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Tocotrienamines and tocopheramines: Reactions with radicals and metal ions

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ABSTRACT

The antioxidant activity of vitamin E (VE) homologs α, γ and δ-tocotrienamines (**4b**–**6b**), never studied before, and α, γ and δ-tocopheramines (**4a**–**7a**) was investigated by means of different total antioxidant capacity (TAC) tests. In all the test model systems, compounds **4a**–**7a** and **4b**–**6b** showed similar or higher TAC values than the parental vitamin E forms and their physiological metabolites. α-Homologs of VE amines showed markedly higher activity than the VE congeners in the TEAC test, which is tailored for liposoluble antioxidants, while γ-homologs of the amine analogs showed higher activity in the FRAP tests. Kinetics analysis of the reaction with DPPH· showed higher second order rate *k* for **4a** than for α-tocopherol (**1a**). α-Tocopherolquinone **1f** was the common main oxidation product for both **1a** and α-tocopheramine (**4a**) exposed to ferric ions or DPPH·, and the implied oxidative deamination of **4a** was accompanied by a nitration reaction of phenolic substrates that were added to the reaction medium. Possible mechanisms of these reactions were studied.

1. Introduction

Vitamin E (VE) is a term that encompasses a group of fat soluble vitamers (four tocopherols and four tocotrienols), derived from the structure of 6-chromanol,[1] which exhibit the biological activity of α-tocopherol **1a** (Fig. 1). In 1942, Smith et al. reported that the amino analog of racemic **1a**, racemic α-tocopheramine **4a**, has approximately the same vitamin and antioxidant properties as **1a** itself.[2] Later on, also (all-*rac*)-*N*-methyl-β- and γ-tocopheramine were reported to show vitamin activity close to **1a**.[3,4] Using an isotope dilution method, it was proved that their vitamin activity was not due to a conversion of the amine analogs into **1a**, and that those analogs shared the same metabolic pathway with the corresponding VE forms, providing carboxyethyl hydroxychromans (CEHCs) and tocopherolquinone as metabolites.[5] More recently, the antioxidant behavior of α- to δ-tocopherol and α- to δ-tocopheramine was compared in different model systems,[6] showing a superior capability of tocopheramines in the quenching of singlet oxygen, while tocopherols were more reactive towards phenoxyl radicals.

Recently, we reported a very simple route for the preparation of all the VE amines in stereoisomerically pure form, to be used as precursors of VE amide analogs.[7] According to this original procedure, α-, γ- and δ-tocotrienamine (**4b**, **5b**, **6b**, respectively, Fig. 1) have been synthesized for the first time. These VE analogs have been recently demonstrated to show in vitro and in vivo anticancer activity.[8,9]

With the present study, we comparatively investigated the antioxidant activity of all these amine analogs. Based on the recent work by Muller et al.[10] three electron-transfer assays were used to comparatively evaluate antioxidant capacity of VE and VE amine compounds, namely the Trolox equivalent antioxidant capacity (TEAC) assay, the 2,2-diphenyl-1-picrylhydrazyl (DPPH·) assay and the ferric ion reducing antioxidant parameter (FRAP). In addition, we carried out a preliminary study on the oxidation of **4a** that disclosed an interesting nitrating activity of electron rich aromatic amines under oxidative conditions.

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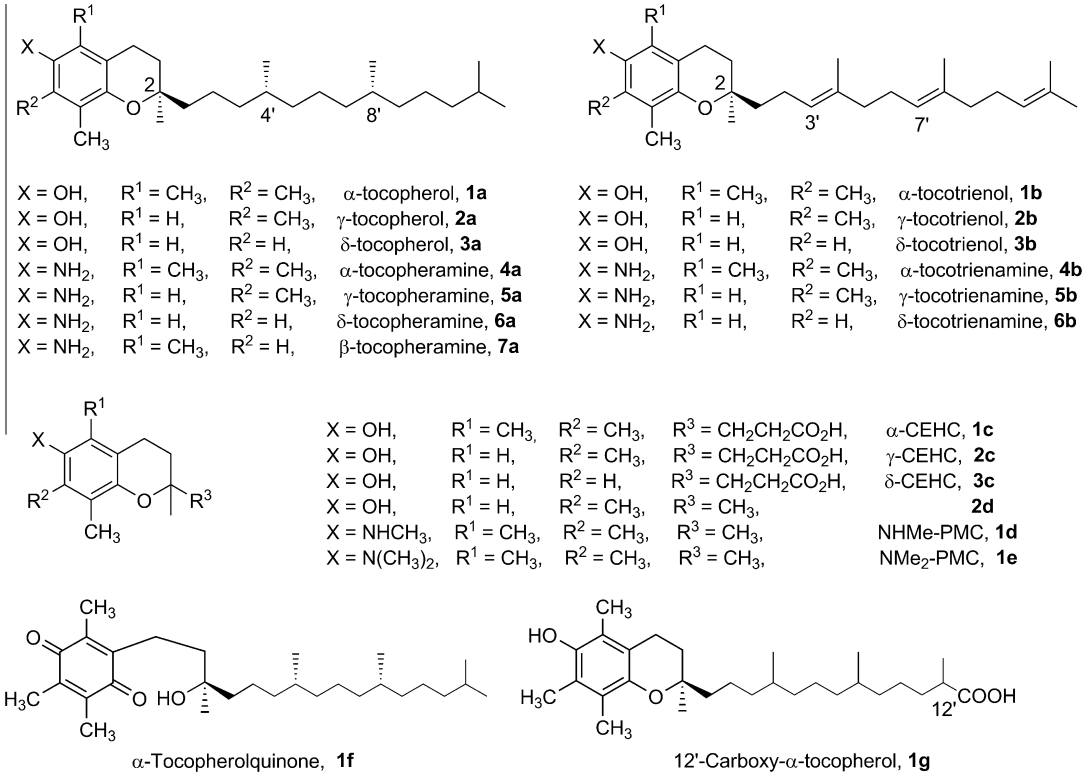

Stereochemistry: (2*R*,4'*R*,8'*R*) for tocopherols, tocopheramines and **1f**, (2*R*,3'*E*,7'*E*) for tocotrienols and tocotrienamines, (2*R*,4'*RS*,8'*RS*,12'*RS*) for **1g**, (2*RS*) for CEHCs **1c-3c**.

Figure 1. Structures of vitamin E compounds assayed and α-tocopherolquinone.

2. Results and discussion

2.1. Antioxidant tests

The TEAC assay is based on the reduction of the radical cation from 2,2′-azinobis-(3-ethylbenzothiazoline)-6-sulfonic acid ($ABTS^{\cdot+}$). This is a mixed electron-transfer and hydrogen atom-transfer assay that has been described to be more suitable than other TAC probes in assessing fat soluble antioxidants.[11] The results of this assay expressed as Trolox equivalents (Fig. 2, upper panel) showed that VE amines have higher antioxidant power than all the other VE compounds assessed in this study. Among the amine compounds, antioxidant data of the α-homolog was higher when compared to γ- and δ-homologs, in agreement with previous results,[10] indicating that the degree of methylation as well as the character of the side chain made little difference in determining the antioxidant capacity of tocopherols and tocotrienols in this assay.

The FRAP test is a pure electron-transfer assay that determines the capability of antioxidant compounds to reduce ferric ions that are detected by the formation of a complex with the probe di-2,4,6-tripyridyl-s-triazine (TPTZ).[12,13] Differences among the assayed compounds were negligible in this test except for the γ-homolog of VE amines that showed appreciably higher reduction capacity (Fig. 2, middle panel).

Reduction of $DPPH^{\cdot}$, a stable organic nitrogen radical, (Fig. 2, lower panel) showed that antioxidant activity of both VE and VE amines paralleled the degree of methylation of the chroman ring with the order of magnitude α > γ > δ, independently of structural features of the side chain and without differences between VE and VE amines. This parallels the expected electron donating capability of chromanols even if hydrogen transfer could be also involved in this test.[14]

Although the three tests provided different reducing activities, which is expected because of chemical differences of the assay systems,[10,14] all VE amines investigated showed similar or higher antioxidant activity than their tocopherol and tocotrienol counterparts.

In the copper-mediated ascorbic acid oxidation test, a fairly concentration-dependent antioxidant effect (lower oxidation rate of ascorbic acid) was demonstrated for all the tocopheramine derivatives following the order: **6a** > **5a** > **4a**. Baseline oxidation rate for the copper-induced ascorbic acid depletion test (calculated using the vehicle ethanol as sample) was 11.4 ± 0.9 nM s^{-1}. A considerable decrease of the oxidation rate was observed for VE amines when applied at concentrations $\geqslant$10 μM, and this effect was significant vs **1a** only at the final concentration of 100 μM, with a percentage of reduction of 19.5 ± 8.3, 35.0 ± 4.9 and 44.7 ± 3.2 for the homologs **4a**, **5a** and **6a**, respectively (p <0.05 for **5a** and **6a**).

Altogether these results demonstrate that VE amines can be a valuable alternative to natural forms of VE when used as antioxidants in different model systems based on radical- or metal-dependent oxidation reactions. Although this evidence was suggested in earlier studies [see[6] and references therein], here we show for the first time a semi-quantitative comparison of antioxidant power of all the series of VE amine analogs, including tocotrienamines, never investigated before.

2.2. Stopped-flow analysis of $DPPH^{\cdot}$ reaction with 1a and 4a

Besides steady-state tests, the reaction kinetics was investigated by stopped flow spectroscopy (Fig. 3). The reaction of $DPPH^{\cdot}$ with tocopheramines/tocotrienamines and **1a** was studied in ethanol. By means of the kinetic isolation technique (10- to 20-fold

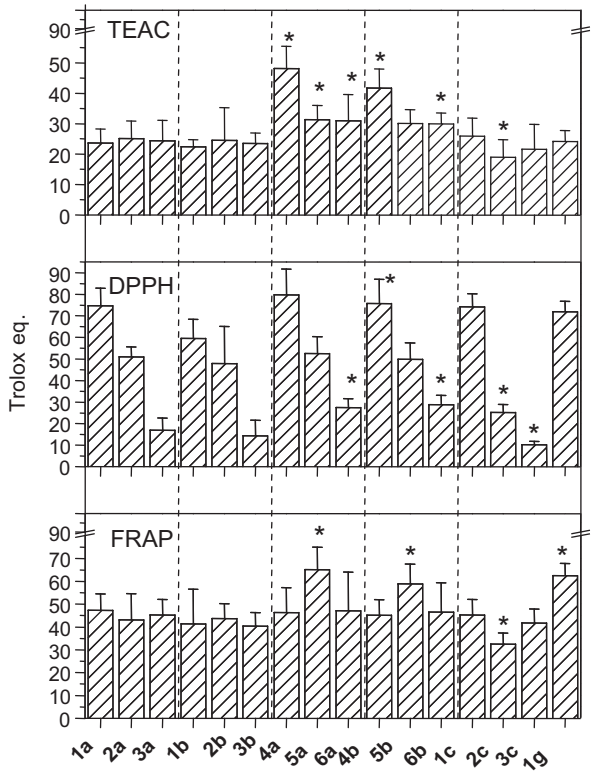

Figure 2. Steady-state electron-transfer capacity of VE amines and their corresponding VE forms. Compounds **1a**–**6a**, **1b**–**6b**, **1c**–**2c** and **1g** were investigated with three total antioxidant capacity tests (from top to bottom left panels): the trolox equivalent antioxidant capacity (TEAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH·) bleaching and the ferric reducing ability (FRAP) test. Further details on these analyzes methods are reported in the text. * p <0.05 with respect to the corresponding form in the tocopherol or tocotrienol series; **1c**–**2c** and **1g** were compared with tocopherols.

excess of the antioxidant over DPPH·) directly the second order rate constants (L mol^{-1} s^{-1}) were obtained (Fig. 3).

The rate constant obtained for **1a** was 280 ± 22 L mol^{-1} s^{-1}. In contrast tocopheramines/tocotrienamines exhibited much higher rate constants, such as 4287 ± 1371 L mol^{-1} s^{-1} for **4a**. The obtained rate constants for **1a** were in the range of data (245 ± 15 L mol^{-1} s^{-1}) obtained in a previous study.[15] For tocopheramines and tocotrienamines so far no rate constants with DPPH· were available. The permethylated **4a** and **4b** showed nearly

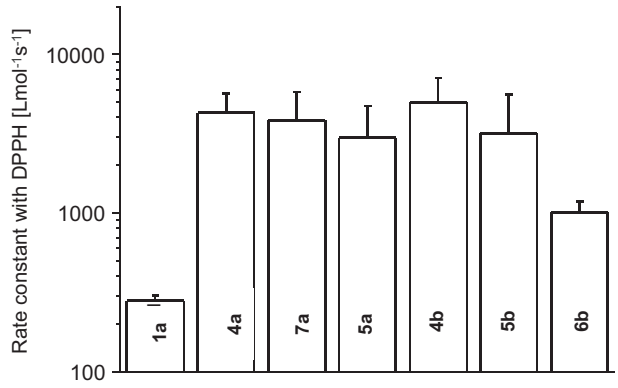

Figure 3. Second order rate constants for the reaction of **1a** and selected tocopheramines/tocotrienamines with DPPH·. The rate constants were obtained from the decay of the DPPH· absorption at 518 nm in ethanol in the presence of an at least 10-fold excess of the antioxidant over DPPH·. Data represent mean values ± SD of at least five experiments.

identical rate constants. Related to the α-congeners of VE amines, respectively, the hypomethylated derivatives showed decreased rate constants, but still higher rates than **1a**. This in line with the observation of Mukai et al.[16] that the rate constants for tocopherol congeners in the reaction with an aroxyl radical increase with the degree of methylation.

2.3. Iron- and DPPH·-mediated deamination of VE amines

At first sight, those similarities in the antioxidant behavior of VE and VE amines may suggest that they share the same mechanism of action. Indeed, their structures are very closely related, and they form the same oxidation end product, the tocopherolquinone.[2] DPPH·-mediated and ferric ion-mediated oxidation of **1a** and **4a** were investigated in order to verify whether these vitamers form α-tocopherolquinone (**1f**, Fig. 1) as oxidation product in both reaction systems.

2.3.1. Reaction with DPPH·

Tocopherolquinone **1f** was produced during the DPPH·-mediated oxidation of **1a** or **4a** (1:1 molar ratio, Fig. 4), in similar yields. GC–MS analysis was used to confirm the conversion of **4a** to **1f** in this reaction (not shown) and in the Fe-induced oxidation reaction (Fig. 7, right panel). In order to detect possible reaction products of DPPH·, the mixtures of DPPH· (0.5 mM) with **1a**/**4a** (0.5 mM) were subjected to HPLC analysis after 20 min reaction (Fig. 5). While the formation of nitrated DPPH· or DPPH-H was not directly observed, the HPLC analysis revealed a product (retention time 4 min), which was only observed after stoichiometric reduction of DPPH· with **4a** but not with **1a**. A LC/MS analysis gave MS peaks at 228 and 263 m/z. So far these data suggest a breakdown product of DPPH· (such as a nitrated diphenyl hydrazine derivative or trinitroaniline), which arises only in the presence of **4a**.

This raised the question which intermediates of tocopheramine oxidation could be observed. Attempts to detect and identify aminyl radicals of **4a** at room temperature failed so far. However, it was possible to show that upon photooxidation of the N-methylated α-tocopheramine model compound **1d** (Fig. 1) in the presence of oxygen, a fairly stable radical could be detected by ESR spectroscopy (Fig. 6 left panel). Simulation of the spectrum based on a hypothetic nitroxyl radical agrees with the experimental spectrum. The splitting pattern for this nitroxyl radical of **1d** shows similarities to the spectrum obtained by Murphy et al.[17] during oxidation of *N*-methyl-γ-tocopheramine.

Since time resolution of ESR spectroscopy was too low to detect similar intermediates also for **4a** during oxidation with DPPH· and other oxidants, possible intermediates were studied by optical stopped flow spectroscopy. Figure 6 (right panel) shows UV/vis spectroscopy profiles obtained between 300 and 650 nm of the products formed during the reaction between DPPH· radical and **1a** and **4a** in acetonitrile. The results are consistent with the formation of the chromanoxyl radical from **1a** (λ_{max} at 428 nm; Fig. 6, right upper panel) in agreement with previous findings.[15] During the reaction of **4a** with DPPH· a reaction intermediate of **4a** with λ_{max} at 474 nm (Fig. 6, right lower panel) was observed in the time windows of 100–200 ms after mixing. Although UV/vis absorption is not suitable for proper identification of the intermediate, it is interesting in this context that nitroxyl radicals of other compounds have an absorption maximum between 400 and 500 nm.[18] These data suggest that intermediates of tocopheramines with higher oxidation states of nitrogen are possibly involved in their oxidation by DPPH· and other oxidants.

2.3.2. Reaction with ferric iron

In the case of **1a**, the ferric ion-catalyzed reaction proceeded to completion very rapidly producing **1f** in the presence of 1:1 molar

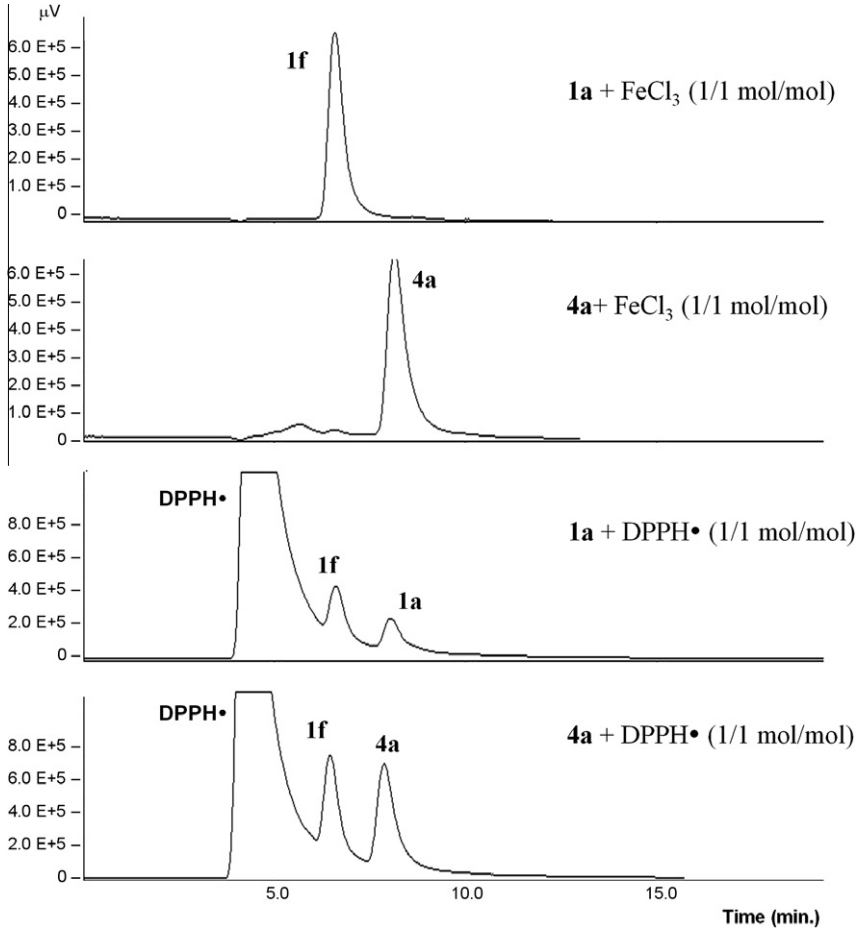

Figure 4. HPLC-ECD analysis of the reaction between $FeCl_3$ (top)/DPPH· (bottom) and **1a** and **4a**. (right) HPLC-ECD analysis of **1a** and **4a** oxidation products formed during the reaction with DPPH·. Peak identity was confirmed using authentic external standards and by GC–MS analysis that was carried out as described in the text.

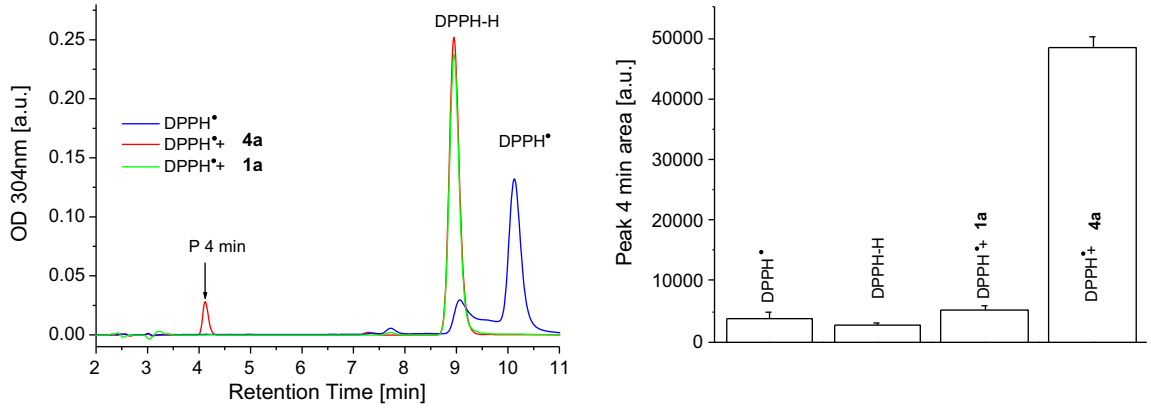

Figure 5. HPLC analysis of the DPPH· related reaction products obtained from the reaction of DPPH·· with **1a**/**4a**. (left) Chromatogram obtained by UV detection at 304 nm of DPPH· (starting compound), DPPH· + **1a** and DPPH· + **4a**. In addition, the major reduction product (DPPH-H) and a unknown DPPH· reaction product (P 4 min) are indicated. (right) Peak area of the reaction product eluting at 4 min in different reagents and the reaction mixtures of DPPH· + **1a** and DPPH· + **4a**. Data represent mean values ± SD of five experiments.

ratio (or higher, i.e., 1:4 and 1:40) between the vitamer and the oxidant (Figs. 4 and 7). Compound **1f** was also obtained in the oxidation of **4a**, but only with a partial conversion at 1:40 **4a** to $FeCl_3$ molar ratio (approx. 45% conversion as estimated by HPLC-ECD, Fig. 4, bottom left panel), while a molar ratio of 1:4 was not sufficient to form **1f** from **4a**. This yield of **1f** lower than that obtained with **1a** is in apparent contrast with FRAP data (Fig. 2, lower panel) where **4a** and **1a** produced the same extent of one-electron reduction. This can be possibly explained by the different reaction conditions of these two experiments. While **1f** formation (Fig. 7) was assessed in unbuffered ethanol (pH 6.3), the reduction of Fe^{3+}–TPTZ complex in the FRAP test was carried out in acetate buffer 300 mM, pH 3.6, with a 1:500 molar ratio between vitamin E compounds and ferric ions.[12,13]

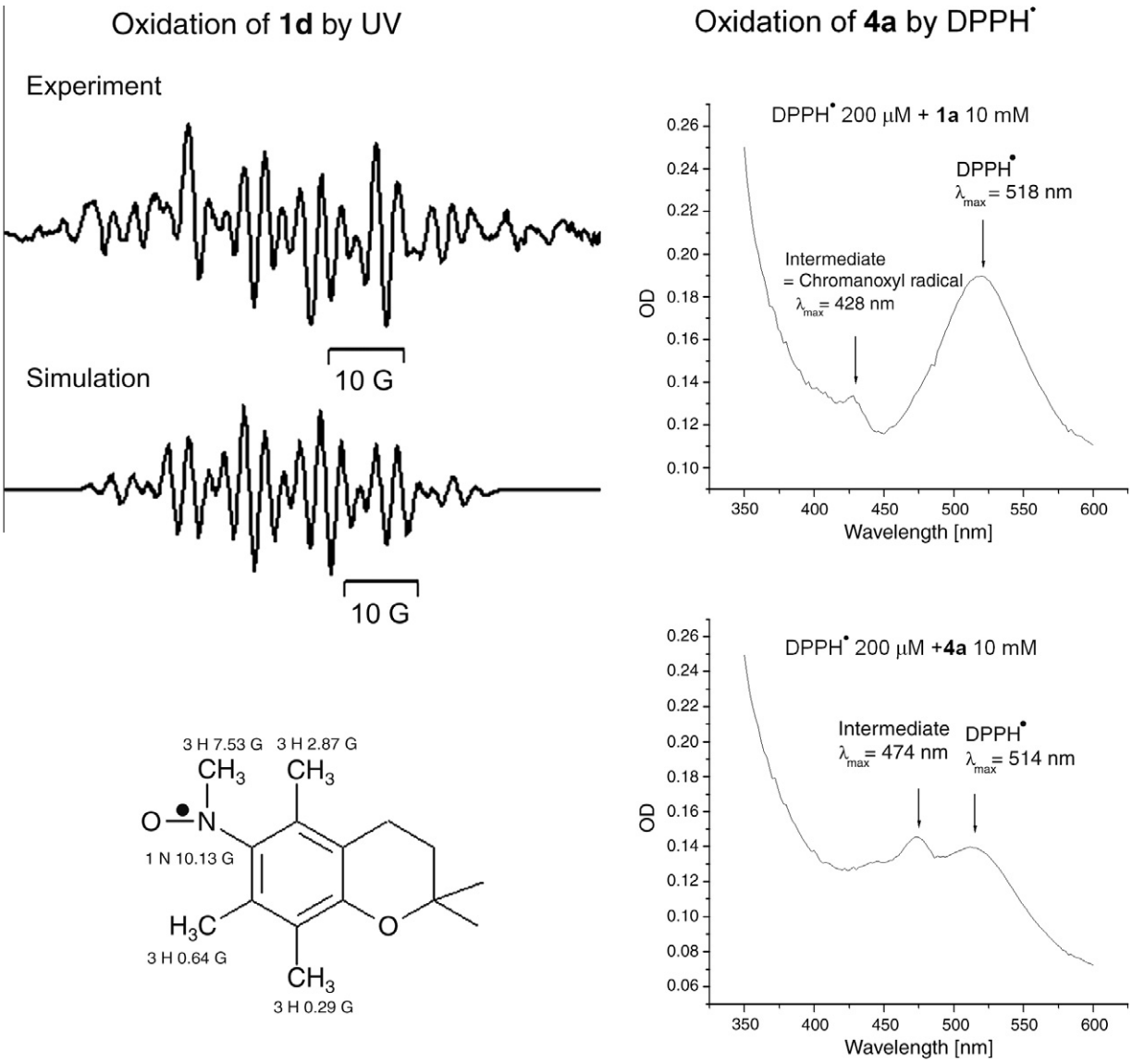

Figure 6. Intermediates observed during reaction between DPPH·· and tocopheramines. (left) ESR spectrum of the photooxidation product of **1d**. The simulated ESR spectrum is based on the structure of the nitroxyl radical intermediate of **1d**. The coupling constants (Gauss) used for the simulation are listed adjacent to the respective atoms. (right) Stopped flow UV/vis spectra obtained during the reaction of DPPH· with **1a** (upper) and **4a** (lower). The spectra show the absorption maximum of DPPH· at 518/514 nm, which is rapidly decaying in this reaction. In the wavelength region below in the case of **1a** the chromanoxyl radical (λ_{max} 428 nm) and in the case of **4a** an intermediate with λ_{max} of 474 nm is detected.

2.4. Phenol substrate nitration in the iron-mediated deamination of 4a

Since both metal- and radical-dependent oxidation reactions lead to tocopheramine deamination to form **1f**, we preliminarily investigated the fate of the nitrogen species released into the reaction medium, starting from the hypothesis that the oxidation of the amino group induced the release of a radical species which could correspond or evolve to NO_x species. The analogy with the nitro radical scavenging ability of tyrosine[19] suggested the use of phenolic compounds in order to trap possible tocopheramine derived radicals or NO_x species during the $FeCl_3$ induced oxidation of **4a**. Under 1:10 and 1:100 **4a** to $FeCl_3$ molar ratio (in 50:50 v/v H_2O/EtOH), phenol, 2,6-dimethylphenol and 2,6-di-*tert*-butylphenol were all found to form the corresponding nitration products. In this series of substrates, increasing steric hindrance in proximity to the phenol group reduced the conversion rate into corresponding nitration products with results in the following order of magnitude: phenol > 2, 6-dimethylphenol > 2,6-di-*tert*-butylphenol (Table 1).

On the other hand, if the phenol group was blocked, as in anisole and 2,6-dimethoxybenzene, no nitration product was observed. From these data, it seems that the nitration reaction is a radical process, in which the formation of an intermediate phenoxyl radical is critically involved. If the phenoxyl radical cannot be formed, like in anisole, or its formation is difficult because of steric hindrance, the reaction does not occur or proceeds to a lesser extent, respectively. Traces of nitrated phenol (i.e., *o*- and *p*-nitrophenol, 2,6-dimethyl-4-nitrophenol) were observed in the reaction on methoxy analogs employing a 100:1:1 ratio, indicating that an O-demethylation reaction occurred and the resulting free phenol was then nitrated.

We did not find any nitroso-, azo-, azoxy- or nitro-tocopheryl analogs originating from direct oxidation of the 6-amino group in **4a**.

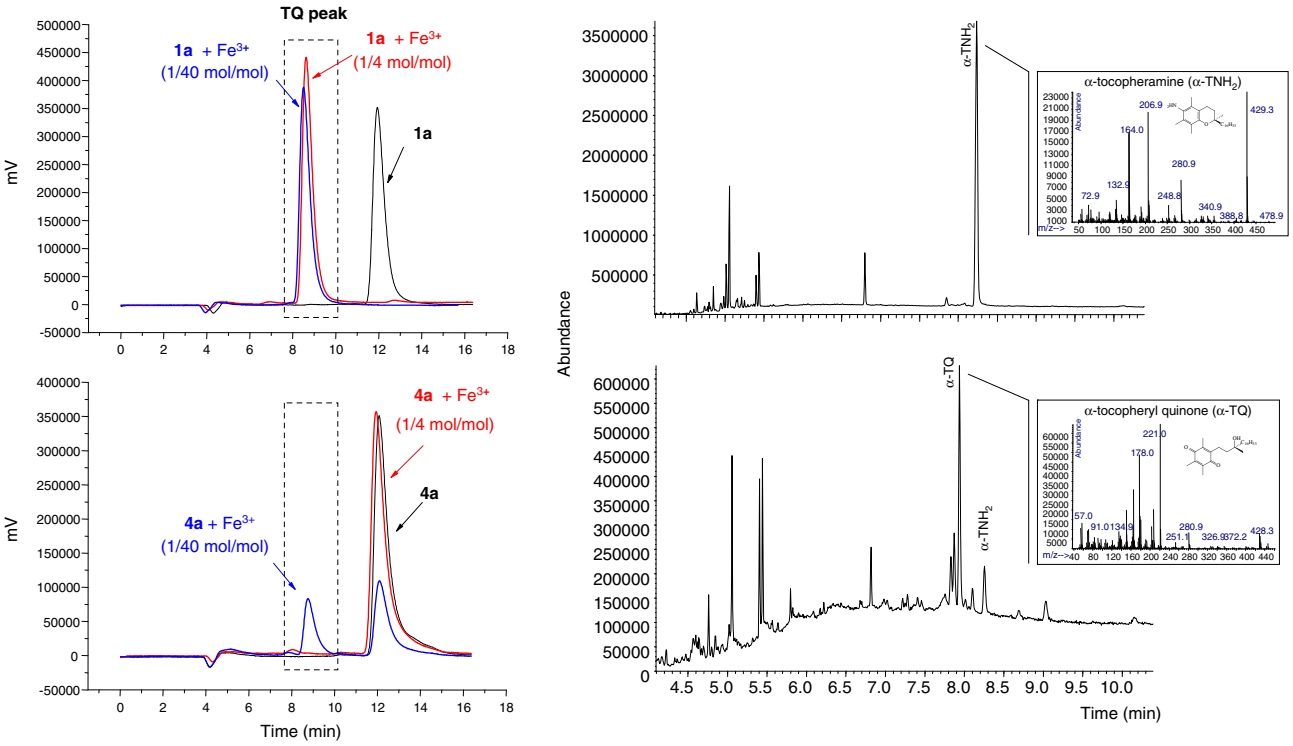

Figure 7. HPLC-ECD (left) and GC–MS (right) analysis of the oxidation products formed during the ferric ion-catalyzed reaction of **1a** and **4a**. **1a** and **4a** were exposed to a molar excess of $FeCl_3$ (1:40 or 1:4 mol/mol) in ethanol and immediately assessed by HPLC-ECD or GC–MS analysis as described in detail in the text.

Table 1
Nitration of phenolic substrates during the ferric ion-catalyzed oxidation of **4a**

Compound	Nitration product[a] (%)	Nitration product[b] (%)
PhOH	12	26
PhOMe	0	2
$HOC_6H_3(Me)_2$-(2,6)	4	9
$MeOC_6H_3(Me)_2$-(2,6)	0	Traces
$HOC_6H_3(t\text{-}Bu)_2$-(2,6)	0.2	0.4

Data were mean values of triplicate experiments.
[a] $FeCl_3$/**4a**/phenolic 10:1:1.
[b] $FeCl_3$/**4a**/phenolic 100:1:1.

In order to investigate if the observed reaction was specific for **4a** or shared with other aromatic amines, aniline and 4-aminoanisole were used in place of **4a** in the presence of phenol. In the case of aniline, no detectable nitration product was observed. On the other hand, 4-aminoanisole, a model compound possessing higher similarity to **4a**, gave 2% and 6% conversion into nitrophenol employing $FeCl_3$ to 4-aminoanisole to phenol 10:1:1 and 100:1:1 molar ratios, respectively.

Those results suggested that, in the presence of phenoxyl radicals generated under oxidative conditions, aromatic primary amines bearing electron donating groups can transfer the nitrogen to another phenolic substrate, the end product being a nitro derivative. The proposed formation of radical intermediates is currently under further investigation by ESR and stopped flow techniques.

The nitration reaction was also preliminarily investigated in apolar conditions using **2a**, its analog 6-hydroxy-2,2,7,8-tetramethylchroman **2d** (Fig. 1) and salicylic acid as phenolic substrates. Oxidation reactions were carried out in hexane using activated Ag_2O as oxidizing agent. In all these cases nitration products were detectable, the best results obtained by the exposure of **4a** to Ag_2O in *n*-hexane with a fivefold molar excess of the oxidant species. For salicyclic acid, these conditions produced a 33% of conversion into the corresponding nitration product 5-nitro-salicyclic acid. When applying an 1:1 **4a** to salicylic acid ratio, 8% of the salicylic acid was nitrated so that the two outcomes are consistent. In the case of **2a** and its model compound **2d**, the yield of the corresponding nitration products was 13% and 3%, respectively, in the case of 1:1 **4a** to phenol ratio.

3. Conclusions

Tocopheramines and, for the first time, tocotrienamines were comparatively assessed for their antioxidant function using three TAC tests, namely TEAC, DPPH· bleaching and FRAP test. In all these single-electron tests, VE amines showed similar or higher antioxidant capacity than the parental tocopherols, tocotrienols and chromanol metabolites. α-Tocopheramine and α-tocotrienamine showed markedly higher activity than the congeners in the TEAC test, which is tailored for liposoluble antioxidants and is a mixed electron-transfer and hydrogen atom-transfer assay, while gamma forms of the amine analogs showed high activity in the FRAP tests which is a pure electron-transfer test. Kinetics analysis of DPPH· reduction, showed higher second order rate *k* for VE amines than **1a**. Deamination of tocopheramines occurred during either ferric ions or DPPH· reactions with the formation of tocopherolquinone as oxidation product. At the same time, present phenolics were nitrated, proving that the amino group was released and transformed into nitrating species that in turn were trapped by the phenol. The nitration of phenolic substrates depended on the presence of a free phenolic hydroxyl group, and the steric hindrance of this phenolic hydroxyl group influenced the nitration yield. The data suggest a radical mechanism for the deamination of VE amine compounds, involving phenoxyl radicals in the nitration mechanism.

This study clearly demonstrates that VE amines are synthetic analogs with superior antioxidant activity in the VE family of compounds. Although the detailed antioxidant mechanism of those compounds remains undisclosed, we here identified that in both

metal- and radical-catalyzed reactions, VE amines exert nitrating activity against phenolic substrates. This is a new finding that deserves further investigation to ascertain its biological relevance and a possible role in the antioxidant function of VE amines.

These results in this study may help to better understand vitamin E activity as well as the anticancer and anti-inflammatory properties of these VE analogs.

4. Experimental section

4.1. Chemicals and general experimental

Synthesis and characterization of VE amines were described in Ref. 7. All the test compounds were stored as powders or oils in sealed amber vials under nitrogen at −20 °C until use. Stock solutions were prepared by dissolving the VE and VE analogs in absolute ethanol, divided in aliquots and maintained under the same storage conditions. Before each experimental session, compound purity and actual concentration of stock solutions were verified by UV spectroscopy and HPLC analysis (see below). (2*R*,4′*R*,8′*R*)-α-, γ- and δ-tocopherol, salicylic acid, diphenyl picryl hydrazyl radical (DPPH·) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) were from Sigma Chemicals (Milan, Italy). CEHC metabolites were prepared as described in Ref. 20. Metabolite **1g** was kindly provided by Dr. Marc Birringer, Department of Human Nutrition, Institute of Nutrition, Friedrich Schiller University, Jena, Germany. Other reagents and solvents were commercially available and used without further purification. Reagent-grade solvents were used for all extractions and workup procedures. *n*-Hexane, diethyl ether, ethyl acetate and petroleum ether used in chromatography were distilled before use. TLC was performed using Merck silica get 60 F254 pre-coated plates. Flash chromatography was performed using Baker silica gel (40 μm particle size). All products were purified to homogeneity and verified by TLC/GC analysis. Melting points, determined on a Kofler-type micro hot stage with Reichert-Biovar microscope, are uncorrected. NMR spectra were recorded at 300.13 MHz for ^{1}H and at 75.47 MHz for ^{13}C NMR in $CDCl_3$ if not otherwise stated. Chemical shifts, relative to TMS as internal standard, are given in δ values, coupling constants in Hz. ^{13}C resonances were assigned by means of APT, HMQC and HMBC spectra. Elemental analyzes were performed at the Microanalytical Laboratory of the Institute of Physical Chemistry at the University of Vienna.

4.2. Statistics

Data were presented as mean ± SD of three series of experiments run in triplicate. Differences between TAC data were assessed by Student Newman–Keuls post-hoc ANOVA and a level of $p < 0.05$ was accepted as significant.

4.3. Vitamin E analysis

Analysis of VE compounds was performed by HPLC-ECD (electrochemical detector) and GC–MS as described before.[21,22] HPLC analysis was carried out under isocratic conditions on a MetaSil AQ 3μ, C18 150 × 4.6 mm column (Varian Inc.) using 10 mM sodium perchlorate in 98:2 MeOH/H_2O as mobile phase. The ECD detector was composed of a glassy carbon cell with working electrode set at 750 mV oxidation potential against a AgCl reference electrode. For redox-silent compounds, UV detection was operated using a Jasco UV975 detector module set at 210 nm and mounted in series before the ECD detector. GC–MS analysis was performed on a 7890A GC system equipped with a 5975C VL triple-axis mass spectrometry detector (Agilent Technologies, Inc.). Helium gas was used as carrier gas at constant flow of 1.2 ml/min. Injector and transfer line temperature were 250 °C and 290 °C, respectively. A split–splitless inlet set to pulsed splitless mode was used for injection. Starting oven temperature was set at 50 °C and compound separation was obtained with a linear temperature ramp of 50 °C/min from 50 °C to 320 °C; this final temperature was held for 5 min. MS source temperature was set at 230 °C and Electron Ionization (EI) was operated at constant voltage with the electro multiplier set at 1200 V and 4 min solvent delay. Total ion current was performed and the collected data were analyzed with Enhanced Chemstation software (Agilent Technologies, Inc.) that was supported with NIST library for MS spectra analysis.

4.4. Antioxidant activity assays

Comparative analysis of the antioxidant activity of VE compounds was performed according to the approach recently described by Muller et al.[10] Three tests based on single electron or H-atom transfer were used to assess antioxidant activity of VE and VE amine compounds, namely TEAC, DPPH· and FRAP. Assay details are described in Ref. 10. To adapt the FRAP assay to the investigation of lipophilic antioxidants, minor changes to the original method of Benzie and Strain[12] were introduced according to the protocol developed in Ref. 13.

The antioxidant capacity of tocopheramines was also assessed in comparison with **1a** using the copper-induced ascorbic acid depletion assay. Briefly, all exposures were performed in triplicate in UV 96 well flat-bottomed plates (Greiner bio-one) at a final volume of 200 μl. Exposures were initiated by the addition of 20 μl of a concentrated stock of ascorbate (2 mM, pH adjusted to 7 by NaOH) to obtain a starting ascorbate concentrations of 200 μM in each well that contained 160 μl of VE suspension and 20 μl of Chelex-100 resin treated water containing 2 μM Cu+ ions that were preliminarily verified to produce 1st order oxidation kinetics of ascorbate. Immediately prior to the addition of ascorbate to each assay well, the plate was pre-incubated for 10 min at 37 °C in a plate reader (Spectra Max 190), and during the exposure the plate was maintained at this temperature. After addition of ascorbate, the concentration remaining in each well was monitored every 2 min for a period of 2 h by measuring the absorbance at 265 nm. The initial rate of ascorbic acid depletion was measured in triplicate experiments and DTPA was used as metal chelator to verify the specificity of the analysis.

4.5. Stopped flow reaction of 1a, tocopheramines and tocotrienamines with diphenyl picryl hydrazyl radical (DPPH·)

Stock solutions of antioxidants (ca. 2 mM), DPPH· (200 μM) were prepared in ethanol (HPLC-grade). The concentrations of the individual antioxidant stock solutions were determined via their extinction coefficients reported in Ref. 7. This concentration divided by two provides the starting concentration of the antioxidant after mixing ($C_{Antioxidant}$).

For stopped flow experiments a stopped flow mixing accessory (RX 2000, Applied Photophysics) was used in conjunction with a diode array photometer (Multispec 1501, Shimadzu). The two syringes were loaded with 2 ml each of the DPPH· solution and the antioxidant solution. After initiation of the data acquisition at the photometer for the range of 350–600 nm in 0.1 s intervals the mixing was initiated by the pneumatic drive accessory. The time point $t = 0$ s was obtained from the spike of the appearance of the DPPH· absorption. From the 3D scans the time traces of 518 nm minus 600 nm were extracted for kinetic analysis of the DPPH· decay. The second order rate constant (k) was directly obtained by nonlinear regression according to the following formula:

$$OD = OD_{\text{total decay}} \times e^{-k \times c_{\text{Antioxidant}} \times (\text{time} - \text{time}_{\text{offset}})} + OD_{\text{offset}}$$

Data were collected in at least three different experimental sessions. To detect intermediates of antioxidants formed during the reaction with DPPH·, stock solutions of **1a** (10 mM), **4a** (10 mM), DPPH· (200 μM) in acetonitrile (HPLC-grade) were used in the described rapid mixing procedure.

4.6. HPLC analysis of reaction products of DPPH· with 1a and 4a

Aerobic solutions of DPPH· (0.5 mM) were mixed with **1a** (0.5 mM) or **4a** (0.5 mM) and incubated for 20 min at room temperature. The solvent of the mixture was evaporated in a stream of argon and the solid residue was dissolved in the HPLC eluent. HPLC analysis was performed on a Waters LC-1 Module. The column (Hibar, LiChrospher 100, RP-18 (5 μm), 250-4) was eluted with acetonitrile/H_2O (70/30 v/v) at a rate of 0.8 ml/min and 25 °C using inject volumes of 10 μl. The DPPH·-related compounds were detected by an UV-detector at 304 nm. Peaks of DPPH·, DPPH-H were identified by standard compounds. The DPPH· reaction product at a retention time of 4 min was collected from 10 HPLC runs and then subjected to LC/MS. LC/MS was done on a Dionex Ultimate 3000 system with a dual micro-flow pump, autosampler, temperature controlled column compartment and UV-DAD. The flow was split before the MS, so that only 200 μl/min entered the ESI source. MS was performed on an Agilent MSD 6320 XCT ion trap in negative mode ESI (capillary voltage: 3500 V, dry temperature: 300 °C, dry gas flow: 10 l/min, nebulizer pressure: 25 psi). The scan range was set to 300–500 *m*/*z* and five scans were averaged for one mass spectrum (Smart ICC target: 20,000, maximum accumulation time: 500 ms, smart parameter target mass: 400 *m*/*z*).

4.7. Detection of intermediates during the oxidation of 1d by ESR spectroscopy

The α-tocopheramine model compound 2,2,5,7,8-pentamethylchroman-6-amine (NH_2–PMC, PMC = pentamethylchromane) was prepared according to,[7] starting from 2,2,5,7,8-pentamethylchroman-6-ol instead of **1a**. To obtain the N-methylated derivatives, NH_2–PMC (223 mg, 1.02 mmol) was dissolved in DMSO (2.5 ml) under an argon atmosphere. NaOH (300 mg, 7.5 mmol) and CH_3I (216 mg, 1.53 mmol, 1.53 equiv) were added consecutively, and the mixture was stirred at room temperature. After 5 h the reaction was quenched with water, and the mixture was extracted three times with dichloromethane. The organic extract was washed four times with deionized water and once with brine. The organic extract was dried over $MgSO_4$, filtered and evaporated in vacuo. The residue (204 mg, yellow oil) was purified by column chromatography (5 g silica gel, *n*-hexane/ethyl acetate 5:1), providing *N*,*N*-dimethyl-(2,2,5,7,8-pentamethylchroman-6-yl)-amine **1e** (NMe_2–PMC) in 37% yield (94 mg) as a yellow oil, and *N*-methyl-(2,2,5,7,8-pentamethylchroman-6-yl)-amine **1d** in 36% yield (85 mg) as a colorless oil.

A solution of **1d** (50 mM) in CH_2Cl_2 was prepared and transferred to a quartz flat cell, which was immersed in liquid nitrogen. Then the flat cell was rapidly transferred to the standard resonator of the ESR instrument (Brucker EMX) and irradiated using a high pressure mercury lamp. The ESR spectra were recorded with the following parameters: microwave frequency 9.78 GHz, modulation frequency 100 kHz, modulation amplitude 2 G, time constant 41 ms, center field 3491 G, sweep 80 G, sweep time 10 s, receiver gain 9×10^4, scans 1. Simulation of the spectra was performed using the program WINSIM.[23]

4.7.1. Methyl-(*N*,2,2,5,7,8-pentamethylchroman-6-yl)-amine (1d, NHMe–PMC)

TLC: R_f = 0.19 (*n*-hexane/ethyl acetate 5:1 (v/v)); ^{1}H NMR ($CDCl_3$) δ: 1.30 (s, 6H, H-2a), 1.78 (t, $^3J_{HH}$ = 6.8 Hz, H-3), 2.08 (s, 3H, H-7a), 2.13 (s, 3H, H-8b), 2.17 (s, 3H, H-5a), 2.59 (t, 2H, $^3J_{HH}$ = 6.8 Hz, H-4), 2.80 (s, 6H, N–CH_3); ^{13}C NMR ($CDCl_3$) δ: 12.0 (C-8b), 14.2 (C-5a), 15.1 (C-7a), 21.2 (C-4), 27.0 (C-2a), 33.0 (C-3), 43.0 (*N*-CH_3), 72.6 (C-2), 100.0 (C-4a), 117.0 (C-8), 133.3 (C-7), 135.0 (C-5), 141.4 (C-6), 188.0 (C-8b); EI-MS *m*/*z* 233 (100%), 177 (95%), 178 (41%), 149 (36%), 134 (19%), 234 (16%), 148 (13%); Anal. Calcd for $C_{16}H_{25}ON$: C, 77.68; H, 10.19; N, 5.66. Found: C, 77.68; H, 10.32; N, 5.60.

4.7.2. Dimethyl-(*N*,*N*,2,2,5,7,8-pentamethylchroman-6-yl)-amine (1e, NMe_2–PMC)

TLC: R_f = 0.72 (*n*-hexane/ethyl acetate 5:1 (v/v)); ^{1}H NMR ($CDCl_3$) δ: 1.30 (s, 6H, H-2a), 1.80 (t, $^3J_{HH}$ = 6.8 Hz, H-3), 2.12 (s, 3H, H-8b), 2.19 (s, 3H, H-7a), 2.24 (s, 3H, H-5a), 2.63 (t, $^3J_{HH}$ = 6.8 Hz, H-4), 2.65 (s, 3H, *N*–CH_3), 2.81 (br, 1H, NH); ^{13}C NMR ($CDCl_3$) δ: 12.4 (C-8b), 13.6 (C-5a), 14.5 (C-7a), 21.7 (C-4), 27.2 (C-2a), 33.4 (C-3), 37.0 (*N*-CH_3), 72.9 (C-2), 117.5 (C-4a), 122.9 (C-8), 127.4 (C-5), 129.3 (C-7), 139.1 (C-6), 148.4 (C-8a); EI-MS *m*/*z* 247 (100%), 192 (43%), 148 (30%), 191 (27%), 176 (19%), 248 (17%), 190 (14%), 232 (13%), 177 (10%); Anal. Calcd for $C_{15}H_{23}ON$: C, 77.21; H, 9.93; N, 6.00. Found: C, 77.14; H, 10.00; N, 5.92.

4.8. Nitration of phenolic substrates

Nitration of phenolics (**2a**, **2d**, salicylic acid) upon oxidation of **4a** was preliminarily investigated using $FeCl_3$ as the oxidant (10:1 and 100:1 molar ratio with VE compounds) in 50:50 v/v H_2O/EtOH. Nitration studies in hexane were carried out with Ag_2O as the oxidant, using either salicylic acid, **2a** or its analog **2d** as the phenol derivatives and **4a** as the aniline derivatives. Tocopheramine and oxidants were simultaneously introduced in the reaction tube. In the case of **2a** and **2d**, a 4:1-ratio was also used, and the γ-chromanol substrates were introduced 5 min later than the oxidant to minimize their direct oxidation. The structure of the nitration products was confirmed by UV and NMR spectroscopy.

4.8.1. 5-Nitro-γ-tocopherol[24]

Red oil. TLC: R_f = 0.65 (*n*-hexane/diethyl ether, v/v = 9:1); UV (EtOH): λ_{max} (nm) = 265, 315, 418; ^{1}H NMR ($CDCl_3$) δ: 1.67 (t, 2H, ^3J = 6.9 Hz, H-3), 2.11 (s, 3H, H-7a/8b), 2.14 (s, 3H, H-7a/8b), 2.96 (t, 2H, ^3J = 6.8 Hz, H-4), 10.65 (s, 1H, –OH); ^{13}C NMR ($CDCl_3$) δ: 11.87 (C-7a), 13.05 (C-8b), 21.83 (C-4), 23.99 (C-2a), 31.14 (C-3), 75.02 (C-2), 113.12 (C-4a), 125.43 (C-7), 134.22 (C-5), 137.12 (C-8), 144.03 (C-8a), 148.00 (C-6), isoprenoid side chain: 19.76 (C-4a′), 19.84 (C-8a′), 21.09 (C-2′), 22.67 (C-13′), 22.74 (C-12a′), 24.50 (C-6′), 24.65 (C-10′), 28.00 (C-12′), 32.64 (C-8′), 32.75 (C-4′), 37.33 (C-7′), 37.46 (C-5′), 37.47 (C-9′), 37.52 (C-3′), 39.32 (C-11′), 39.69 (C-1′); Anal. Calcd for $C_{28}H_{47}O_4N$: C, 72.84; H, 10.26; N, 3.03. Found: C, 72.78; H, 10.42; N, 2.94.

4.8.2. 6-Hydroxy-5-nitro-2,2,7,8-tetramethylchroman

Reddish-colored crystals, mp: 79–81 °C; TLC: Rf = 0.50 (*n*-hexane/ethyl acetate, v/v = 30:1); UV (EtOH): λ_{max} (nm) = 265, 315, 418; IR (KBr): 2977, 2929, 1595, 1532, 1444, 1276, 1173; ^{1}H NMR ($CDCl_3$) δ: 1.24 (s, 6H, H-2a), 1.67 (t, 2H, ^3J = 6.8 Hz, H-3), 2.11 (s, 3H, H-7a/8b), 2.15 (s, 3H, H-7a/8b), 2.95 (t, 2H, ^3J = 6.8 Hz, H-4), 10.60 (s, 1H, –OH); ^{13}C NMR ($CDCl_3$) δ: 11.92 (C-7a), 13.18 (C-8b), 21.87 (C-4), 26.74 (C-2a), 32.31 (C-3), 73.22 (C-2), 113.04 (C-4a), 125.16 (C-7), 133.98 (C-5), 136.90 (C-8), 145.18 (C-8a), 148.22 (C-6); MS (ESI Q-TOF) *m*/*z*: calcd for

$C_{13}H_{17}O_4N$ $(M+H)^+$ 252.1230. Found: 252.1208. Anal. Calcd for $C_{13}H_{17}O_4N$: C, 62.14; H, 6.82; N, 5.57. Found: C, 62.40; H, 6.73; N, 5.43.

4.8.3. 5-Nitro-salicylic acid (2-hydroxy-5-nitrobenzoic acid)

Mp: 227–229 °C; TLC: $R_f = 0.44$ (toluene/ethyl acetate, v/v = 8:2); ^1H NMR (DMSO-d_6) δ: 7.14 (d, 1H, $^3J = 9.4$ Hz, H-3), 8.32 (dd, 1H, $^3J = 9.4$ Hz, $^4J = 1.8$ Hz, H-4), 8.55 (d, 1H, $^4J = 1.8$ Hz, H-6); ^{13}C NMR (DMSO-d_6) δ: 117.3 (C-3), 119.4 (C-1), 123.0 (C-6), 124.9 (C-4), 129.6 (C-5), 162.7 (C-2), 165.2 (COOH). Anal. Calcd for $C_7H_5O_5N$: C, 45.91; H, 2.75; N, 7.65. Found: C, 45.86; H, 2.91; N, 7.66.

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