken in order to explore this hypothesis.

The addition of phenylselenyl group on the succinate moiety controls the lipophilicity, conformational flexibility, size, and acidity of molecules within the functional domain. Thus, these factors may underlie the enhancement of pro-apoptotic activity of **7**–**9** compared to that of **10**–**12** through a caspase-mediated pathway. Recent studies have shown that increase of lipophilicity reduces apoptogenic activity of tocopheryl dicarboxylates.[41] Although the phenylselenyl substitution on succinate monoesters decreases water solubility and amphiphilicity of **7**–**9**, the pro-apoptotic activity is increased which is in contrast to the above-mentioned studies. This difference may be attributed to the presence of selenium or to the specific structural changes such as reduced conformational flexibility due to the presence of the phenyl ring. The lack of studies on other tocopheryl succinate derivatives, substituted on the succinyl moiety itself, does not allow an exploration to what extent each

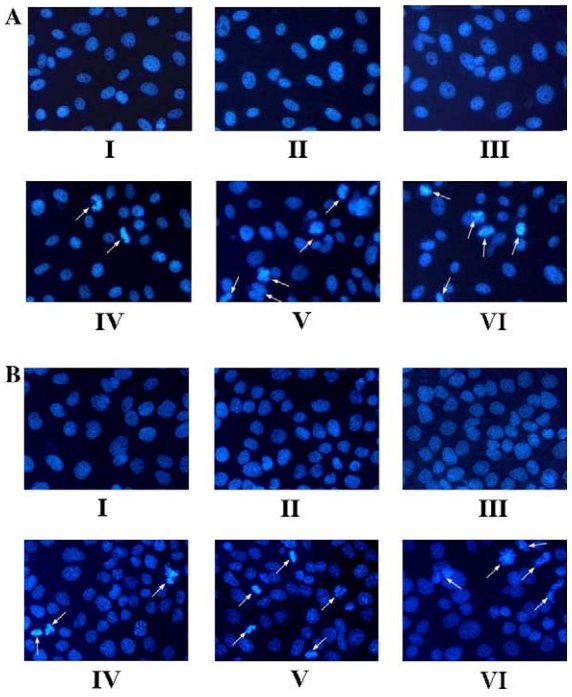


Figure 4. Morphologic representation of apoptosis by nuclear staining with DAPI. Cells were seeded on plastic chamber slides and treated as indicated with each compound at IC_{50} concentrations. Apoptotic cell death is assessed based on nuclear morphology. Apoptotic rings, apoptotic bodies, and condensed chromatin (arrows) were visualized by fluorescence microscopy, in three random fields, each of 250 cells, per slide. Panels A and B correspond to PC-3 and DU-145, respectively. (I) Untreated control; (II) **5**; (III) **4**; (IV) **7**; (V) **8**; (VI) **9**.

one of these factors contributes to the pro-apoptotic activity. However, the resistance of prostate cancer cells to **5** and **4** suggests that the structural changes on the succinic moiety play an important role in the enhancement of apoptotic properties of these molecules. Further, generation of biologically active moieties after cell internalization of **7**–**9** according to the model previously described[20] strongly suggests that even though they might be subjected to enzymatic decomposition by esterases, vitamin E monoesters have significant anticancer activity both in vitro and in vivo similar to that of more stable compounds such as ether derivatives.[42] The biological activity of **7**–**9** implies that these compounds are stable enough to transfer selenium at the cellular level, improving the bioavailability of selenium, thus, increasing the selenium content in the cell, an effect not induced by **5** alone.

3. Conclusions

α-, γ-Tocopheryl and γ-tocotrienyl phenylselenyl succinate as well as their succinate analogues were synthesized and their pro-apoptotic properties on prostate cancer cell were evaluated. To our knowledge this is the first time that selenium is combined with succinate vitamin E components in the same molecule. ^{1}H NMR spectra in $CDCl_3$ show the presence of two isomers, **7a**–**9a** and **7b**–**9b**, for each vitamin E component. In vitro cancer cell growth assays and assessments of mechanisms of cancer cell death show that **10**–**12** are more potent apoptogenic compounds than their non-esterified equivalents, while **7**–**9** are more potent apoptogenic than either **5** or **10**–**12**. However, **5** and **4** neither elicit any biological responses in cancer cell systems nor modify the effect of **10** at equimolar concentrations. This effect may be attributed to a lower bioavailability of these selenium molecules compared to **7**–**9**. The enhancement of biological activity of **7**–**9** monoesters strongly implies that selenium is transferred to the site of action of succinate vitamin E esters. These significant augmentations in apoptogenicity are attributed to structural modifications of the succinic moiety and lead to activation of the caspase apoptotic cascade. Whether these pathways are associated to the ones triggered by **10** alone or are unique to structural modifications within **10** and **5** in these novel compounds is currently under investigation.

The new strategy of using conjugates of succinylated tocopherols and tocotrienols with selenium to probe the biological responses and mechanism of cell death induced by the structural and chemical modifications of the functional domain has been proven to be successful. The structure of the functional domain is proposed to be responsible for the pro-apoptotic properties and, together with the structure of the chromanol ring, determines the signaling pathways to be modulated by vitamin E analogues. Significant evidence is provided that the phenylselenyl moiety enhances the pro-apoptotic and antiproliferative properties of vitamin E succinates. In order to further elucidate the structure–function relationship driving these responses, additional work is undertaken involving the study of new molecules containing other substituents on the succinate moiety and modified signaling domains.

4. Materials and methods

4.1. Materials

Compounds **10**, **1a**,**c**, and **2c** were provided by YASOO Health Inc. (Nicosia, Cyprus). Tocopherols and tocotrienol used in this paper were extracted from rice and are natural isomers, more specifically the *RRR*-isomers for tocopherol and the *R*-isomer for tocotrienol. Sodium borohydrite, bromosuccinic acid, acetic anhydrite, zinc chloride, 2,2-diphenyl-1-picrylhydrazyl free radical DPPH$^{\cdot}$, ethylenediaminetetraacetate disodium salt (EDTA), Trizma hydrochloride (Tris–HCl) and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), TLC silica gel 60 F254 plates, and TLC silica gel high purity grade 70–230 mesh 60 Å were purchased from Sigma-Aldrich (Taufkirchen, Germany). Sodium hydroxide in pellets, triethylamine, and hydrochloric acid 37% were purchased from Merck. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, Fungizone, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), phosphate-buffered saline (PBS), trypsin (0.05%), and trypan blue were purchased from Invitrogen (Paisley, UK). All chemicals were used

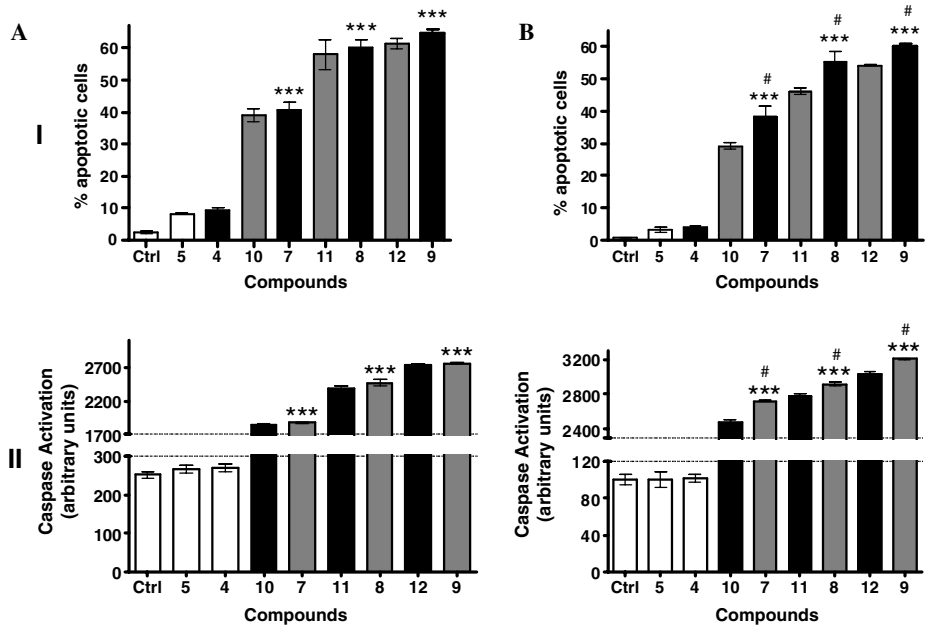


Figure 5. Quantification of apoptosis. The efficacy of vitamin E components and newly synthesized compounds **4**, **5**, and **7**–**12** to induce apoptotic cell death was assessed by subjecting cells treated with equimolar concentrations (0.060 mM) of each compound over 72 h to (I) trypan blue exclusion test and (II) DEVD-caspase proteolytic activity assay. (I) Apoptotic cells were distinguished by revealing their rough membranes, different shapes and sizes as well as their nuclear condensation. Evenly blue cells were considered as dead. Compounds **7**–**9** monoesters are assessed against **5** (*) and **10**–**12** monoesters (#). Treatments were performed in triplicate and repeated twice. Results are given as mean values of two experiments ±SD. (II) Cell lysates were subjected to assessment of caspase-3 activity, using the caspase-3-specific fluorogenic substrate z-DEVD-AFC as described in Section 4. Data show that **7**–**12** induced cell death associated with increase in caspase-3 activity. Arbitrary units of proteolytic activity elicited by equimolar concentrations of **7**–**9** are assessed against **5** (*) and **10**–**12** monoesters (#). Single symbol indicates $0.01 < p \leqslant 0.05$. Double symbol indicates $0.005 < p \leqslant 0.01$. Triple symbol indicates $p < 0.005$. Comparisons of **7**–**9** monoesters with **5** yield $p < 0.0001$. All bars are represented with ±SD.

without further purification. The androgen insensitive prostate cancer cell lines, DU-145 (grade II, dehydroepiandrosterone negative) and PC-3 (grade IV, androgen receptor negative), were purchased from the American Type Culture Collection (ATCC). 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) was purchased from Roche (Mannheim, Germany), and z-asp-glu-val-asp-7-amino-4-trifluoro-methyl-coumarin (z-DEVD-AFC) was from Camiya Biomedical Co (Seattle, WA, USA). Dithiothreitol, sucrose, Nonidet P-40 substitute (NP-40), and glycerol were bought from Fluka. Tissue culture plasticware was purchased from Bibby Sterilin (Staffordshire, UK). Methanol and toluene were dried over magnesium and CaH_2, respectively, and were distilled before use. Synthesis and distillation of the molecules were performed under high-purity nitrogen using standard schlenk techniques. C, H and N analyses were conducted by Desert Analytics (Tucson, AZ, USA). High-purity gases N_2 and CO_2 (99.995% and 99.9%, respectively) were used. Electronic spectra were recorded on a UV–vis spectrophotometer Shimazu, model 1600 A. Measurements of optical density were performed on a Perkin-Elmer Wallac 1420 microplate spectrophotometer equipped with Multilabel Counter Software 3.00.

4.1.1. Synthesis of bromosuccinic anhydrite (3). Bromosuccinic acid (5.00 g, 25.4 mmol) and excess acetic anhydrite (5.18 g, 50.7 mmol) were mixed and refluxed for 2 h. The solvent was evaporated under vacuum to give a viscous brown liquid. The yield was 4.54 g (100%). ^{1}H NMR δ ($CDCl_3$) (ppm): 4.89 (dd, 1H, $C^{2''}(H_X)$Br), 3.74 (dd, 1H, $C^{3''}(\mathbf{H}_A)(H_B)$), 3.27 (dd, 1H, $C^{3''}(H_A)(\mathbf{H}_B)$). $^{13}C\{^1H\}$ NMR δ ($CDCl_3$) (ppm): 168.67 ($C^{1''}$), 168.02 ($C^{4''}$), 40.06 ($C^{2''}$), 33.94 ($C^{3''}$). The numbering is according to Scheme 4.

4.1.2. Synthesis of 4-methyl-2-phenylselenyl succinate (4). $NaBH_4$ (0.20 g, 5.0 mmol) was slowly added within 30 min to a methanol (10 mL) solution of diphenyldiselenide (0.44 g, 1.4 mmol) at 0 °C under nitrogen atmosphere. The yellow solution turned colorless and the mixture was stirred additionally for 1 h at 0 °C. Consequently, a deoxygenated solution of **3** (0.50 g, 2.9 mmol) in methanol (5 mL) was added. White solid was precipitated and stirring was continued for 2 h at room temperature. Methanol was evaporated under vacuum and the yellow solid residue was dissolved in *n*-hexane (10 mL). The product was extracted with H_2O (3× 10 mL) and the aquatic phase was acidified with 6 N HCl forming a yellow oil, which was extracted with CH_2Cl_2 (3× 10 mL). The organic phase was dried with Na_2SO_4 and the solvent was evaporated under vacuum resulting in 0.71 g (87%) of yellow oil. Besides the main component, 4-methyl-2-phenylselenyl succinate (**4b**), the product of this reaction also contains the other isomer, 1-methyl-2-phenylselenyl succinate (**4a**), in less than 5%. ^{1}H NMR δ ($CDCl_3$) (ppm): 10.60 (br, 1H, $C^{1''}$(O)OH), 7.59 (m, 2H, $C^{6''}$H, $C^{10''}$H), 7.35 (m, 3H, $C^{7''}$H, $C^{8''}$H, $C^{9''}$H), 3.97 (dd, 1H, $H_XC^{2''}$), 3.67

(s, 3H, $H_3CO(O)C^{4''}$–), 3.04 (dd, 1H, $C^{3''}$ $\mathbf{H_A}H_B$), 2.81 (dd, 1H, $C^{3''}H_A\mathbf{H_B}$). $^{13}C\{^1H\}$ NMR δ ($CDCl_3$) (ppm): 178.40 ($C^{1''}$), 171.50 ($C^{4''}$), 136.62 ($C^{6''}$, $C^{10''}$), 129.69 ($C^{7''}$, $C^{8''}$, $C^{9''}$), 126.79 ($C^{5''}$), 52.54 ($H_3\mathbf{C}O(O)C^{4''}$), 37.21 ($C^{2''}$), 36.95 ($C^{3''}$). ^{77}Se NMR δ ($CDCl_3$) (ppm): 646.95 (d). The numbering is according to Scheme 4.

4.1.3. Synthesis of phenylselenyl succinic acid (5). HCl 12 N (10 mL) was added to **4** (0.70 g, 2.40 mmol) and the mixture was refluxed overnight at 75 °C. The solution was cooled at room temperature and a white solid was precipitated. The solid was filtered, washed with CH_2Cl_2 (2× 3 mL), and dried under vacuum. The yield was 0.55 g (82%). Found: C, 43.23; H, 3.92. $C_{10}H_{10}O_4Se$ $0.25H_2O$ requires: C, 43.26; H 3.81. ^{1}H NMR δ (CD_3CN) (ppm): 7.63 (d, 2H, $C^{6''}H$, $C^{10''}H$), 7.40 (m, 3H, $C^{7''}H$, $C^{8''}H$, $C^{9''}H$), 3.87 (dd, 1H, $C^{2''}H_X$), 2.77 (m, 2H, $C^{3''}H_2$). $^{13}C\{^1H\}$ NMR δ (CD_3CN) (ppm): 173.20 ($C^{1''}$), 172.20 ($C^{4''}$), 136.20 ($C^{6''}$, $C^{10''}$), 130.00 ($C^{7''}$, $C^{9''}$), 129.40 ($C^{8''}$), 127.2 ($C^{5''}$), 37.00 ($C^{2''}$), 36.60 ($C^{3''}$). ^{77}Se NMR δ (CD_3CN) (ppm): 634.80 (d). The numbering is according to Scheme 4.

4.1.4. Synthesis of phenylselenyl succinic anhydrite (6). Compound **5** (0.55 g, 2.00 mmol) and excess acetic anhydrite (10 mL) were stirred at 30 °C for 2 h. Acetic anhydrite was evaporated under vacuum resulting in 0.51 g (100%) of a brown solid. ^{1}H NMR δ (CD_3CN) (ppm): 7.68 ($C^{6''}H$, $C^{10''}H$), 7.42 ($C^{7''}H$, $C^{8''}H$, $C^{9''}H$), 4.39 (dd, 1H, $H_XC^{2''}$), 3.53 (dd, 1H, $C^{3''}\mathbf{H_A}H_B$), 2.94 (dd, 1H, $C^{3''}H_A\mathbf{H_B}$). ^{13}C NMR δ (CD_3CN) (ppm): 173.70 ($C^{1''}$), 172.73 ($C^{4''}$), 137.03 ($C^{6''}$, $C^{10''}$), 130.56 ($C^{7''}$, $C^{9''}$), 129.95 ($C^{8''}$), 127.65 ($C^{5''}$) 37.41 ($C^{2''}$), 36.26 ($C^{3''}$). ^{77}Se NMR δ (CD_3CN) (ppm): 668.52 (d). The numbering is according to Scheme 4.

4.1.5. Synthesis of α-tocopheryl-2-phenylselenyl succinate (7). Compound **6** (0.58 g, 2.3 mmol) and $ZnCl_2$ powder (0.17 g, 1.3 mmol) were added to a dry toluene solution (20 mL) of **1a** (0.30 g, 0.70 mmol). The whole mixture was stirred and refluxed under N_2 for one day. Next the mixture was filtered and the solvent was removed under vacuum. The oily residue was dissolved in 1–2 mL $CHCl_3$ and was passed through a well-packed chromatography column using as eluents first *n*-hexane, then a mixture of chloroform–*n*-hexane (5:1) and finally chloroform. The solvent was evaporated yielding 0.27 g (55%) of brownish oil containing **7a** and **b**, whose ratio varied from 40% to 60% in each component. TLC (hexane–$CHCl_3$, 1:5) $R_{1\alpha}$ = 0.69, R_7 = 0.11. Found: C, 68.91; H, 8.84. $C_{39}H_{58}O_5Se{\cdot}0.3n$-hexane requires: C, 68.86; H, 8.81. ^{1}H NMR δ ($CDCl_3$) (ppm): (**7b**) 11.10 (br, 1H, $HO(O)C^{1''}$), 7.71 (d, 2H, $C^{6''}H$, $C^{10''}H$), 7.36 (m, 3H, $C^{7''}H$, $C^{8''}H$, $C^{9''}H$), 4.02 (dd, 1H, $C^{2''}H_X$), 3.12 (dd, 1H, $C^{3''}(\mathbf{H_A})(H_B)$), 3.02 (dd, 1H, $C^{3''}(H_A)(\mathbf{H_B})$), 2.47 (m, 2H, C^4H_2), 1.99 (s, 3H, $C^{12}H_3$), 1.90 (s, 3H, $C^{13}H_3$), 1.86 (s, 3H $C^{14}H_3$), 1.70 (m, 2H, C^3H_2), 1.38 (m, 3H, $C^{4'}H$, $C^{8'}H$, $C^{12'}H$), 1.16 (s, 3H, $C^{11}H_3$), 1.35–0.9 (m, 18H, $C'H_2$-phytyl), 0.78 (m, 12H, H_3C-phytyl); (**7a**) 11.1 (br, 1H, $HO(O)C^{4''}$), 7.71 (d, 2H, $C^{6''}H$, $C^{10''}H$), 7.36 (m, 3H, $C^{7''}H$, $C^{8''}H$, $C^{9''}H$), 4.15 (dd, 1H, $C^{2''}H_X$), 3.05 (dd, 1H, $C^{3''}(\mathbf{H_A})(H_B)$), 2.85 (dd, 1H, $C^{3''}(H_A)(\mathbf{H_B})$), 2.47 (m, 2H, C^4H_2), 2.01 (s, 3H, $C^{12}H_3$), 1.90 (s, 3H, $C^{13}H_3$), 1.86 (s, 3H, $C^{14}H_3$), 1.70 (m, 2H, C^3H_2), 1.38 (m, 3H, $C^{4'}H$, $C^{8'}H$, $C^{12'}H$), 1.16 (s, 3H, $C^{11}H_3$), 1.35–0.9 (m, 18H, –$C'H_2$-phytyl), 0.78(m, 12H, H_3C-phytyl). $^{13}C\{^1H\}$ NMR δ ($CDCl_3$) (ppm): (**7b**) 177.93 ($C^{1''}$), 170.16 ($C^{4''}$), 149.93 (C^9), 140.75 (C^6), 136.66 ($C^{6''}$,$C^{10''}$), 129.78 ($C^{7''}$, $C^{9''}$), 129.40 ($C^{8''}$), 127.65 (C^7), 127.03 (C^5), 125.33 ($C^{5''}$), 123.50 (C^{10}), 117.87 (C^8), 75.48 (C^2), (39.81, 37.81, 37.72, 33.19, 33.11, 25.22, 21.45) ($C'H_2$-phytyl), 36.89 ($C^{3''}$), 37.12 ($C^{2''}$), 31.46 (C^3), 28.39 ($C^{4'}$, $C^{8'}$, $C^{12'}$), 24.86 (C^{11}), (23.14, 23.05) ($H_3\mathbf{C}C^{4'}$, $H_3\mathbf{C}C^{8'}$), 20.98 (C^4), (20.10) ($(H_3\mathbf{C})_2C^{12'}$), 13.37 ($H_3\mathbf{C}C^{14}$), 12.53 ($H_3\mathbf{C}C^{13}$), 12.22 ($H_3\mathbf{C}C^{12}$); (**7a**) 176.93 ($C^{4''}$), 171.53 ($C^{1''}$), 149.93 (C^9), 140.67 (C^6), 135.98 ($C^{6''}$, $C^{10''}$), 129.70 ($C^{7''}$, $C^{9''}$), 129.40 ($C^{8''}$), 127.38 (C^7), 126.86 (C^5), 125.93 ($C^{5''}$), 123.50 (C^{10}), 117.87 (C^8), 75.48 (C^2), (39.81, 37.81, 37.72, 33.19, 33.11, 25.12, 21.45) ($C'H_2$-phytyl), 37.52 ($C^{3''}$), 36.27 ($C^{2''}$), 31.46 (C^3), 28.39 ($C^{4'}$, $C^{8'}$, $C^{12'}$), 24.86 (C^{11}), (23.14, 23.05) ($H_3\mathbf{C}C^{4'}$, $H_3\mathbf{C}C^{8'}$), 20.98 (C^4), (20.10) ($(H_3\mathbf{C})_2C^{12'}$), 13.37 ($H_3\mathbf{C}C^{14}$), 12.53 ($H_3\mathbf{C}C^{13}$), 12.22 ($H_3\mathbf{C}C^{12}$). ^{77}Se NMR δ ($CDCl_3$) (ppm): (**7b**) 652.10 (d), (**7a**) 635.70 (d). The numbering is according to Scheme 4.

4.1.6. Synthesis of γ-tocopheryl-2-phenylselenyl Succinate (8). Compounds **1c** and **6** were used in the same mole ratio like in the synthesis of **7**, and similar preparation and purification procedures were followed. The yield was 50% in the brownish oil containing **8a** and **b** whose ratio varied from 40% to 60% in each component. TLC (hexane–$CHCl_3$, 1:5) R_{1c} = 0.59, R_8 = 0.31. Found: C, 67.79; H, 8.25. $C_{38}H_{56}O_5Se$ requires: C, 67.94; H, 8.40. ^{1}H NMR δ ($CDCl_3$) (ppm): (**8b**) 9.70 (br, 1H, $HO(O)C^{1''}$), 7.70 (d, 2H, $C^{6''}H$, $C^{10''}H$), 7.33 (m, 3H, $C^{7''}H$, $C^{8''}H$, $C^{9''}H$), 6.51 (s, 1H, C^5H), 4.09 (dd, 1H, $C^{2''}H_X$), 3.12 (dd, 1H, $C^{3''}(\mathbf{H_A})(H_B)$), 2.96 (dd, 1H, $C^{3''}(H_A)(\mathbf{H_B})$), 2.69 (m, 2H, C^4H_2), 2.17 (s, 3H, $C^{13}H_3$), 2.11 (s, 3H $C^{14}H_3$), 1.73 (m, 2H, C^3H_2), 1.51 (m, 3H, $C^{4'}H$, $C^{8'}H$, $C^{12'}H$), 1.29 (s, 3H, $C^{11}H_3$), 1.45–1.00 (m, 18H, $C'H_2$-phytyl), 0.86 (m, 12H, H_3C-phytyl); (**8a**) 9.70 (br, 1H, $HO(O)C^{4''}$), 7.70 (d, 2H, $C^{6''}H$, $C^{10''}H$), 7.33 (m, 3H, $C^{7''}H$, $C^{8''}H$, $C^{9''}H$), 6.55 (s, 1H, C^5H), 4.16 (dd, 1H, $C^{2''}H_X$), 3.09 (dd, 1H, $C^{3''}(\mathbf{H_A})(H_B)$), 2.94 (dd, 1H, $C^{3''}(H_A)(\mathbf{H_B})$), 2.69 (m, 2H, C^4H_2), 2.17 (s, 3H, $C^{13}H_3$), 2.11 (s, 3H, $C^{14}H_3$), 1.73 (m, 2H, C^3H_2), 1.51 (m, 3H, $C^{4'}H$, $C^{8'}H$, $C^{12'}H$), 1.29 (s, 3H, $C^{11}H_3$), 1.45–1.00 (m, 18H, $C'H_2$-phytyl), 0.86 (m, 12H, H_3C-phytyl). $^{13}C\{1H\}$ NMR δ ($CDCl_3$) (ppm): (**8b**) 178.74 ($C^{1''}$), 170.49 ($C^{4''}$), 150.12 (C^9), 141.82 (C^6), 136.80 ($C^{6''}$, $C^{10''}$), 129.79 ($C^{7''}$, $C^{9''}$), 127.86 ($C^{8''}$), 127.41 (C^7), 119.13 (C^5), 126.35 ($C^{5''}$), 118.90 (C^{10}), 118.84 (C^8), 76.46 (C^2), (40.69, 39.82, 37.89, 37.73, 29.83, 28.43, 25.64, 24.66, 22.68) ($C'H_2$-phytyl), 37.09 ($C^{3''}$), 37.25 ($C^{2''}$), 31.44 (C^3), 33.18 ($C^{4'}$, $C^{8'}$, $C^{12'}$), 24.66 (C^{11}), (23.20, 22.86) ($H_3\mathbf{C}C^{4'}$, $H_3\mathbf{C}C^{8'}$), 21.45 (C^4), 20.17 ($(H_3\mathbf{C})_2C^{12'}$), 13.06 (H_3C^{14}), 12.39 (H_3C^{13}); (**8a**) 177.73 ($C^{4''}$), 171.80 ($C^{1''}$), 150.12 (C^9), 141.73 (C^6), 136.47 ($C^{6''}$, $C^{10''}$), 129.57 ($C^{7''}$, $C^{9''}$), 127.86 ($C^{8''}$), 127.05 (C^7), 118.93 (C^5), 126.71 ($C^{5''}$), 118.90 (C^{10}), 118.84 (C^8), 76.46 (C^2), (40.69, 39.82, 37.89, 37.73, 29.83, 28.43, 25.64, 24.66, 22.68) ($C'H_2$-phytyl), 37.37 ($C^{3''}$), 36.63 ($C^{2''}$), 31.44 (C^3), 33.18 ($C^{4'}$, $C^{8'}$, $C^{12'}$), 24.66 (C^{11}), (23.20, 22.86) ($H_3\mathbf{C}C^{4'}$, $H_3\mathbf{C}C^{8'}$), 21.45 (C^4), 20.17 ($(H_3\mathbf{C})_2C^{12'}$), 13.06 (H_3C^{14}), 12.39 (H_3C^{13}). ^{77}Se NMR δ (CDCl3) (ppm):

(**8a**) 652.50 (d), (**8b**) 640.40 (d). The numbering is according to Scheme 4.

4.1.7. Synthesis of γ-tocotrienyl-2-phenylselenyl succinate (9). Similar synthesis and purification procedures like those described above were followed for the preparation and isolation of **9**. The yield was 53% in the brownish oil containing **9a** and **b** whose ratio varied from 40% to 60%. TLC (hexane–$CHCl_3$, 1:5) $R_{2c} = 0.51$, $R_9 = 0.26$. Found: C, 68.56; H, 7.42. $C_{38}H_{50}O_5Se$ requires: C, 68.55; H, 7.57. ^1H NMR δ ($CDCl_3$) (ppm): (**9b**) 9.64 (br, 1H, HO(O)C$^{1''}$), 7.68 (d, 2H, C$^{6''}$H, C$^{10''}$H), 7.35 (m, 3H, C$^{7''}$H, C$^{8''}$H, C$^{9''}$H), 6.52 (s, 1H, C^{5}H), 4.09 (dd, 1H, C$^{2''}H_X$), 3.15 (dd, 1H, C$^{3''}$(**H$_A$**)(H$_B$)), 2.89 (dd, 1H, C$^{3''}$ (H$_A$)(**H$_B$**)), 2.70 (m, 2H, C$^4H_2$), 2.15 (s, 3H, C$^{13}H_3$), 2.00 (s, 3H C$^{14}H_3$), 1.34 (s, 3H, C$^{11}H_3$), 1.83–1.05 (m, 2H, C$^3H_2$; 6H, C′H_2-methylfarnesyl, 6H, C′H-methylfarnesyl; 3H, H_3CC**H**-methylfarnesyl) 0.92 (m, 12H, H_3C-methylfarnesyl); (**9a**) 9.64 (br, 1H, HO(O)C$^{4''}$), 7.68 (d, 2H, C$^{6''}$H, C$^{10''}$H), 7.35 (m, 3H, C$^{7''}$H, C$^{8''}$H, C$^{9''}$H), 6.55 (s, 1H, C^{5}H), 4.16 (dd, 1H, C$^{2''}H_X$), 3.18 (dd, 1H, C$^{3''}$(**H$_A$**)(H$_B$)), 2.81 (dd, 1H, C$^{3''}$(H$_A$)(**H$_B$**)), 2.70 (m, 2H, C$^4H_2$), 2.15 (s, 3H, C$^{13}H_3$), 2.0 (s, 3H C$^{14}H_3$), 1.34 (s, 3H, C$^{11}H_3$), 1.83–1.05 (m, 2H, C$^3H_2$; 6H, C′H_2-methylfarnesyl, 6H, C′H-methylfarnesyl; 3H, H_3CC**H**-methylfarnesyl), 0.92 (m, 12H, H_3C-methylfarnesyl). ^{77}Se NMR δ (CDCl3) (ppm): (**9a**) 652.40 (d), (**9b**) 640.80 (d). The numbering is according to Scheme 4.

4.1.8. Synthesis of γ-tocopheryl succinate (11). Compound **11** was synthesized and purified in a similar manner to that described above, except that succinic anhydrite (0.091 g, 0.91 mmol) was added to the **1c**/toluene solution. The yield was 51% in the brownish oil containing **11**. TLC (hexane–$CHCl_3$, 1:5) $R_{1c} = 0.60$, $R_{11} = 0.24$. Found: C, 74.40; H, 10.98. $C_{32}H_{52}O_5$ requires: C, 74.38; H, 10.14. ^1H NMR δ ($CDCl_3$) NMR δ ($CDCl_3$) (ppm): 9.72 (br, 1H, HO(O)C$^{4''}$), 6.57 (s, 1H, C^{5}H), 2.86 (d, 2H, C$^{2''}H_2$), 2.84 (d, 2H, C$^{3''}H_2$), 2.70 (m, 2H, C$^4H_2$), 2.11 (s, 3H, C$^{13}H_3$), 2.02 (s, 3H C$^{14}H_3$), 1.53 (m, 2H, C$^3H_2$), 1.33 (m, 3H, C$^{4'}$H, C$^{8'}$H, C$^{12'}$H), 1.14 (s, 3H, C$^{11}H_3$), 1.30–1.00 (m, 18H, C′H_2-phytyl), 0.86 (m, 12H, H_3C-phytyl). $^{13}C\{1H\}$ NMR δ ($CDCl_3$) (ppm): 178.26 (C$^{1''}$), 171.69 (C$^{4''}$), 150.04 (C^9), 141.86 (C^6), 127.43 (C^7), 119.15 (C^5), 118.86 (C^{10}), 118.68 (C^8), 77.48 (C^2), (40.66, 40.46, 39.79, 31.81, 30.11, 29.38, 28.39, 25.21, 24.85) (C′H_2-phytyl), 37.69 (C$^{3''}$), 37.86 (C$^{2''}$), 31.42 (C^3), 33.15 (C$^{4'}$, C$^{8'}$, C$^{12'}$), 24.60 (C^{11}), (23.13, 22.86) (H_3**C**C$^{4'}$, H_3**C**C$^{8'}$), 21.41 (C^4), 20.16 ((H_3**C**)$_2$C$^{12'}$), 13.01 (H_3C^{14}), 12.36 (H_3C^{13}). The numbering is according to Scheme 4.

4.1.9. Synthesis of γ-tocotrienyl succinate (12). Compound **12** was synthesized and purified in a similar manner like that described above for **11**. The yield was 53% in the brownish oil containing **12**. TLC (hexane–$CHCl_3$, 1:5) $R_{2c} = 0.53$, $R_{12} = 0.20$. Found: C, 75.33; H, 9.12. $C_{32}H_{46}O_5$ requires: C, 75.26; H, 9.08. ^1H NMR δ ($CDCl_3$) NMR δ ($CDCl_3$) (ppm): 9.66 (br, 1H, HO(O)C$^{4''}$), 6.57 (s, 1H, C^{5}H), 2.81 (d, 2H, C$^{2''}H_2$), 2.79 (d, 2H, C$^{3''}H_2$), 2.75 (m, 2H, C$^4H_2$), 2.11 (s, 3H, C$^{13}H_3$), 2.01(s, 3H C$^{14}H_3$), 1.32 (s, 3H, C$^{11}H_3$), 1.84–0.83 (m, 2H, C$^3H_2$; 6H, C′H_2-methylfarnesyl, 6H, C′H-methylfarnesyl; 3H, H_3CC**H**-methylfarnesyl) 0.77 (m, 12H, H_3C-methylfarnesyl). The numbering is according to Scheme 4.

4.2. NMR spectroscopy

NMR spectra were recorded on a Bruker Avance 300 spectrometer operating at 300 MHz for ^1H and 75 MHz for ^{13}C. The ^1H and ^{13}C NMR spectra were recorded using a sweep width of 6000 and 15000 Hz, respectively, and a pulse angle of 30°. The 2D $\{^1H\}$ NMR COSY-45 experiments (pulse sequence 90°–t_1–45°) were conducted using 256 increments (each consisting of 16 scans) covering the full spectrum (8.00 ppm in both dimensions). The standard NOESY pulse sequence (90°–t_1–90°–t_m–90°) was used in the 2D $\{^1H\}$ EXSY-NOESY measurements and these spectra were acquired in 512 increments (with 16 scans each) covering 6.00 ppm of the spectra in both dimensions and 0.25 s mixing time. The phase sensitive HMQC sequence enriched with BIRD filter and GARP decoupling (90°) was applied at inverse H, C correlation for the 2D $\{^1H, ^{13}C\}$ HMQC spectra. Sensitive enhanced HMBC pulse sequence was used for 2D $\{^1H, ^{13}C\}$ HMBC with low-pass J-filter to suppress one bond correlations without decoupling during acquisition. The delay for evolution of long-range couplings was set to 50, 70, and 150 ms.

4.3. Free radical scavenging by DPPH·

The rate of DPPH· disappearance was measured at 515 nm during an interval from 1 s to 30 min. Stock solutions (20.5 μM) of DPPH· were freshly prepared in dry methanol previously distilled over magnesium. Stock solutions of the selenium derivatives (12 mM) were prepared in dry methanol and kept at room temperature. The final concentrations of the tested compounds were in the range 60–300 μM, while that of DPPH· was 20 μM. The samples were incubated at 25 °C for 4 min, and the reaction was initiated by the addition of DPPH·. The measurements were done in triplicate. The method of Espin et al.[34] who studied the radical scavenging capacity (RSC) of phenolic compounds by kinetic analysis was followed in the present study. Second-order rate constants were calculated to determine the RSC of antioxidants. In this study, the decay of DPPH· from the medium has been assumed to follow pseudo-first-order kinetics, under the conditions of the reaction $[DPPH^{\cdot}]_0$, $[AH]_0$, wherein one of the reactants is in large excess compared to the other, so that the concentration of the minor component decreased exponentially.[43,44] The [DPPH·] concentration is calculated from Eq. 2:

$$[DPPH^{\cdot}] = [DPPH^{\cdot}]_0 e^{-k_{obsd}t}, \quad (2)$$

where [DPPH·] is the radical concentration at time t, and $[DPPH^{\cdot}]_0$ is the radical concentration at time zero, and k_{obsd} is the pseudo-first-order rate constant. The pseudo-first-order rate constant k_{obsd} was linearly dependent on the concentration of antioxidants [AH], and from the slope of their plot, second-order rate constants (k_2) were

calculated to evaluate the radical scavenging capacity of each compound. The results of representative experiments are given as means ±SD.

4.4. Cell culture

4.4.1. Stock cultures and experimental media. PC-3 and DU-145, androgen unresponsive prostate cancer cells, were maintained in DMEM containing 3.7 g/L of $NaHCO_3$, supplemented with 10% (v/v) FBS and 1% penicillin, streptomycin, and Fungizone at 37 °C, and were equilibrated under a humidified atmosphere of 5% CO_2. Stock cultures were seeded at a density of 2×10^5 cells/mL, allowed to multiply to confluence and passaged using 0.05% trypsin.

Stock solutions of **1a**,**c**, **2c**, **4**, **5** and the **7**–**12** monoesters were prepared in pure ethanol and were diluted into the culture medium so that the final concentration of ethanol was 0.1%. The same amount of ethanol was added to the control sample. Stock solutions were kept at 4 °C.

4.4.2. Cell viability assays and quantification of cell death. The efficacy of compounds to modulate cancer cell growth was assessed in: (i) crystal violet proliferation assay and (ii) trypan blue exclusion test, assessing membrane integrity breakdown.

(i) Cell suspensions (5×10^3 cells/100 μL/well) were seeded in 96-well microtiter plates and allowed to adhere to the plate for 48 h. Cells were then incubated with 0.020, 0.040, 0.060, 0.080, and 0.100 mM of **1a**,**c**, **2c**, **4, 5**, and **7**–**12**, at 37 °C, 5% CO_2 for 72 h. Cells were subsequently subjected to crystal violet assay which employed an aqueous solution containing 0.2% crystal violet and 2% ethanol (0.20 g crystal violet in 100 mL of deionized water plus 2.00 mL of ethanol). Viability was determined by staining cells with the crystal violet solution and assessing the OD_{620} of the cell lysates correlating to cell number, by using a microplate reader.

(ii) Trypan blue distinguishes apoptotic cells with high sensitivity.[36] Cell suspensions (1×10^6/3 mL/well) were plated in 6-well plates and treated with the appropriate concentrations of test compounds as in (i). Treated cells were harvested, pelleted, and resuspended in culture medium. Cell suspension (10 μL) was mixed with an equal volume of trypan blue and cells were counted on a hematocytometer under a light microscope. Apoptotic cells were distinguished by revealing their rough membranes, different shapes and sizes as well as their nuclear condensation. Evenly blue cells were considered as dead. Treatments were performed in triplicate and repeated twice. Results are given as mean values of two experiments ±SD.

4.4.3. DAPI staining of nuclei. Cells were seeded on plastic chamber slides and treated as indicated. After two washes with PBS, cells were fixed in methanol for 5 min, washed again with PBS, and incubated with 1.00 mg/mL DAPI in PBS for 15 min at 37 °C. After several washes with deionized water, the slides were mounted with PBS/glycerol. DAPI staining was visualized by fluorescence microscopy. Apoptotic cells were recognized based on nuclear morphology and by counting the number of pyknotic nuclei from three random fields, each of 250 cells, per slide.

4.4.4. Measurement of DEVD-caspase activity. DEVD-caspase was assayed by cleavage of z-DEVD-AFC, a fluorogenic substrate based on the peptide sequence at the caspase-3 cleavage site of poly (ADP–ribose) polymerase as described by Nicholson et al.[40] and modified by others.[39,45,46] Cells (5×10^5) grown in 24-well plates were incubated with IC_{50} concentrations of the compounds for 72 hrs, washed once with PBS, and resuspended in 200 μL of lysis buffer containing 5.00 mM Tris–HCl, 5.00 mM EDTA, and 0.5% NP-40, pH 7.50. After 15 min in lysis buffer at 4 °C, insoluble material was pelleted at 15,000*g* and an aliquot of the lysate was tested for protease activity. Each assay contained 8.00 μM of substrate in 1.00 mL of protease buffer (50 mM HEPES, 10% sucrose, and 10.0 mM DTT, 0.1% CHAPS, pH 7.40) with 20 μL of cell lysate. Control samples contained the same final concentration of pure ethanol (0.10% v/v). After 4 h at room temperature, fluorescence was quantified (excitation 400 nm, emission 505 nm) by using a microplate reader. Results are given as means for triplicate measurements from a representative experiment repeated at least twice ±SD.

4.4.5. Statistical analysis. Comparison of the effect of the different compounds on absorbance at the appropriate wavelength (crystal violet assay, DEVD-caspase activity) or on the number of apoptotic cells (trypan blue test) was made by analysis of variance (ANOVA). Post hoc comparisons were made with one-sample Student's *t* test for 95% confidence.

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