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# Does conjugation of antioxidants improve their antioxidative/anti-inflammatory potential?

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ABSTRACT

A series of symmetric and asymmetric spermine (SPM) conjugates with *all-trans*-retinoic acid (ATRA), acitretin (ACI), (*E*)-3-(trioxsalen-4′-yl)acrylic acid (TRAA) and L-DOPA, amides of ACI, L-DOPA and TRAA with 1-aminobutane, benzylamine, dopamine and 1,12-diaminobutane as well as hybrid conjugates of *O*,*O*′-dimethylcaffeic acid (DMCA) with TRAA or *N*-fumaroyl-indole-3-carboxanilide (FICA) and 2-(2-aminoethoxy)ethanol were synthesized and their antioxidant properties were studied. The reducing activity (RA)% of the compounds were evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging assay and found to be in the range 0–92(20 min)%/96(60 min)% at 100 μM, the most powerful being the conjugates L-DOPA-SPM-L-DOPA (**8**, RA = 89%/96%) and L-DOPA-dopamine (**13**, RA = 92%/92%). Conjugate DMCA-NH$(CH_2CH_2O)_2$-FICA (**14**) was the most powerful LOX inhibitor with $IC_{50}$ 33.5 μM, followed by the conjugates ACI-NHCH$_2$Ph (**10**, $IC_{50}$ 40.5 μM), ACI-SPM-TRAA (**7**, $IC_{50}$ 41.5 μM), DMCA-NH$(CH_2CH_2O)_2$-TRAA (**15**, $IC_{50}$ 65 μM), **13** ($IC_{50}$ 81.5 μM) and ACI-dopamine (**11**, $IC_{50}$ 87 μM). The most potent inhibitors of lipid peroxidation at 100 μM were the conjugates **15** (98%) and ACI-SPM-ACI (**4**, 97%) whereas all other compounds showed activities comparable or lower than trolox. The most interesting compounds, namely ATRA-SPM-ATRA (**3**), **4**, **10**, **11** and **15**, as well as unconjugated compounds such as ATRA and dopamine, were studied for their anti-inflammatory activity in vivo on rat paw oedema induced by Carrageenan and found to exhibit, for doses of 0.01 mmol/mL of conjugates per Kg of rat body weight, weaker anti-inflammatory activities (3.6–40%) than indomethacin (47%) with conjugate **3** being the most potent (40%) in this series of compounds. The cytocompatibility of selected compounds was evaluated by the viability of RAMEC cells in the presence of different concentrations (0.5–50 μM) of the compounds. Conjugates **3** ($IC_{50}$ 2.6 μM) and **4** ($IC_{50}$ 4.7 μM) were more cytotoxic than the corresponding unconjugated retinoids ATRA ($IC_{50}$ 18.3 μM) and ACI ($IC_{50}$ 14.6 μM), whereas conjugate **15** ($IC_{50}$ 12.9 μM) was less cytotoxic than either DCSP ($IC_{50}$ 11.3 μM) or the *tert*-butyl ester of TRAA ($IC_{50}$ 2.9 μM).



## 1. Introduction

Hydroxycinnamic acid compounds are widely distributed in the plant kingdom as esters of organic acid or glycosides and they are bound to protein and other cell wall polymers. They present potential antioxidative properties involving free radical-scavenging activity, metal ion chelation, and inhibitory actions on specific enzymes that induce free radical and lipid hydroperoxide formation.[1,2] Therefore, their antioxidant actions could prevent oxidant rancidity in foods and oxidant damages in vivo, relating to diseases such as cancer, diabetes, and cardiovascular, Alzheimer's, and Parkinson's diseases. Persistently high levels of ROS can modify essentially biological molecules, such lipids, proteins and DNA. It is consistent that rates of ROS production are increased in most diseases. Oxidative stress has been associated with several human diseases such as cancer, neurodegenerative syndromes and inflammation.

Spermine (SPM) is a natural antioxidant, which is found in all living organisms. Research shows that SPM has an important function in those areas of the body that are exposed to a high level of oxygen usage. This pertains to the skin, the brain, sperm cells and the lungs in particular. Spermine's role is that of protecting cells against free radicals and their destructive effects.[3]

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Today, there is an increase interest in the combination of two pharmacophores on the same scaffold. This procedure leads to hybrid molecules or conjugates. Hybrid drugs, just as their name implies, combine two drugs in a single molecule with the goal of creating a chemical entity more medically effective than its individual components.[4] These combo drugs can indeed be more powerful than either of their precursors.

Kukoamines A (KukA) (Fig. 1) and B (KukB) are SPM alkaloids, actually SPM conjugates with dihydrocaffeic acid (DHCA), which were isolated from the medicinal plant *Lycium chinense*.[5] More recently, alkaloids of the kukoamine type, for example, KukA, were identified in a range of solanaceous species, including potato.[6] KukA shows hypotensive activity,[5a] and is a potent and selective inhibitor of trypanothione reductase (TryR),[7] a crucial enzyme for the survival of pathogenic trypanosomatid parasites from oxidative stress.

We have recently examined the antioxidative properties of a series of KukA analogs and found that KukA was the most potent inhibitor of soybean lipoxygenase (LOX) whereas its analog di(*O*,*O*′-dimethylcaffeoyl)spermine (DCSP, Fig. 1), a conjugate of SPM with *O*,*O*′-dimethylcaffeic acid (**1**), inhibited 100% the lipid peroxidation at 100 μM using the water-soluble azo compound 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) assay. Both compounds had almost identical anti-inflammatory activity, which was comparable to that of the usual standard indomethacin.[8] In addition, other types of compounds were examined for their antioxidative properties, namely analogs of the antipsoriatic drugs trioxsalen (TRX) and acitretin (ACI) (Fig. 1). In the former family of compounds, the most potent LOX inhibitor was the analog *tert*-butyl 3-(trioxsalen-4′-yl) acrylate (TRAB) which also showed high lipid peroxidation inhibition (80% at 100 μM using the AAPH assay) and almost identical anti-inflammatory activity to indomethacin.[9] In the latter family of compounds, although ACI showed a very low LOX activity, it inhibited at 85% (at 100 μM) lipid peroxidation induced by AAPH and was the most powerful anti-inflammatory agent in the series with activity higher (ca. 1.3 times) than indomethacin. Similar inhibition of lipid peroxidation to acitretin was presented by the analog *N*-fumaroylindole-3-carboxanilide (FICA) (Fig. 1), which however had a much more powerful effect in inhibiting LOX.[10]

There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious effects of free radicals in the human body, and to prevent the deterioration of fats and other constituents of foodstuffs. Taking into consideration that conjugation of caffeic acid analogs[8] or acidic retinoids like *all-trans*-retinoic acid (ATRA) and ACI[11] with SPM results in molecular entities with altered or superior biological properties compared to the combination of the unconjugated molecules, we decided to examine the effect of conjugation of various bioactive molecules on their antioxidative activity. It should be noted that attachment of lipophilic moieties on the amino function(s) of PAs usually leads

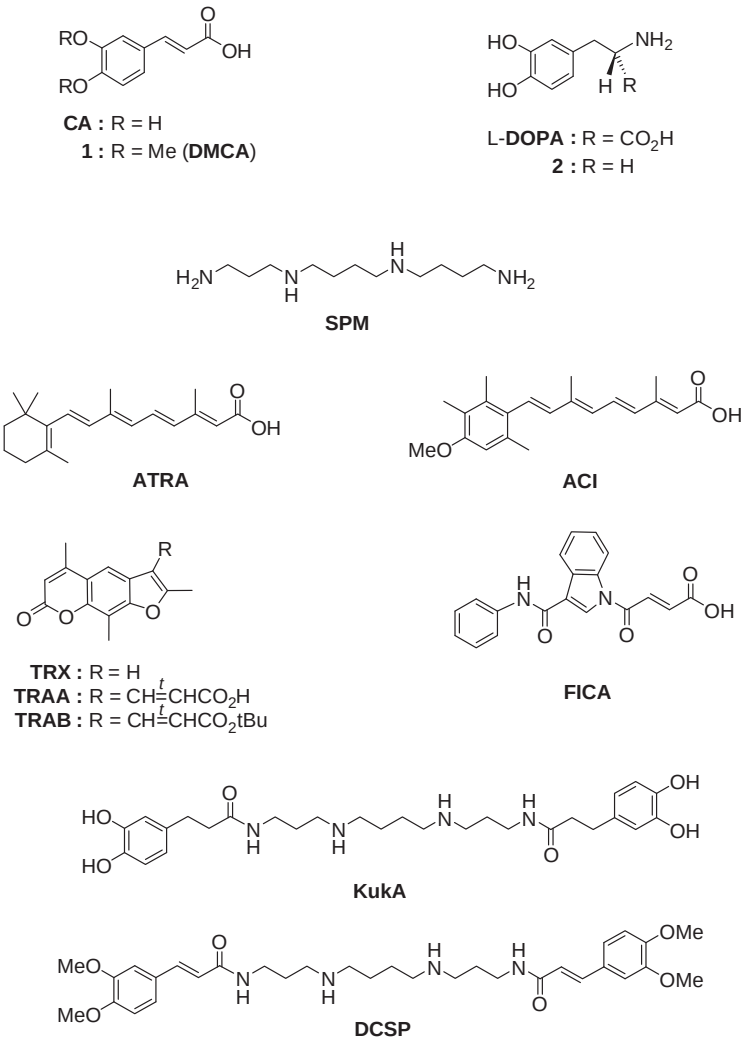



**Figure 1.** Structures of compounds encountered in the present work.

to increased toxicity (SPM to a greater extent than SPD).[12] However, we have chosen to use SPM over spermidine (SPD) as the PA because the SPD conjugates we have tested so far were invariably less active than the corresponding SPM conjugates[8,11] and on the other hand PAs present antioxidative activity which is correlated to the number of the amino groups in the molecule (SPM more efficient than SPD).[13] For this purpose, we have synthesized symmetric conjugates of SPM with (a) the acidic retinoids ATRA and ACI (conjugates **3** and **4**, respectively),[11] (b) the trioxsalen analog 3-(trioxsalen-4′-yl)acrylic acid (TRAA) (conjugate **5**)[14] and (c) the known powerful antioxidant L-DOPA (conjugate **8**), as well as hybrid conjugates of TRAA with the afore mentioned acidic retinoids (conjugates **6** and **7**, respectively) (Fig. 2). An antioxidant effect of dopamine has been reported[15,16] in the literature on lipid peroxidation (LPO) in rat brain homogenates. L-DOPA and many other catechol compounds, including norepinephrine, alpha-methyldopamine, and catechol itself, but not monophenols, exhibit a similar property. L-DOPA was found to be potent in free radical-scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. Hydroxyl radicals (OH) and superoxide anion radicals were effectively scavenged by L-DOPA.[17]

In addition, in an effort to examine the significance of the secondary free amino groups in the conjugates, we thought of including in our study the conjugate **9** (Fig. 3), considered as an analog of SPM conjugate **3**. Furthermore, we included in the present study simpler conjugates/amides of ACI, such as compounds **10** and **11**, and of TRAA (compound **12**), the conjugate **13** of L-DOPA and dopamine (**2**) and finally the hybrid conjugates **14** and **15** (Fig. 3) of **1** (a constituent of SPM conjugate DCSP) and the ACI analog FICA or the TRX derived acid TRAA. We considered of interest to examine the possible lipoxygenase (LOX) activity of these compounds, presented in Figures 2 and 3, their potential to inhibit lipid peroxidation of biological membranes and to act as anti-inflammatory agents as well as evaluate their cytocompatibility. Through these compounds, we could hopefully determine (a) the effect of conjugation of polyamines or simpler amines, such as the benzyl and butyl amine, dopamine, 1,12-diaminododecane, 2-(2-aminoethoxy) ethanol, with biologically active molecules, such as retinoids and analogs, psoralen analogs, caffeic acid analogs and L-DOPA, on the biological activity and (b) the particular structural characteristics which might possibly lead to increased antioxidative and/or anti-inflammatory activities and reduced cytotoxicity.

## 2. Results and discussion

### 2.1. Chemistry

The conjugates **3** and **4**[11] and **5** and **7**[14] were synthesized according to published procedures. Conjugate **6** was prepared in analogous manner to conjugate **7**, that is by first coupling compound **16** (TRAA)[14] with $N^1$,$N^4$,$N^9$-tris(*tert*-butoxycarbonyl)spermine ($Boc_3$-SPM),[18] in the presence of *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluoro-phosphate (HBTU), and then removal of the Boc protecting groups by treatment with trifluoroacetic acid (TFA) to obtain the monoacylated SPM derivative **17**, as the corresponding tris-trifluoroacetate salt. This was then selectively acylated by the succinimidyl 'active' ester **18**[11] (Scheme 1) to give conjugate **6** in 45% overall yield.

The synthesis of conjugate **8** started with the preparation of *N*-Trt-L-DOPA (**19**), following a literature procedure for the synthe-

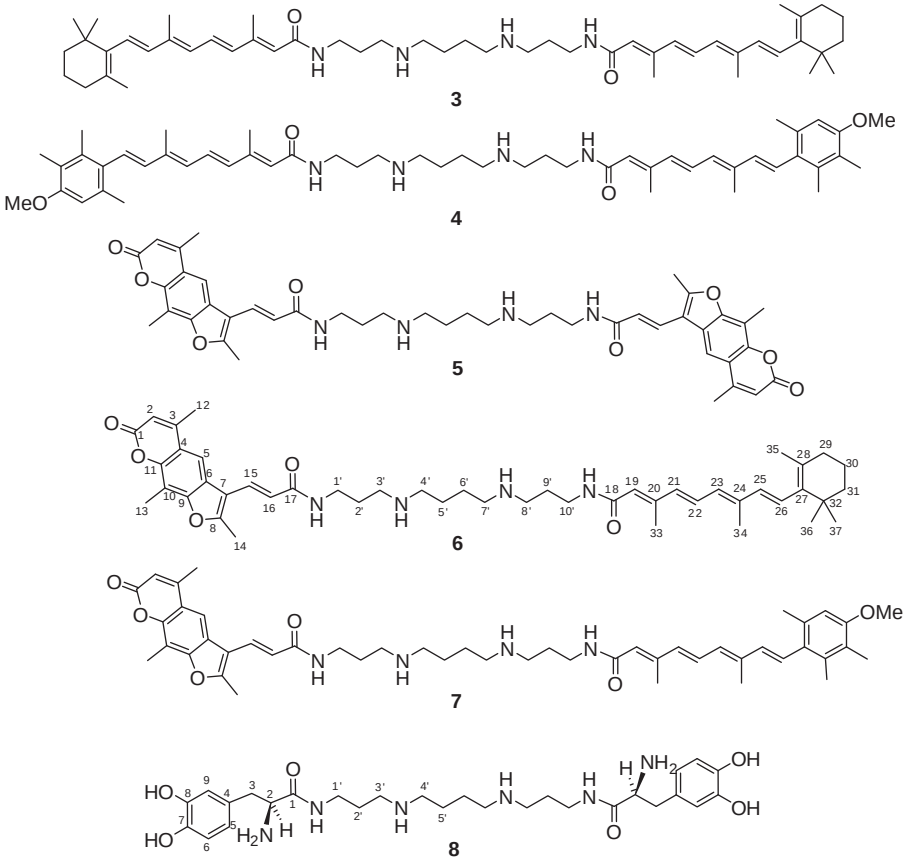



**Figure 2.** Structures of spermine conjugates synthesized in the context of the present work.

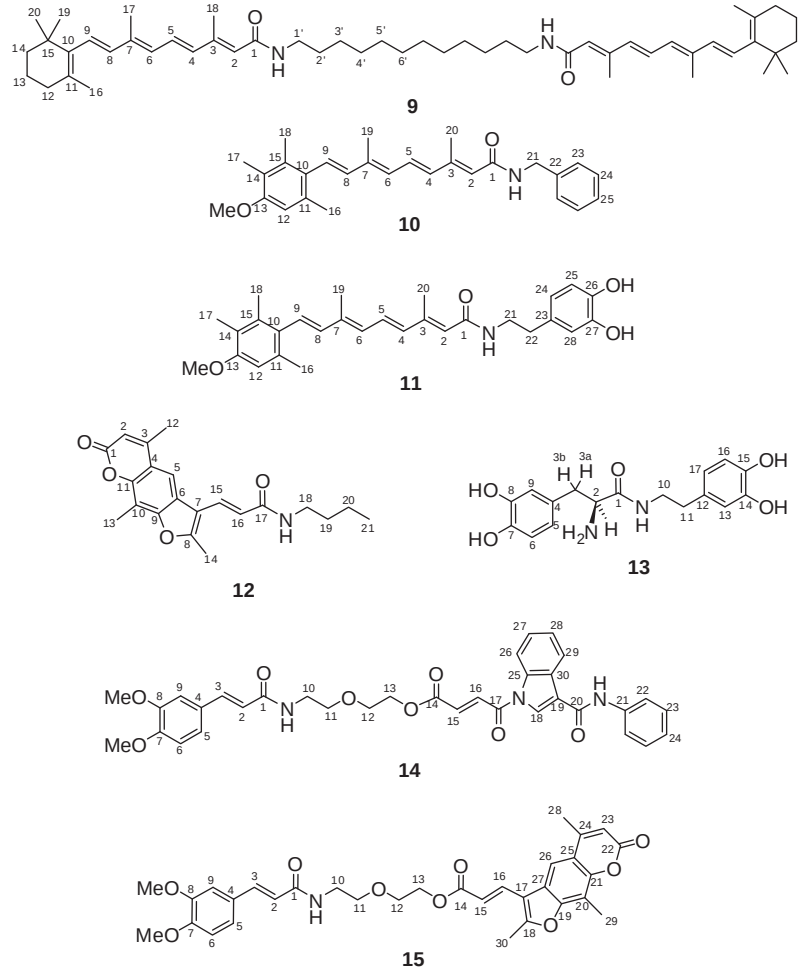



**Figure 3.** Structures of other types of conjugates synthesized in the context of the present work.

sis of L-amino acids N-protected with the triphenylmethyl (trityl, Trt) group.[19] It involved treatment of L-DOPA with $Me_3SiCl$, followed by N-tritylation and carboxyl group deprotection with methanolysis. The product **19** was obtained pure as the corresponding diethylammonium salt in 81% yield. Compound **19** was then converted to the corresponding isolable benzotriazolyl (Bt) ester (**20**), according to a literature procedure for the activation of *N*-tritylamino acids,[20] in 85% yield. This ester was then coupled to $N^4$,$N^9$-ditritylspermine ($N^4$,$N^9$-$Trt_2$-SPM),[21] N-deprotected with TFA and finally treated with 1.4 M HCl/EtOH to give conjugate **8**, as the corresponding tetrakis-hydrochloride salt in 39% overall yield.

Conjugate **9** was readily obtained in 90% yield through coupling the active ester **18** with 1,12-diaminododecane, followed by routine flash column chromatography (FCC) purification. Conjugates **10** and **11** were obtained in 68% and 85% yield, respectively, by coupling active ester **21**[11] with either benzyl amine or dopamine, whereas conjugate **12** was obtained in 78% yield through coupling acid **16** with 1-butylamine. Conjugate **13** was obtained in 85% yield by coupling active ester **20** with dopamine followed by FCC purification. Finally, the conjugates **14** and **15** were obtained by first coupling the active ester **22**[22] with 2-(2-aminoethoxy)ethanol to give amide **23** in 88% yield. This compound was then condensed with either FICA[10] or TRAA[14] under Mitsunobu reaction conditions to afford conjugates **14** and **15** in 64% and 68% yields, respectively, following FCC purification.

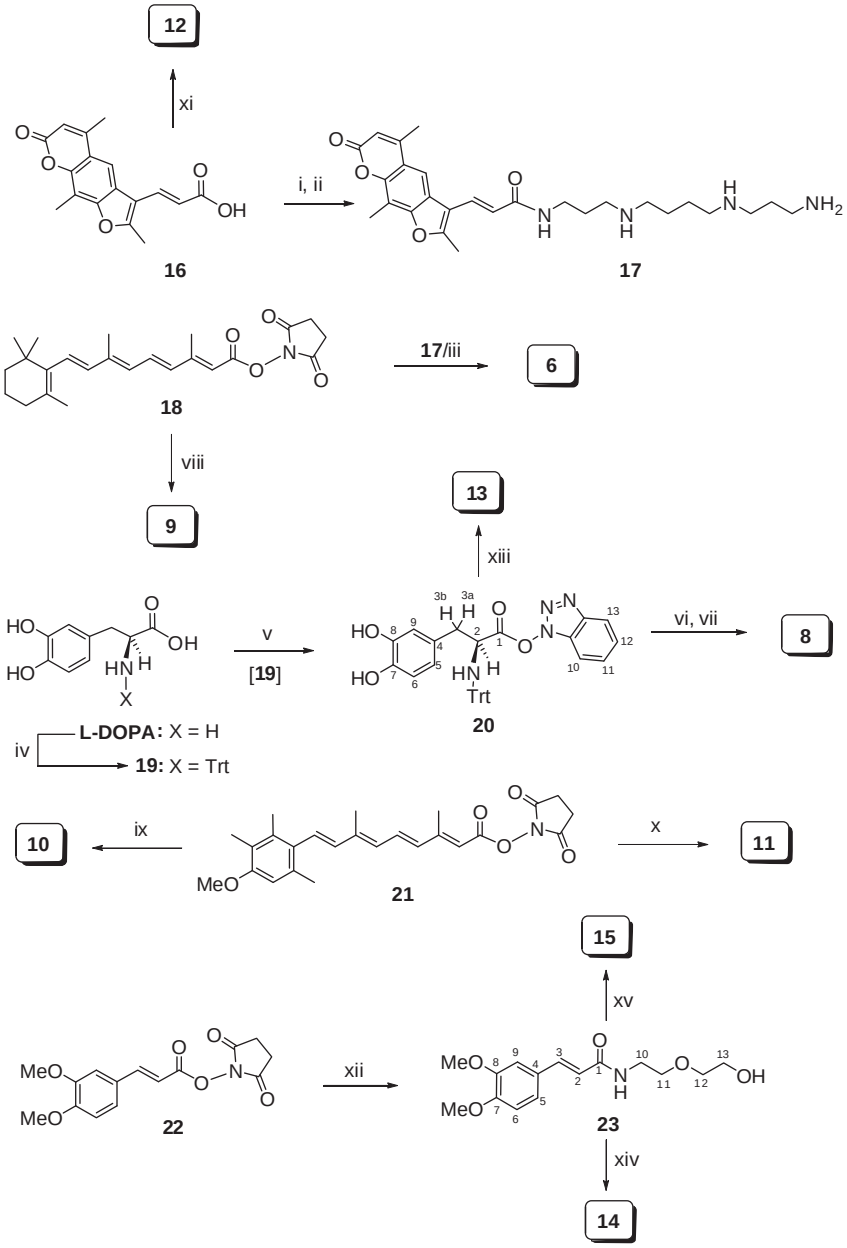
## 2.2. Antioxidant and anti-inflammatory activity

### 2.2.1. In vitro antioxidant activity studies

In the present investigation, the conjugates **3–15** as well as the parent molecules comprising them were studied with regard to their antioxidant ability as well as to their ability to inhibit soybean LOX. Taking into account the multifactorial character of oxidative stress and inflammation, we decided to evaluate the in vitro antioxidant activity of the synthesized molecules using two different antioxidant assays: (a) interaction with the stable free radical 1,l-diphenyl-2-picrylhydrazyl radical (DPPH), (b) interaction with the water-soluble azo compound AAPH.

Both require a spectrophotometric measurement and a certain reaction time in order to obtain reproducible results.[23] DPPH has been used in a radical-scavenging measuring method. DPPH is a stable free radical in an ethanolic solution. In its oxidized form, the DPPH radical has an absorbance maximum centred at about 517 nm.[24] The DPPH method is described as a simple, rapid and convenient method independent of sample polarity for screening many samples for radical-scavenging activity.[25] These advantages made the DPPH method interesting for testing our compounds.

The use of the free radical reactions initiator AAPH is recommended as more appropriate for measuring radical-scavenging activity in vitro, because the activity of the peroxyl radicals produced by the action of AAPH shows a greater similarity to cellular activities such as lipid peroxidation.[26]




**Scheme 1.** Synthesis of conjugates **6**, **8** and **9**–**15**. Reagents and conditions: (i) $Boc_3$-SPM, TEA, HBTU, DMF, 25 °C, 45 min, 61%; (ii) TFA/$CH_2Cl_2$ (1:1), 25 °C, 2 h; (iii) DIEA, $CHCl_3$/DMF (1:1), 25 °C, 5 h, 61%; (iv) (a) $Me_3SiCl$, TEA, $CHCl_3$, 61 °C, 1 h; (b) *i*-PrOH/$CH_2Cl_2$ (1:3), TEA, 0 °C, 15 min then Trt-Cl, 25 °C, 1 h; (c) MeOH, 25 °C, 30 min, 81%; (v) HOBt, DCC, THF, 0–25 °C, 2 h, 85%; (vi) $N^4$,$N^9$-$Trt_2$-SPM, DIEA, $CH_2Cl_2$, 25 °C, 2 h, 47%; (vii) TFA/$CH_2Cl_2$ (2:8), 0 °C, 2 h then 1.4 M HCl/EtOH, 83%; (viii) 1,12-diaminododecane, TEA, $CHCl_3$, 40 °C, 2 h, 90%; (ix) benzylamine, DIEA, $CH_2Cl_2$, 25 °C, 15 min, 68%; (x) dopamine·HCl, DIEA, $CHCl_3$/DMF (5:1), 0–25 °C, 2 h, 85%; (xi) 1-butylamine, DIEA, HBTU, DMF, 25 °C, 15 min, 78%; (xii) 2-(2-aminoethoxy)ethanol, TEA, DMF, 0–25 °C, 45 min, 88%; (xiii) (a) dopamine·HCl, DIEA, $CHCl_3$/DMF (6:1), 0–25 °C, 30 min, 53%; (b) TFA/$CH_2Cl_2$ (3:7), 25 °C, 30 min, 85%; (xiv) FICA, TPP, DIAD, THF, 0–25 °C, 40 min, 64%; (xv) TRAA, TPP, DIAD, THF, 0 to 25 °C, 45 min, 68%.

Antioxidants are defined as substances that even at low concentration significantly delay or prevent oxidation of easy oxidizable substrates. Antioxidants with high antioxidant potential activity will therefore be beneficial for clinical applications. It is well-known that free radicals play an important role in the inflammatory process.[27] Many non-steroidal anti-inflammatory drugs have been reported to act either as inhibitors of free radical production or as radical scavengers.[28] Consequently, compounds with antioxidant properties could be expected to offer protection in rheumatoid arthritis and inflammation and to lead to potentially effective drugs. Thus, we tested the present conjugates with regard to their antioxidant ability and in comparison to well-known antioxidant agents, for example, CA, ascorbic acid, NDGA and trolox.

The interaction/reducing activity (RA) of the examined compounds with the stable, long-lived, free radical DPPH is shown in Table 1. This interaction indicates their radical-scavenging ability in an iron-free system. In the DPPH assay, the dominant chemical reaction involved is the reduction of the DPPH radical by a single electron transfer (ET) from the antioxidant.[29] Particularly effective such antioxidants are the phenoxide anions from phenolic compounds like catechol and derivatives. The highest interactions in the present set of compounds were observed for compounds **8** and **13** (values higher than those for NDGA and comparable to ascorbic acid) and compound **11** (value comparable to NDGA but lower than ascorbic acid). These compounds are characterized by the presence of either L-DOPA (conjugate **8**) or dopamine

**Table 1**
Interaction-reducing activity % with DPPH (RA %); in vitro inhibition of soybean lipoxygenase (LOX) ($IC_{50}$); % inhibition of lipid peroxidation (AAPH %); inhibition % of Carrageenan-induced rat paw oedema (ICPE %)

| Compd[a] | *C* log *P*[35] | RA % 0.1 mM 20/60 min (±SD)[b] | $IC_{50}$ μM or % LOX Inh. @ 0.1 mM/0.05 mM (±SD)[b] | AAPH % @ 0.1 mM (±SD)[b] | ICPE % 0.01 mmol/Kg (±SD)[d] |
|---|---|---|---|---|---|
| CA | 0.82 | 5.5/6 ± 0.3 | 600 μM ± 15 | 17.5 ± 0.8 | |
| DHCA[8,c] | 0.45 | 100/100 ± 3.8 | 16/na ± 0.2 | 47 ± 1.3 | |
| DMCA | 1.90 | 9/10 ± 0.2 | 23/na ± 0.4 | 56 ± 1.6 | |
| SPM[8] | −2.33 | 4/4 ± 0.5 | na/na | na | |
| KukA[8] | −1.79 | 96/100 ± 2.9 | 9.5 μM ± 1.1 | 72 ± 2.3 | 43* ± 0.6 |
| DCSP[8] | 1.42 | 52/58 ± 3.2 | 62 μM ± 3.2 | 100 ± 6.9 | 42** ± 2.6 |
| TRAA[9] | 3.42 | 11/11 ± 0.9 | 9/na ± 0.8 | 73 ± 5.3 | |
| TRAB[9] | 5.03 | 11/11 ± 0.7 | 9.4 μM ± 3.1 | 80 ± 6.2 | 46** ± 1.8 |
| FICA[10] | 2.66 | 1/na ± 0.05 | 52.4 μM ± 2.6 | 85 ± 5.8 | 11* ± 0.7 |
| L-DOPA | −2.82 | 76#/76 ± 4.3 | 23/na ± 1.3 | 65 ± 1.2 | |
| 2 (Dopamine) | 0.17 | 86#/86 ± 5.7 | 100μM ± 4.5 | 43 ± 2.7 | 13* ± 0.8 |
| ATRA | 6.74 | 11/23 ± 0.8 | na/na | 75 ± 3.5 | 11* ± 0.8 |
| ACI[10] | 6.07 | na/na | 1/na ± $10^{-3}$ | 85 ± 1.6 | 63** ± 3.4 |
| **3** | ¥ | na/5 ± 0.3 | 76/na ± 3.8 | 58 ± 4.5 | 40** ± 2.8 |
| **4** | ¥ | 26/38 ± 1.4 | 75/na ± 6.7 | 97 ± 3.8 | 19* ± 1.2 |
| **5** | ¥ | 41/54 ± 2.8 | 57/na ± 2.2 | 33 ± 2.2 | |
| **6** | ¥ | 11/38 ± 0.4 | 43/na ± 3.1 | 45 ± 2.8 | |
| **7** | ¥ | na/na | 41.5μM ± 2.9 | 31 ± 1.9 | |
| **8** | ¥ | 89/96 ± 6.7 | 32/na ± 1.2 | 71 ± 4.1 | |
| **9** | ¥ | na/13.4 ± 0.8 | 23/na ± 0.8 | 41 ± 2.3 | |
| **10** | ¥ | na/2 ± 0.008 | 40.5μM ± 3.5 | 45 ± 1.1 | 3.6* ± 0.8 |
| **11** | ¥ | 79/79 ± 1.4 | 87 μM ± 6.2 | 10 ± 0.7 | 13.7* ± 1.0 |
| **12** | 4.72 | na/8.4 ± 0.7 | 24/na ± 1.4 | 66 ± 1.6 | |
| **13** | −0.31 | 92/92 ± 4.6 | 81.5 μM ± 5.2 | na | |
| **14** | ¥ | na/12 ± 1.0 | 33.5μM ± 2.4 | na | |
| **15** | ¥ | na/15 ± 0.8 | 65 μM ± 2.7 | 98 ± 4.6 | 9* ± 0.3 |
| NDGA | | 81/83 ± 2.4 | 28 μM ± 1.3 | | |
| Trolox | | | | 63 ± 1.2 | |
| Ascorbic acid | | 99/100 ± 1.8 | | | |
| Indomethacin | | | | | 47** ± 3.1 |

Each value represents the mean obtained from 6 animals in two independent experiments ($n = 6 \times 2$). In all cases, significant difference from control: $^{*}p < 0.1$, $^{**}p < 0.01$ (Student's *t*-test); ¥, calculated values are too high to be realistic; na, no activity under the reported experimental conditions; #, immediately, both compounds presented the same high antioxidant activity at 0.05 mM.

[a] Full structures of all compounds in this column are presented in Supplementary data section (Table 1).

[b] Values are means ± SD of three or four different determinations, significant differences from control values $^{*}p < 0.05$.

[c] DHCA stands for dihydrocaffeic acid.

[d] The effect on oedema is expressed as percent of weight increase of hind paw (and as percent of inhibition of oedema) in comparison to controls.

(conjugate **11**) or both substructures (conjugate **13**), for which high interaction values were already observed when used alone (76%/76% for L-DOPA and 86%/86% for dopamine). The interaction, for these compounds remains, with the exception of **8**, highly constant after 60 min. It is worth noting that, obviously, the activity of conjugate **11** comes from its dopamine substructure alone as ACI itself presented zero interaction. Packer[30] also earlier did not find any interaction with DPPH for ACI. The high interaction of compounds **8**, **11** and **13** with the DPPH radical should be attributed to the presence of the easily oxidizable catechol subunits present in the molecule, whereas the higher values for compounds **8** and **13** compared to **11** might be further attributed to the presence of the free amino function(s). DHCA as well as Kuka, incorporating the catechol unit, also presented very high interaction values, whereas for DMCA as well as DCSP, in which the hydroxyl groups of the catechol unit have been converted into methyl ether groups, the reducing ability has been greatly decreased. Interestingly, DMCA interacts more effectively with DPPH than CA, in which the catechol subunit is retained. It therefore seems that the presence of a double bond in position p to one of the hydroxyl groups has an unfavourable effect on the interaction with DPPH and that in such cases better interaction is secured when the hydroxyl groups are masked as methyl ethers.

Compounds **3**–**7**, **9**, **10**, **12**, **14** and **15** presented low, if any, interaction to DPPH at 100 μM after 20 min whereas a small increase in the interaction is generally observed in most cases after 60 min. This can be attributed to the absence of any easily oxidizable functionalities like the ones (two catechol subunits) present in NDGA which is used as a reference compound. Regarding the effect of conjugation on the interaction values of the conjugates compared to the unconjugated molecules, it is apparent that conjugation of ATRA with SPM or 1,12-diaminododecane (conjugates **3** and **9**) leads to lower interaction values than the unconjugated retinoid, an effect which is however reversed with the more electron-rich and thus more easily oxidizable aromatic molecules ACI (conjugate **4**), TRAA (conjugate **5**) and L-DOPA (conjugate **8**). Interestingly, conjugate **9**, lacking the secondary amino functions in its chain, interacts with DPPH slightly better than conjugate **3**. Furthermore concerning the hybrid conjugates **6** and **7**, although conjugation of ATRA and TRAA through SPM (conjugate **6**) caused essentially no improvement in the antioxidative properties of the unconjugated molecules, the conjugation of ACI to TRAA (conjugate **7**) resulted in complete loss of activity.

In our studies, AAPH was used as a free radical initiator to follow oxidative changes of linoleic acid to conjugated diene hydroperoxide. Azo compounds generating free radicals through spontaneous thermal decomposition are useful for free radical production studies in vitro.[31] The water-soluble azo compound AAPH has been extensively used as a clean and controllable source of thermally produced alkyl peroxyl free radicals. In the AAPH assay, the highly reactive alkylperoxyl radicals are intercepted mainly by a hydrogen atom transfer (HAT) from the antioxidant.[29] Therefore, particularly effective HAT agents are compounds with high hydrogen atom donating ability, that is compounds with low heteroatom-H bond dissociation energies and/or compounds from which hydrogen abstraction leads

to sterically hindered radicals as well as compounds from which abstraction of hydrogen leads to C-centred radicals stabilized by resonance. With the exception of compounds **13** and **14** which showed no inhibition, all other compounds caused inhibition of lipid peroxidation (LPO). In particular, compounds **3**, **5**–**7** and **9**–**11** presented inhibition values (10–58%) lower than the common standard trolox (63%). On the other hand, compounds **4**, **8**, **12** and **15** presented values (66–98%) higher than trolox, with most potent inhibitors being the compounds **4** (97%) and **15** (98%). Although the conjugates **8** (71%) and KukA (72%) might exert their antioxidative effect through their easily oxidizable catechol units, the antioxidative effect of the other three compounds should be attributed to other factors. For example, conjugate **4** like ACI may exert its inhibitory effect either by hydrogen atom abstraction from the Me groups of the polyene chain and stabilization of the resulting new C-centred radical through resonance or through interception of the alkylperoxyl radicals by their conjugated tetraene chain. Similar considerations might be applicable to compound **12** and in particular compound **15**. It is interesting to note that the compounds incorporating free phenolic functions are not the most powerful antioxidants in the AAPH assay with their activity ranging from 0 (conjugate **13**) to 72% (KukA). On the contrary, extremely high inhibitory values were obtained for compounds bearing masked phenolic functions as methyl ethers, such as conjugates DCSP (100%), **4** (97%) and **15** (98%).

Regarding the effect of conjugation on the ability of the conjugates to inhibit LPO, the two most powerful and almost equipotent inhibitors of LPO (compounds **4** and **15**) include the structural characteristics of the compounds ACI the former and of DCSP and TRAB the latter, which all have high inhibition values (80–100%). Although, DMCA is not particularly effective (56%), its conjugate DCSP with SPM exhibit the highest anti-LPO activity in the present set of compounds. Also, the conjugate of DHCA with SPM, namely KukA, shows much lower inhibitory activity than DCSP although the unconjugated molecule DHCA shows inhibitory activity (47%) similar to that for DMCA. Since CA itself shows a particularly low inhibitory activity (17.5), it seems that unsaturation of the side-chain and replacement of the hydroxyl groups by methyl ether groups, in the above mentioned cases, improves significantly the inhibitory activity towards LPO. This is indeed apparent when the inhibitory activities of CA and DMCA on one hand and of KukA and DMCA on the other hand are compared. Striking is however the difference when structural characteristics of the molecules DSCP and FICA are combined (conjugate **14**), which both also have very high inhibitory values (100% and 85%, respectively). In that case, complete loss of the inhibitory activity is observed. The same applies to conjugate **13**, where conjugation of L-DOPA (65% inhibition value) and dopamine (43% inhibition value) also resulted to complete loss of the inhibitory ability towards LPO.

Lower inhibition values are also obtained when conjugating TRAA with SPM either alone (compound **5**) or with ATRA (compound **6**) or ACI (compound **7**). Also, conjugation of ATRA with SPM (compound **3**) leads to significantly lower inhibition value, which becomes even lower when SPM is replaced by 1,12-diaminododecane (compound **9**). On the contrary, conjugation of ACI (with higher inhibition value than ATRA) with SPM provides a stronger inhibitor whereas the benzylamide (compound **10**) and the dopaminamide (compound **11**) of ACI resulted in very low inhibition values compared to ACI. In addition, the inhibitory activity of dopamine is totally abolished when conjugated to L-DOPA. However, when L-DOPA is conjugated to SPM the resulting conjugate **8** is slightly more inhibitory than L-DOPA itself. Finally, amidation of TRAA (compound **12**) results in lower inhibitory activity than either the free acid (TRAA) or the corresponding *tert*-butyl ester (TRAB).

Eicosanoids are oxygenated metabolites of arachidonic acid with a broad implication in a diversity of diseases. Upon appropriate stimulation of neutrophils, arachidonic acid (AA) is cleaved from membrane phospholipids and can be converted into leukotrienes (LTs) through 5-lipoxygenase (5-LOX). Leukotriene B4 (LTB4) is a potent mediator of inflammation, amplifying recruitment and activation of inflammatory cells. LTB4 generation is considered to be important in the pathogenesis of neutrophil-mediated inflammatory diseases[32] with a marked relation to the severity of cardiovascular diseases and cancer. Inhibitors of LOX have attracted attention initially as potential agents for the treatment of inflammatory and allergic diseases, certain types of cancer and cardiovascular diseases.[33,34] Goldreich et al.[35] examined the effect of retinol, *all-trans*-retinoic acid and 13-*cis*-retinoic acid on the activity of lipoxygenase-1 and lipoxygenase-2 towards linoleic acid. *All-trans*-retinoic acid and 13-*cis*-retinoic acid inhibited lipoxygenase-1 activity competitively, whereas retinol inhibited lipoxygenase-1 activity in a mixed manner. These findings suggest that retinoids may bind to the active site of the enzyme or simultaneously act as antioxidants.

In this context, we decided to further evaluate the synthesized conjugates for their ability to inhibit soybean LOX by the UV absorbance based enzyme assay.[8] Most of the LOX inhibitors are antioxidants or free radical scavengers.[36] LOXs contain a 'non-haem' iron per molecule in the enzyme active site as high-spin $Fe^{2+}$ in the native state and the high spin $Fe^{3+}$ in the activated state. Some studies suggest a relationship between LOX inhibition and the ability of the inhibitors to reduce $Fe^{3+}$ at the active site to the catalytically inactive $Fe^{2+}$. This inhibition is related to their ability to reduce the iron species in the active site to the catalytically inactive ferrous form,[34] whereas several LOX inhibitors are excellent ligands for $Fe^{3+}$. For the sake of comparison, the inhibitory values of the conjugates Kuka and DCSP as well as of 'parent' unconjugated molecules are included in the study (Table 1). NDGA a known inhibitor of soybean LOX has been used as a reference compound (28μM) and CA (600 μM) as the positive control.

Perusal of the $IC_{50}$'s inhibition values (Table 1) shows that the most potent inhibitors are the compounds **14** ($IC_{50}$ 33.5 μM), **10** ($IC_{50}$ 40.5 μM) and **7** ($IC_{50}$ 41.5 μM), followed by the conjugates **15** ($IC_{50}$ 65 μM), **13** ($IC_{50}$ 81.5 μM) and **11** ($IC_{50}$ 87 μM). The inhibitory effect of the last two mentioned compounds should be mainly attributed to the presence of the dopamine unit because in contrast to dopamine ($IC_{50}$ 100 μM) neither ACI (1% at 0.1 mM) nor L-DOPA (23% at 0.1 mM) present notable inhibitory values. Accordingly, the symmetric spermine conjugate **8** containing L-DOPA is showing slightly more inhibitory activity (32% at 0.1 mM) compared to L-DOPA. This shows that the easily oxidizable catechol unit, which is also an efficient chelator for the $Fe^{3+}$ ion present in the enzyme active site, is not the only factor to account for the difference in activity between the conjugates **8** and **13**. Although lipophilicity is referred as an important physicochemical property for LOX inhibitors,[37] herein the theoretically calculated log *P*[38] values are too high to be realistic and thus, we do not take them into consideration in the discussion of the results.

The inhibitory activity of the other compounds which do not contain an easily oxidizable moiety might be attributed mainly to their ability to attach to the active site of the enzyme and to a lesser degree to their electron donating ability. The common structural element of compounds **11** and **13** is the dopamine moiety and it seems that replacement of ACI by L-DOPA as the acid component slightly improves inhibition. On the other hand, compound **10** and **11** both have ACI as the acid component (they are ACI amides) and differ only in the amine component of the conjugate. It therefore seems that benzylamine is a much better replacement for dopamine. Compound **14** combines characteristics of two molecules (DCSP and FICA) with high inhibition values which reflect to the higher value observed for this conjugate. On the other hand compound **15**, which also combines characteristics of two molecules

(DCSP and TRAB) with high inhibition values, showed an inhibitory value which is a little less than the value for the weaker (DCSP) of the afore mentioned two inhibitors. It is worth noting that the SPM conjugates with ATRA (compound **3**) and ACI (compound **4**) present almost identical interaction values with LOX which are definitely much higher than the unconjugated retinoids, indicating the beneficial nature of the conjugation in these cases. High interaction value is observed by the hybrid conjugate TRAA-SPM-ACI (**7**), which is higher than those for either the symmetric conjugates **4** and **5** or the asymmetric conjugate TRAA-SPM-ATRA (**6**).

Furthermore, replacement of the polyamine chain by an aliphatic chain, as in conjugate **9**, leads to higher interaction values than the unconjugated retinoid but certainly much lower than the corresponding conjugate with the polyamine chain (conjugate **3**), which incorporates two free secondary amino functions. On the other hand, conjugation of L-DOPA with SPM seemed to only slightly improve the inhibition capability of L-DOPA. Furthermore, it seems that amides of the acid TRAA (e.g., compound **12**) are better inhibitors than the free acid (TRAA) but much weaker inhibitors when compared to esters, for example, the *tert*-butyl ester TRAB. Also, the conjugation of L-DOPA with dopamine (conjugate **13**) resulted in a stronger inhibitor than the parent molecules. It therefore seems that in most cases conjugation of antioxidants have a beneficial effect on their inhibitory effect on LOX.

### 2.2.2. Anti-inflammatory activity in vivo

In acute preliminary toxicity experiments, the in vivo examined compounds did not present toxic effects in doses up to 0.1 mmol/kg body weight. For the anti-inflammatory assay, we used a dose of 0.01 mmol/kg equimolar to the administered standard drug indomethacin for the sake of comparison.

For the in vivo screening, the selection of the compounds was generally based on their good inhibitory activity on LOX and anti-lipid peroxidation. Thus, the most interesting compounds in this series, namely compounds **3**, **4**, **10**, **11** and **15** were selected to be examined in vivo by using the functional model of Carrageenan-induced rat paw oedema. Although compound **13** presented comparable to compound **11** LOX inhibition, it was not chosen for further studies because (a) it had no anti-LPO activity, (b) its substructure L-DOPA showed low inhibitory value for LOX and (c) the anti-inflammatory activity of dopamine (the other substructure of **13**) had been already found to be rather low (only 13%). In addition, compound **11** was chosen for further in vivo testing because it contained the substructure of the retinoid drug ACI, which had previously shown high anti-inflammatory activity (63%).[10] Unconjugated molecules were also included in the study for the sake of comparison.

Carrageenan-induced oedema is a non-specific inflammation resulting from a complex of diverse mediators.[39] As shown in Table 1, all conjugates, with the exception of conjugate **3** (40%) which exhibited a comparable effect to indomethacin (47%), showed much less potent anti-inflammatory activity (3.6–19%). It is worth noting that although conjugate **3** was almost four times more active than the unconjugated ATRA, the reverse holds for the conjugate **4**, which is ca. three times less active than ACI. Also, conjugate **3** is ca. two times more active than conjugate **4**, although ATRA itself is almost six times less inhibitory than ACI. A similar trend has been observed with the inhibitory activity of these compounds on the enzymic activity of RNase P, that is although ACI is stronger inhibitor than ATRA, the reverse is true for their symmetric conjugates with SPM.[11] Furthermore, amidation of the very active ACI (compound **10**) leads to a large decrease of its activity (ca. twenty times), which is not as bad (ca. five times less active) when the amino component is changed to dopamine (compound **11**). Finally, conjugation of the substructures of the DCSP and TRAB (compound **15**) also resulted in a high decrease (ca. five times) in the anti-inflammatory activity (46% for TRAB and 42% for DCSP). Therefore, with the exception of the conjugate ATRA-SPM-ATRA (**3**), conjugation of compounds with high anti-inflammatory activities results to a serious decrease in their anti-inflammatory effect.

**Table 2**
Cytotoxicity of compounds on RAMEC rat endothelial cells (24 h incubation)

| Compd[a] | $IC_{50}$[b] (μM) |
|---|---|
| KukA | 3.5 |
| DCSP | 11.3 |
| TRAB | 2.9 |
| ATRA | 18.3 |
| ACI | 14.6 |
| **3** | 2.6 |
| **4** | 4.7 |
| **9** | 2.8 |
| **15** | 12.9 |

[a] Full structures of all compounds in this column are presented in Supplementary data section (Table 2).
[b] Values are means of six different determinations. Viability data are subject to ±5% error.

### 2.2.3. Cytotoxicity studies

For the cytotoxicity studies, the selection of the compounds was generally based on their good anti-inflammatory activity, namely compounds KukA, DCSP, TRAB, ACI, **3** and **4** or their good inhibitory activity on LOX and anti-lipid peroxidation activity, namely conjugate **15**. For the sake of comparison, ATRA and conjugate **9** were also included in the study. The cytocompatibility of these compounds was evaluated by the viability of rat endothelial cells (RAMEC) cells in the presence of different concentrations of the compounds (0.5–50 μM) and is presented in Table 2 in the form of $IC_{50}$ values for the examined compounds.

The retinoids ATRA ($IC_{50}$ 18.3 μM) and ACI ($IC_{50}$ 14.6 μM) were the least cytotoxic for the RAMEC cells in this series of compounds. Their corresponding conjugates with SPM, namely compounds **3** ($IC_{50}$ 2.6 μM) and **4** ($IC_{50}$ 4.7 μM) were however more toxic than the unconjugated molecules. It is interesting to note that although ACI seems to be more toxic than ATRA, the reverse holds true for their conjugates. It would be tempting to hypothesize that the increased toxicity of conjugates compared to the unconjugated retinoids is due to the presence of the SPM chain. This is not however supported by the $IC_{50}$ value (2.8 μM) for the conjugate **9** which contains a conjugating chain of identical length to that of SPM but lacks the two secondary amino functions of SPM. It seems that the main cause of increased toxicity is the conjugation per se and not the presence of SPM. Also, a comparison of the $IC_{50}$ values of the conjugates KukA ($IC_{50}$ 3.5 μM) and DCSP ($IC_{50}$ 11.3 μM) shows that the main cause of increased toxicity should be attributed to the change in the non-SPM part of the molecules and in particular to the presence of the free phenolic hydroxyls. Finally, the conjugate **15**, incorporating structural characteristics of conjugate DCSP ($IC_{50}$ 11.3 μM) and of ester TRAB ($IC_{50}$ 2.9 μM), presented lower toxicity ($IC_{50}$ 12.9 μM) than either of these compounds. Therefore, conjugation of antioxidant/anti-inflammatory agents results in variable cytotoxicity on RAMEC rat endothelial cells depending primarily on the structure of the unconjugated molecule(s) and to a much lesser degree to the structure of the conjugating entity. In the cases examined, the presence of the SPM moiety does not seem to play a particularly important role in increasing cytotoxicity.

## 3. Conclusions

In general, conjugation of molecules with an interest as antioxidants and/or anti-inflammatory agents leads to compounds which generally present, with a few exceptions, higher inhibition on LOX and less potent anti-inflammatory activity. Regarding their ability to interact with AAPH, the highest activity was observed for conjugates **4** and **15** which both incorporate moieties (ACI, TRAB, DCSP) characterized by high interaction values with AAPH. Both compounds had low interaction values with DPPH whereas on the contrary the highly interacting with DPPH conjugate **13** exhibited zero interaction with AAPH, thus showing that lipid peroxidation activity is not always accompanied by DPPH radical-scavenging ability and vice-versa as it has been also previously referred.[10,40] This can be attributed to the different chemical reactions involved in the two assays.[29] In the DPPH assay, a reduction of the long-lived N-centred radical mainly takes place through ET from the antioxidant. Particularly effective such antioxidants are the phenoxide anions from phenolic compounds, such as the catechol unit containing compounds DHCA, KukA, L-DOPA, dopamine and the conjugates **8**, **11** and **13** as well as the reference compound NDGA. On the other hand in the AAPH assay, the highly reactive alkyl peroxyl radical is neutralized mainly by HAT from the antioxidant. Compounds incorporating phenol moieties in the present set of compounds seem not to be particularly effective HAT agents. Obviously, compounds acting as particularly effective HAT agents are the conjugates DCSP, **4** and **15**. The ability of conjugates to interact with either DPPH or AAPH varies and depends on the structure and the reducing ability of the unconjugated molecules. The incorporation of compounds like L-DOPA or dopamine, containing the highly oxidizable catechol unit, in conjugates provides compounds with increased interaction values against DPPH and inhibitory activity on LOX but generally very low inhibition of LPO and low anti-inflammatory potency. With the exception of conjugates DCSP and **15** which presented cytotoxicity on cells comparable to that of the well-known retinoids ATRA and ACI, the other conjugates KukA, **3**, **4** and **9** and the ester TRAB which were tested exhibited increased cytoxicity, indicating that the safety of use of these compounds for biomedical applications may be compromised at high doses.

## 4. Experimental section

### 4.1. Materials and methods

Melting points were determined with a Buchi SMP-20 apparatus and are uncorrected. IR spectra were recorded for KBr pellets on a Perkin–Elmer 16PC FT-IR spectrophotometer. $^1$H NMR spectra were obtained at 400.13 MHz and $^{13}$C NMR spectra at 100.62 MHz on a Bruker DPX spectrometer; TMS was used as reference. ESI-MS spectra were recorded on a Waters micromass ZQ spectrometer equipped with a quadropole analyzer, using MeOH as solvent. Optical rotations were measured at 25 °C with a Schmidt–Haensch Polartronic D polarimeter with a 0.5 dm cell. Analytical RP-HPLC were performed on a Waters system (2695 Alliance). Elution of the compounds was determined from the absorbance at 254 nm (Waters 2996 Diode array detector). Compound purity was assessed using a Lichrosphere™ RP 8e C8 column (5 μm, 125 × 4 mm) and a linear gradient of 10–60% acetonitrile (containing 0.08% TFA) in water (containing 0.08% TFA) for compounds **6**, **8** and **9** or a linear gradient of 10–40% acetonitrile (containing 0.08% TFA) in water (containing 0.08% TFA) for compound **13** over 30 min at a flow rate of 1 mL/min. Microanalyses were performed on a Carlo Erba EA 1108 CHNS elemental analyzer in the Laboratory of Instrumental Analysis of the University of Patras. Flash column chromatography was performed on Merck silica gel 60 (230–400 mesh) and TLC on Merck 60$F_{254}$ films (0.2 mm) precoated on aluminium foil. Spots were visualized with UV light at 254 nm, charring agents or ninhydrine. The eluent systems used were: (A) PhMe/EtOAc (9:1), (B) PhMe/EtOAc (8:2), (C) PhMe/EtOAc (6:4), (D) PhMe/EtOAc (1:1), (E) EtOAc, (F) $CHCl_3$/MeOH (98:2), (G) $CHCl_3$/MeOH (97:3), (H) $CHCl_3$/MeOH (9:1), (I) $CHCl_3$/MeOH/concd $NH_3$ (7:3:0.3).

All the chemicals used were of analytical grade and commercially available from Aldrich or Fluka. ACI and SPM were purchased from CHEMOS GmbH and ACROS ORGANICS, respectively. Compounds such as **16** (TRAA),[14] FICA,[10] **18**,[11] **21**,[11] DMCA[22] and **22**[22] were available in our laboratory from previous research projects. Finally, conjugates **3**,[11] **4**,[11] **5**[14] and **7**[14] were synthesized, for the purpose of the present study, according to literature protocols.

### 4.2. $N^1$-(*All-trans*-retinoyl)-$N^{12}$-[3-(trioxsalen-4′-yl)acryloyl]spermine (6)

To an ice-cold suspension of **16** (0.08 g, 0.27 mmol), $Boc_3$-SPM (0.14 g, 0.28 mmol) and TEA (0.09 mL, 0.66 mmol) in DMF (0.7 mL), HBTU (0.11 g, 0.30 mmol) was added and the resulting solution was stirred for 45 min at ambient temperature. Then, the reaction mixture was diluted with $CHCl_3$ and washed with a 5% aqueous solution of $NaHCO_3$ and $H_2O$. The organic layer was dried over $Na_2SO_4$ and evaporated to dryness to leave an oily residue, which upon FCC purification, using the eluant system E gave the anticipated fully protected spermine analog (0.09 g, 74%) as a yellow oil. This was then treated with an ice-cold solution of 50% TFA/$CH_2Cl_2$ (0.5 mL) and stirred at this temperature for 2 h. Volatile components were removed under vacuo and the residue was triturated with $Et_2O$ and left refrigerated overnight to give compound **17** as the corresponding tris-trifluoroacetate salt.[14] This compound was used as such into the next experiment as follows.

To an ice-cold solution of **17** (0.17 g, 0.2 mmol) and **18** (0.05 g, 0.13 mmol) in DMF/$CHCl_3$ (1:1, 0.7 mL), DIEA (0.11 mL, 0.65 mmol) was added dropwise. The resulting solution was stirred at this temperature for 10 min and then left to attain ambient temperature were it kept for further 5 h. Then, the reaction mixture was diluted with $CHCl_3$ and washed once with an 5% aqueous solution of $NaHCO_3$ and twice with $H_2O$. The organic layer was dried over $Na_2SO_4$ and evaporated to dryness to leave an oily residue. Conjugate **6** (0.09 g, 61%) was obtained pure, after FCC purification using solvent system I for elution, as an orange foam. $R_f$ (I): 0.34; IR (thin film, $CHCl_3$, cm$^{-1}$): 1718, 1684, 1676, 1654, 1648, 1636, 1600, 1577, 1400, 1240, 1120, 1101; ESI-MS: *m/z* 787.6 [M+Na], 765.6 [M+H], 441.4 [(M+H)−$C_{20}H_{22}N_2O_4$], 409.3 [(M+H)−$C_{23}H_{36}N_2O$]; RP-HPLC: $t_R$ = 18.08 min; $^1$H NMR (DMSO-$d_6$, $\delta$): 8.31 (s, 1H, N*H*), 8.26 (t, 1H, *J* = 5.6 Hz, N*H*CO), 7.99 (t, 1H, *J* = 5.6 Hz, N*H*CO), 7.81 (s, 1H, H-5), 7.48 (d, 1H, *J* = 16.0 Hz, H-15), 7.09 (dd, 1H, *J* = 15.2 and 11.6 Hz, H-22), 6.66 (s, 1H, H-2), 6.55 (d, 1H, *J* = 16.0 Hz, H-16), 6.29 (d, 2H, *J* = 15.2 Hz, H-21 and H-25), 6.12 (d, 1H, *J* = 11.6 Hz, H-23), 6.10 (d, 1H, *J* = 16.0 Hz, H-26), 5.89 (s, 1H, H-19), 3.33–3.24 (two overlapping q, 4H, *J* = 6.4 Hz, H-1′ and H-10′), 3.13 and 3.12 (two overlapping t, 4H, *J* = 6.8 Hz, H-3′ and H-8′), 2.62–2.55 (m, 4H, H-4′ and H-7′), 2.58 (s, 3H, H-12), 2.53 (s, 3H, H-13), 2.43 (s, 3H, H-14), 2.32 (s, 3H, H-33), 1.96 (s, 3H, H-34), 1.80 (s, 1H, N*H*), 1.72 (s, 3H, H-35), 1.65 (quint., 2H, *J* = 6.8 Hz, H-30), 1.55 (quint., 4H, *J* = 6.8 Hz, H-2′ and H-9′), 1.48–1.39 (m, 8H, H-5′, H-6′, H-29 and H-31), 0.93 (s, 6H, H-36 and H-37); $^{13}$C NMR (DMSO-$d_6$, $\delta$): 167.6, 166.7, 161.3, 159.1, 156.3, 154.9, 153.4, 149.5, 148.2, 138.4, 138.3, 136.2, 136.0, 134.0, 130.0, 129.5, 128.9, 128.3, 123.0, 122.9, 122.1, 121.1, 116.7, 113.2, 112.9, 112.7, 110.2, 109.6, 49.3, 49.2, 47.4, 47.3, 43.7, 38.3, 37.8, 29.8 (two C), 28.8, 27.6, 21.5, 19.5, 17.5, 14.1, 13.8, 13.0, 12.0, 8.5.

### 4.3. Synthesis of $N^1$,$N^{12}$-bis(3,4-dihydroxy-L-phenylalanyl)spermine (8)

#### 4.3.1. Synthesis of *N*-trityl-L-DOPA (19)

To a suspension of L-DOPA (0.59 g, 3 mmol) in anhydrous $CHCl_3$ (9 mL), $Me_3SiCl$ (1.71 mL, 13.5 mmol) was added and the reaction mixture was refluxed for 20 min. TEA (1.87 mL, 13.5 mmol) was then added with cooling and then reflux was continued for further 45 min. The resulting solution was cooled to 0 °C and treated dropwise with a solution of *i*-PrOH (0.34 mL, 4.5 mmol) in $CH_2Cl_2$ (1 mL), followed by the sequential addition of TEA (0.42 mL, 3 mmol) and TrtCl (0.83 g, 3 mmol). The thus obtained reaction mixture was stirred for 1 h at ambient temperature, $CH_3OH$ (7 mL) was added, left at ambient temperature for 30 min and then evaporated to dryness. The residue was treated with a 5% aqueous citric acid and extracted twice with $Et_2O$. The organic layer was washed with 1 N NaOH and water and the aqueous phases were combined, cooled to 0 °C and then acidified with 5% aqueous citric acid solution. The organic material was extracted twice with EtOAc and the combined organic layers were washed with $H_2O$ and brine. Pure Trt-L-DOPA (**19**) (1.07 g, 81%) was thus obtained as a foam and had: $R_f$ (H): 0.20; MS (ESI, 30 eV): *m/z* 440.41 [M+H], 243.41 [Trt]; The latter was converted to the corresponding crystalline diethylammonium salt according to the following procedure: A solution of Trt-L-DOPA in EtOAc (20 mL) was treated with $Et_2NH$ (0.32 mL, 3 mmol) and left to crystallize at ambient temperature for overnight. The precipitate was filtered to give Trt-L-DOPA.DEA (0.86 g, 80%), after recrystallization from acetone, as a light brown solid. This salt had mp 159 °C; $[\alpha]_D^{25}$ +14.2 (*c* 1.0, MeOH); $R_f$ (H): 0.37; IR (KBr, $cm^{-1}$): 3490, 3418, 3056, 2980, 2924, 1616, 1526, 1378, 1260, 774, 744, 706; ESI-MS: *m/z* 440.5 [M+H], 243.5 [Trt]; $^1H$ NMR (DMSO-$d_6$, $\delta$): 7.41–7.35, 7.26–7.21 and 7.18–7.12 (three m, 15H, Trt), 6.56 (d, 1H, *J* = 2 Hz, H-6), 6.51 (d, 1H, *J* = 8.0 Hz, H-9), 6.32 (dd, 1H, *J* = 2.0 and 8.0 Hz, H-5), 3.11 (t, 1H, *J* = 5.2 Hz, H-2), 2.69 (q, 4H, *J* = 7.2 Hz, $(CH_3CH_2)_2N$), 2.40 (dd, 1H, *J* = 4.8 and 13.2 Hz, H-3b), 1.99 (dd, 1H, *J* = 4.8 and 13.2 Hz, H-3a), 1.06 (t, 6H, *J* = 7.2 Hz, $(CH_3CH_2)_2N$); $^{13}C$ NMR (DMSO-$d_6$, $\delta$): 177.0, 147.3, 144.9, 143.7, 130.5, 129.2, 128.0, 126.5, 121.2, 118.1, 115.2, 71.5, 59.1, 41.9 (2C), 12.6.

#### 4.3.2. Synthesis of benzotriazolyl *N*-Trt-L-DOPA (20)

Trt-L-DOPA.$Et_2NH$ (0.51 g, 1 mmol), was partitioned between EtOAc and an ice-cold 5% aqueous citric acid solution. The organic layer was separated and the aqueous re-extracted with EtOAc. The combined organic layers were washed once with $H_2O$, dried and evaporated to dryness to leave Trt-L-DOPA as a brown foam. This was then dissolved in THF (3 mL), cooled to 0 °C and treated sequentially with HOBt (0.20 g, 1.5 mmol) and DCC (0.23 g, 1.1 mmol). The resulting suspension was stirred at this temperature for 30 min and then left to attain ambient temperature, where it was stirred further for 2 h to complete the reaction. Then, a few drops of AcOH and $H_2O$ were added and the mixture was stirred additional for 30 min to destroy excess DCC. DCU was then filtered off and the filtrate was evaporated to dryness to leave an oily residue. This was then dissolved in EtOAc, washed with an ice-cold 5% aqueous solution of $NaHCO_3$ and $H_2O$. The organic layer was dried over $Na_2SO_4$ and evaporated to dryness. Pure active ester **20** (0.47 g, 85%) was finally obtained after FCC purification, using the eluant system B, as light brown foam. $[\alpha]_D^{25}$ −8.48 (*c* 0.7, $CHCl_3$); $R_f$ (B): 0.36; IR (KBr, $cm^{-1}$): 3332, 1820, 1768, 1600, 1446, 1282, 774, 744, 706; ESI-MS: *m/z* 595.4 [M+K], 579.6 [M+Na], 557.2 [M+H], 243.5 [Trt]; $^1H$ NMR (DMSO-$d_6$, $\delta$): 8.92 and 8.91 (two s, 2H, O*H*), 8.10 (d, 1H, *J* = 8.0 Hz, H-10), 7.54–7.47 (m, 7H, Trt and H-11), 7.38–7.31 (m, 6H, Trt), 7.27–7.17 (m, 4H, Trt and H-12), 6.86 (d, 1H, *J* = 8.0 Hz, H-13), 6.69 (d, 1H, *J* = 8.0 Hz, H-6), 6.44 (d, 1H, *J* = 1.6 Hz, H-9), 6.40 (dd, 1H, *J* = 1.6 and 8.0 Hz, H-5), 3.80 (dt, 1H, *J* = 6.4 and 8.8 Hz, H-2), 3.68 (d, 1H, *J* = 6.4 Hz, N*H*), 2.76 (dd, 1H, *J* = 8.8 and 13.2 Hz, H-3a), 2.16 (dd, 1H, *J* = 8.8 and 13.2 Hz, H-3b); $^{13}C$ NMR (DMSO-$d_6$, $\delta$): 171.3, 146.6, 146.0 (three C), 145.9, 145.6, 133.6, 132.3, 128.9 (six C), 128.1 (six C), 127.1, 126.8 (three C), 125.8, 124.7, 120.8, 120.3, 119.5, 117.2, 71.7, 57.7, 40.2 (buried under the solvent).

#### 4.3.3. Preparation of conjugate 8

To a solution of $N^4$,$N^9$-bistritylspermine (0.14 g, 0.21 mmol) and active ester **20** (0.22 g, 0.4 mmol) in $CH_2Cl_2$ (0.5 mL), DIEA (0.09 mL, 0.5 mmol) was added dropwise. The resulting solution was stirred at ambient temperature for 2 h. The reaction mixture was then diluted with $CH_2Cl_2$, washed once with a 5% aqueous solution of $NaHCO_3$ and twice with $H_2O$, dried over $Na_2SO_4$ and evaporated to dryness. The anticipated bisamide (0.23 g, 72%) was obtained pure, after FCC purification, using the solvent system F for elution, as pale brown foam. It had $R_f$ (F): 0.19; IR (KBr, $cm^{-1}$): 3408, 1654, 1638, 1508, 1174, 774, 746, 706; ESI-MS: *m/z* 1530.1 [M+H], 1287.5 [(M+H)−Trt], 243.6 [Trt].

The bisamide (0.2 g, 0.14 mmol) was treated with an ice-cold solution of 20% TFA/$CH_2Cl_2$ (1 mL) and kept at this temperature for 2 h. Then, volatile components were removed under vacuo and the residue was triturated with $Et_2O$ and left refrigerated overnight. The resulting tetra-trifluoroacetate salt was converted to its corresponding tetrahydrochloride salt upon treatment with a 1.5 M solution of HCl in EtOH, followed by evaporation and trituration with $Et_2O$. Conjugate **8** (0.065 g, 83%) was obtained, after filtration under vacuo, as a white solid. It had $[\alpha]_D^{25}$ +24.3 (*c* 0.7, MeOH); IR (KBr, $cm^{-1}$): 3566, 3548, 3480, 3414, 3231, 1636, 1616, 620; ESI-MS: *m/z* 561.3 [M+H]; RP-HPLC: $t_R$ = 13.65 min; $^1H$ NMR (DMSO-$d_6$, $\delta$): 9.09 (br s, 4H, N$H_2^+$), 8.92 (s, 4H, O*H*), 8.70 (t, 2H, *J* = 5.6 Hz, N*H*CO), 8.32 (br s, 6H, N$H_3^+$), 6.70 (d, 2H, *J* = 8.0 Hz, H-6), 6.64 (d, 2H, *J* = 1.6 Hz, H-9), 6.49 (dd, 2H, *J* = 1.6 and 8.0 Hz, H-5), 4.19–4.12 (m, 4H, H-1′), 3.82 (t, 2H, *J* = 7.2 Hz, H-2), 2.85–2.83 (m, 8H, H-3′ and H-4′), 2.81–2.75 (m, 4H, H-3), 1.82–1.70 (m, 8H, H-2′ and H-5′); $^{13}C$ NMR (DMSO-$d_6$, $\delta$): 168.6 (two C), 145.6 (two C), 144.9 (two C), 126.1 (two C), 120.8 (two C), 117.4 (two C), 116.2 (two C), 54.4 (two C), 46.5 (two C), 44.9 (two C), 39.2 (two C, burried under the solvent), 36.9 (two C), 36.4 (two C), 25.9 (two C).

### 4.4. Synthesis of $N^1$,$N^{12}$-bis(*all-trans*-retinoyl)-1,12-aminododecane (9)

To a suspension of 1,12-diaminododecane (0.026 g, 0.13 mmol) in TEA (0.09 mL, 0.66 mmol) and $CHCl_3$ (1 mL), succinimidyl ester **18** (0.12 g, 0.3 mmol) was added. The resulting suspension was heated at 40 °C for 2 h. The reaction mixture was diluted in $CHCl_3$ and washed twice with $H_2O$, dried over $Na_2SO_4$ and evaporated to dryness to leave a residue. Pure **9** (0.09 g, 90%) was obtain, after FCC purification using initially the solvent system D and then the solvent system B as eluents, as an orange foam and had $R_f$ (B): 0.28; IR (KBr, $cm^{-1}$): 3412, 1718, 1654, 1638; ESI-MS: *m/z* 803.1 [M+K], 787.4 [M+Na], 765.3 [M+H]; RP-HPLC: $t_R$ = 17.50 min $^1H$ NMR (DMSO-$d_6$ $\delta$): 7.69 (unresolved t, 2H, N*H*CO), 6.81 (dd, 2H, *J* = 12 and 16 Hz, H-5), 6.22 (d, 2H, *J* = 16 Hz, H-4), 6.18 (d, 2H, *J* = 16 Hz, H-8), 6.12 (2H, d, *J* = 12 Hz, H-6), 6.08 (d, 2H, *J* = 16 Hz, H-9), 5.77 (s, 2H, H-2), 2.61–2.42 (m, 4H, H-1′), 2.19 (s, 6H, H-16), 1.95 (unresolv. t, 4H, H-12), 1.89 (s, 6H, H-17), 1.62 (s, 6H, H-18), 1.53 (t, 4H, *J* = 6.0 Hz, H-14), 1.41–1.38 (m, 8H, H-13 and H-2′), 1.25 (br s, 16H, H-3′, H-4′, H-5′ and H-6′), 0.96 (s, 12H, H-19 and H-20); $^{13}C$ NMR (DMSO-$d_6$, $\delta$): 161.6 (two C), 159.9 (two C), 141.9 (two C), 137.6 (two C), 137.0 (two C), 133.9 (two C), 133.8 (two C), 130.5 (two C), 129.9 (two C), 129.1 (two C), 110.6 (two C), 42.1 (two C), 39.8 (two C, burried under solvent), 34.2 (two C), 33.8 (two C), 33.1 (two C),

29.6 (two C), 29.5 (four C), 28.9 (four C), 27.0 (two C), 21.7 (two C), 19.2 (two C), 14.6 (two C), 13.0 (two C).

### 4.5. Syntheses of acitretin and trioxsalen amides

#### 4.5.1. Synthesis of acitretin benzylamide (10)

To a solution of **21** (0.05 g, 0.12 mmol) and DIEA (0.03 mL, 0.18 mmol) in $CH_2Cl_2$ (0.2 mL), benzylamine (0.014 mL, 0.13 mmol) was added dropwise. After 15 min, a yellow solid precipitated out. Additional $CH_2Cl_2$ was added to dissolve the precipitate and thus obtained solution was washed sequentially with a 5% aqueous solution of citric acid, $H_2O$, a 5% aqueous solution of $NaHCO_3$ and $H_2O$, dried over $Na_2SO_4$ and evaporated to dryness to afford pure **10** (0.034 g, 68%) as yellow solid. It had mp 165–169 °C; $R_f$ (A): 0.28; IR (KBr, $cm^{-1}$): 3320, 1630, 1616, 1586, 1536, 1120, 966; ESI-MS: *m/z* 438.2 [M+Na], 416.3 [M+H], 309.4 [(M+H)−$PhCH_2NH_2$]; $^1$H NMR (DMSO-$d_6$, $\delta$): 8.45 (t, 1H, *J* = 5.8 Hz, N*H*CO), 7.32 (t, 2H, *J* = 7.2 Hz, H-24), 7.26 (d, 2H, *J* = 7.2 Hz, H-23), 7.23 (t, 1H, *J* = 7.2 Hz, H-25), 6.96 (dd, 1H, *J* = 11.6 and 15.6 Hz, H-5), 6.69 (s, 1H, H-12), 6.67 (d, 1H, *J* = 15.6 Hz, H-4), 6.34 (d, 1H, *J* = 15.8 Hz, H-8), 6.31 (d, 1H, *J* = 11.6 Hz, H-6), 6.27 (d, 1H, *J* = 15.8 Hz, H-9), 5.91 (s, 1H, H-2), 4.31 (d, 2H, *J* = 5.8 Hz, H-21), 3.75 (s, 3H, OC*H*$_3$), 2.31 (s, 3H, H-20), 2.25 (s, 3H, H-16), 2.18 (s, 3H, H-18), 2.06 (s, 6H, H-17 and H-19); $^{13}$C NMR (DMSO-$d_6$, $\delta$): 165.5, 156.1, 147.0, 140.1, 138.2, 138.1, 137.0, 135.6, 134.0, 131.2, 129.8, 129.6, 128.7 (2C), 128.0, 127.8 (2C), 127.2, 123.3, 122.0, 110.6, 55.8, 42.4, 21.6, 17.7, 13.6, 13.1, 12.2; Anal. Calcd for $C_{28}H_{33}NO_2$: C, 80.93; H, 8.00; N, 3.37. Found: C, 80.58; H, 8.22; N, 3.59.

#### 4.5.2. Acitretin 2-(3,4-dihydroxyphenyl)ethylamide (11)

To an ice-cold suspension of dopamine hydrochloride (0.04 g, 0.21 mmol) in $CHCl_3$/DMF 5:1 (0.18 mL), DIEA (0.08 mL, 0.46 mmol) was added and the resulting suspension was stirred for further 10 min at this temperature. Then, succinimidyl ester **21** (0.05 g, 0.12 mmol) was added and the resulting reaction mixture was stirred at 0 °C for 5 min and at room temperature for further 2 h. Progressively, the reaction mixture was turned into a solution. After completion of the reaction, the mixture was diluted with $CHCl_3$, washed sequentially with a 5% aqueous solution of $NaHCO_3$, $H_2O$, a 5% aqueous solution of citric acid and $H_2O$, dried over $Na_2SO_4$ and evaporated to dryness to give a residue. Pure **11** (0.047 g, 85%) was obtained as yellow solid, after FCC purification using as eluant the solvent system H. It had mp 152–155 °C; $R_f$ (H): 0.36; IR (KBr, $cm^{-1}$): 3472, 3414, 1636, 1616, 1120; ESI-MS: *m/z* 484.3 [M+Na], 462.3 [M+H], 309.3 [(M+H)–$(HO)_2Ph(CH_2)_2NH_2$]; $^1$H NMR (DMSO-$d_6$, $\delta$): 8.55 (br s, 2H, O*H*), 7.84 (t, 1H, *J* = 5.2 Hz, N*H*CO), 6.93 (dd, 1H, *J* = 11.6 and 14.8 Hz, H-5), 6.68 (s, 1H, H-12), 6.66 (d, 1H, *J* = 16.0 Hz, H-8), 6.64 (d, 1H, *J* = 8.4 Hz, H-25), 6.59 (d, 1H, *J* = 1.6 Hz, H-28), 6.45 (dd, 1H, *J* = 1.6 and 8.4 Hz, H-24), 6.32 (d, 1H, *J* = 11.2 Hz, H-6), 6.31 (d, 1H, *J* = 14.8 Hz, H-4), 6.26 (d, 1H, *J* = 16.0 Hz, H-9), 5.83 (s, 1H, H-2), 3.76 (s, 3H, OC*H*$_3$), 3.25 (H-21, burried under $H_2O$), 2.56 (t, 2H, *J* = 7.6 Hz, H-22), 2.28 (s, 3H, H-20), 2.25 (s, 3H, H-16), 2.18 (s, 3H, H-18), 2.07 (s, 3H, H-17) 2.06 (s, 3H, H-19); $^{13}$C NMR (DMSO-$d_6$, $\delta$): 166.5, 156.0, 146.4, 145.5, 144.0, 138.2, 138.0, 137.1, 135.6, 133.9, 131.2, 130.8, 129.8, 129.3, 127.9, 123.6, 122.0, 119.6, 116.4, 115.9, 110.6, 55.8, 41.0, 35.1, 21.6, 17.6, 13.6, 13.0, 12.2; Anal. Calcd for $C_{29}H_{35}NO_4$: C, 75.46; H, 7.64; N, 3.03. Found: C, 75.62; H, 7.44; N, 2.92.

#### 4.5.3. *N*-Butyl-3-(trioxsalen-4′-yl)acrylamide (12)

To an ice-cold suspension of **16** (0.04 g, 0.13 mmol), 1-butylamine (0.014 mL, 0.14 mmol) and DIEA (0.05 mL, 0.26 mmol) in DMF (0.25 mL), HBTU (0.07 g, 0.19 mmol) was added. The resulting solution was stirred at ambient temperature for 30 min to complete the reaction. The mixture was diluted with $CHCl_3$ washed sequentially with a 5% aqueous solution of citric acid, $H_2O$, a 5% aqueous solution of $NaHCO_3$ and $H_2O$, dried over $Na_2SO_4$ and evaporated to afford pure **12** (0.036 g, 78%) as a white solid. Compound **12** had mp 224–226 °C; $R_f$ (C): 0.13; IR (KBr, $cm^{-1}$): 3282, 1736, 1656, 1614, 1602, 1104; ESI-MS: *m/z* 729.1 [2M+Na], 707.2 [2M+H], 354.3 [M+H], 281.3 [(M+H)−$PhCH_2NH_2$]; $^1$H NMR (DMSO-$d_6$, $\delta$): 8.14 (t, 1H, *J* = 5.6 Hz, N*H*CO), 7.77 (s, 1H, H-5), 7.47 (d, 1H, *J* = 16.0 Hz, H-15), 6.70 (d, 1H, *J* = 16.0 Hz, H-16), 6.35 (s, 1H, H-2), 3.23 (q, 2H, *J* = 6.4 Hz, H-18), 2.58 (s, 3H, H-12), 2.52 (s, 3H, H-13), 2.43 (s, 3H, H-14), 1.49 (quint., 2H, *J* = 6.8 Hz, H-19), 1.33 (sextet, 2H, *J* = 7.2 Hz, H-20), 0.91 (t, 3H, *J* = 7.2 Hz, H-21); $^{13}$C NMR (DMSO-$d_6$, $\delta$) 165.5, 160.3, 159.3, 154.3, 154.2, 149.1, 128.4, 122.6, 122.2, 116.6, 113.4, 113.0, 112.6, 108.8, 38.8, 38.7, 32.0, 20.1, 19.3, 14.2, 12.9, 8.7; Anal. Calcd for $C_{21}H_{23}NO_4$: C, 71.37; H, 6.56; N, 3.96. Found: C, 71.58; H, 6.24; N, 4.09.

### 4.6. Synthesis of *N*-[2-(3,4-dihydroxyphenyl)ethyl]-3,4-dihydroxy-L-phenylalaninamide (13)

To an ice-cold suspension of dopamine hydrochloride (0.04 g, 0.22 mmol) in $CHCl_3$/DMF (6:1, 0.23 mL), DIEA (0.08 mL, 0.46 mmol) was added and the resulting suspension was stirred for further 10 min at that temperature. Then, compound **20** (0.1 g, 0.18 mmol) was added and the reaction mixture was progressively turned into a clear solution. Stirring was continued for 30 min, at ambient temperature, to complete the reaction. The reaction mixture was then diluted with $CHCl_3$ and washed sequentially with a cooled 5% aqueous solution of citric acid, $H_2O$, a cooled 5% aqueous solution of $NaHCO_3$ and $H_2O$, dried over $Na_2SO_4$ and evaporated to dryness to leave an oily residue. Pure N-tritylated **13** (0.055 g, 53%) was obtained as a white foam, after FCC purification using as eluent the solvent system H. This compound had $R_f$ (H): 0.29; IR (thin film, $CHCl_3$, $cm^{-1}$): 3334, 1638, 1602, 1446, 1284, 780, 748, 706; ESI-MS: *m/z* 596.9 [M+Na], 243.2 [Trt].

Protected **13** (0.05 g, 0.087 mmol) was then diluted in an ice-cold solution of 30% TFA/$CH_2Cl_2$ (3 mL) and was stirred at that temperature for 30 min. Then, solvents were evaporated, the residue was triturated with $Et_2O$ and refrigerated overnight. Pure **13** (0.033 g, 85%) was thus obtained, following filtration under vacuo, as the corresponding trifluoroacetate salt. The latter was converted to its hydrochloride salt (a white solid) upon treatment with a 1.5 M solution of HCl in EtOH, which had $[\alpha]_D^{25}$ +16.2 (*c* 0.5, MeOH); $R_f$ (I): 0.17; IR (KBr, $cm^{-1}$): 3408, 1674, 1638, 1616, 1524, 1446, 1286, 1198; ESI-MS: *m/z* 665.3 [2M+H], 333.2 [M+H]; RP-HPLC: $t_R$ = 4.43 min $^1$H NMR (DMSO-$d_6$, $\delta$): 8.88, 8.85, 8.76 and 8.71 (four s, 4H, O*H*), 8.36 (t, 1H, *J* = 5.6 Hz, N*H*CO), 8.02 (br s, 3H, N$H_3^+$), 6.66 (d, 1H, *J* = 8.0 Hz, H-6), 6.63 (d, 1H, *J* = 8.4 Hz, H-16), 6.61 (d, 1H, *J* = 2.0 Hz, H-9), 6.57 (d, 1H, *J* = 2.0 Hz, H-13), 6.45 (dd, 1H, *J* = 2.0 and 8.0 Hz, H-5), 6.41 (dd, 1H, *J* = 2.0 and 8.4 Hz, H-17), 3.25 (H-10, burried under $H_2O$), 3.13 (sextet, 1H, *J* = 6.0 Hz, H-2), 2.82 (dd, 1H, *J* = 6.0 and 13.6 Hz, H-3a), 2.70 (dd, 1H, *J* = 6.0 and 13.6 Hz, H-3b), 2.48 (t, 2H, *J* = 7.6 Hz, H-11); $^{13}$C NMR (DMSO-$d_6$, $\delta$): 168.3, 145.7, 145.6, 145.0, 144.1, 130.2, 126.0, 120.7, 119.7, 117.2, 116.4, 116.0, 115.9, 54.4, 41.1, 37.1, 34.7.

### 4.7. Preparation of conjugates 14 and 15

#### 4.7.1. (*E*)-*N*-(5-Hydroxy-3-oxopentyl)-3,4-dimethoxycinnamide (23)

To an ice-cold solution of **22** (0.31 g, 1 mmol) and 2-(2-aminoethoxy)ethanol (0.10 mL, 1 mmol) in DMF (1.7 mL), TEA (0.28 mL) was added dropwise. The resulting solution was stirred at that temperature for 10 min and then left to attain ambient temperature where it was kept for further 45 min. After completion of the reaction, the mixture was diluted with $CHCl_3$ and washed once with a 5% aqueous solution of $NaHCO_3$ and twice with $H_2O$. The organic layer was dried

over $Na_2SO_4$ and evaporated to dryness to leave an oily residue. Pure **23** (0.26 g, 88%) was obtained, as a colourless oil after FCC purification and using solvent system H as eluant. Compound **23** had $R_f$ (H): 0.37; IR (thin film, $CHCl_3$, $cm^{-1}$): 3356, 3288, 1660, 1598, 1554, 1514, 1260, 1138; ESI-MS: *m/z* 318.4 [M+Na], 296.4 [M+H], 191.5 [(M+H)–$HO(CH_2)_2O(CH_2)_2NH_2$]; $^1H$ NMR ($CDCl_3$, $\delta$): 7.56 (d, 1H, *J* = 16.0 Hz, H-3), 7.05 (d, 1H, *J* = 8.0 Hz, H-6), 7.01 (s, 1H, H-9), 6.81 (d, 1H, *J* = 8.0 Hz, H-5), 6.51 (br s, 1H, N*H*CO), 6.32 (d, 1H, *J* = 16.0 Hz, H-2), 3.88 (s, 6H, O$CH_3$), 3.76 (t, 2H, *J* = 4.0 Hz, H-12), 3.67–3.56 (m, 6H, H-10, H-11 and H-13), 2.43 (br s, 1H, O*H*); $^{13}C$ NMR ($CDCl_3$, $\delta$): 161.8, 145.8, 144.3, 136.3, 123.1, 117.2, 113.7, 106.3, 105.0, 67.5, 65.2, 57.0, 51.2, 51.1, 34.8.

#### 4.7.2. General procedure for coupling 23 with the carboxylic acids FICA and TRAA

To an ice-cold suspension of the carboxylic acids FICA or TRAA (0.3 mmol), **23** (0.09 g, 0.3 mmol) and TPP (0.09 g, 0.36 mmol) in THF (1 mL), DIAD (0.07 mL, 0.36 mmol) was added dropwise and the resulting solution was stirred at ambient temperature to complete the reaction. The reaction mixtures were directly chromatographed (FCC) to afford pure conjugates **14** and **15** respectively.

**4.7.2.1. (*E,E*)-*N*-[11-Aza-14-(3,4-dimethoxyphenyl)-5,8-dioxa-4,12-dioxotetradeca-2,13-dienoyl] indole-3-carboxanilide (14).** Reaction time: 40 min; Elution system D; Yield: 0.12 g (64%), yellow solid; mp 115–117 °C; $R_f$ (D): 0.25; IR (KBr, $cm^{-1}$): 3474, 3412, 3280, 1734, 1720, 1654, 1616, 1600, 1542, 1438, 1184, 1106, 754, 720, 694; ESI-MS: *m/z* 634.1 [M+Na], 612.0 [M+H]; $^1H$ NMR (DMSO-$d_6$, $\delta$): 10.14 (s, 1H, PhN*H*CO), 8.93 (s, 1H, H-18), 8.45–8.40 (m, 1H, H-29), 8.31–8.26 (m, 1H, H-26), 8.07 (t, 1H, *J* = 5.6 Hz, N*H*CO), 7.90 (d, 1H, *J* = 16.0 Hz, H-15), 7.76 (unresolv. dd, 2H, H-22), 7.67–7.64 (m, 2H, H-27 and H-28), 7.57–7.54 (m, 3H, H-23 and H-24), 7.32 (d, 1H, *J* = 16.0 Hz, H-3), 7.10 (d, 1H, *J* = 1.6 Hz, H-9), 7.08 (d, 1H, *J* = 16.0 Hz, H-16), 7.04 (dd, 1H, *J* = 1.6 and 8.4 Hz, H-5), 6.88 (d, 1H, *J* = 8.4 Hz, H-6), 6.54 (d, 1H, *J* = 16.0 Hz, H-2), 4.42–4.36 (m, 2H, H-12), 3.75 and 3.72 (two s, 6H, O$CH_3$), 3.76–3.73 (m, 2H, H-13), 3.56 (t, 2H, *J* = 5.6 Hz, H-11), 3.50 (2H, H-11, buried under water); $^{13}C$ NMR (DMSO-$d_6$, $\delta$): 165.9, 165.1, 163.4, 162.1, 150.5, 149.3, 139.2, 135.8, 134.5, 133.8, 133.7, 132.7, 132.5, 129.2 (two C), 129.1, 126.2, 125.5, 124.8, 122.3, 121.7, 120.7 (two C), 120.3, 116.9, 116.6, 112.1, 110.5, 69.6, 68.3, 65.0, 55.9, 55.8, 39.1; Anal. Calcd for $C_{34}H_{33}N_3O_8$: C, 66.77; H, 5.44; N, 6.87. Found: C, 66.56; H, 5.23; N, 7.03.

**4.7.2.2. 5-(3,4-Dimethoxycinnamoylamido)-3-oxapentyl 3-(trioxsalen-4′-yl)acrylate (15).** Reaction time: 45 min; Elution system G; Yield: 0.12 g (68%), white solid; mp 105–110 °C; $R_f$ (G): 0.27; IR (KBr, $cm^{-1}$): 3274, 3216, 1734, 1710, 1636, 1598, 1510, 1260, 1236, 1104, 722, 696; ESI-MS: *m/z* 598.1 [M+Na], 576.1 [M+H]; $^1H$ NMR (DMSO-$d_6$, $\delta$): 8.01 (t, 1H, *J* = 5.6 Hz, N*H*CO), 7.86 (s, 1H, H-26), 7.73 (d, 1H, *J* = 16.0 Hz, H-16), 7.24 (d, 1H, *J* = 16.0 Hz, H-3), 6.94 (s, 1H, H-9), 6.93 (d, 1H, *J* = 8.0 Hz, H-6), 6.82 (d, 1H, *J* = 8.0 Hz, H-5), 6.63 (d, 1H, *J* = 16.0 Hz, H-15), 6.47 (d, 1H, *J* = 16.0 Hz, H-2), 6.35 (br s, 1H, H-23), 4.36–4.32 (m, 2H, H-12), 3.74 (s, 3H, O$CH_3$), 3.75–3.72 (m, 2H, H-13), 3.68 (s, 3H, O$CH_3$), 3.56 (t, 2H, *J* = 5.6 Hz, H-11), 3.50 (2H, H-10, buried under water), 2.62 (s, 3H, H-28), 2.54 (s, 3H, H-29), 2.45 (s, 3H, H-30); $^{13}C$ NMR (DMSO-$d_6$, $\delta$): 166.9, 165.8, 161.3, 160.3, 156.6, 154.6, 154.3, 150.3, 149.3, 149.1, 139.1, 134.8, 133.6, 132.7, 132.5 (two C), 129.9, 122.0, 121.7, 117.6, 114.1, 112.5, 111.8, 110.0, 68.4 (two C), 63.9, 55.9, 55.7, 19.4, 13.0, 8.6; Anal. Calcd for $C_{32}H_{33}NO_9$: C, 66.77; H, 5.78; N, 2.43. Found: C, 66.98; H, 5.62; N, 2.31.

### 4.8. General biological assays

DPPH, NDGA and CA were purchased from the Aldrich Chemical Co. Milwaukee, WI, (USA). Soybean LOX, linoleic acid sodium salt and indomethacin were obtained from Sigma Chemical, Co. (St. Louis, MO, USA) and Carrageenan, type K, was commercially available. For the in vivo experiments, male and female Fischer-344 rats (180–240 g) were used. For the in vitro tests a Lambda 20 (Perkin–Elmer) UV–vis double beam spectrophotometer was used. Each in vitro experiment was performed at least in triplicate and the standard deviation of absorbance was less than 10% of the mean.

### 4.9. In vitro assays

#### 4.9.1. Determination of the reducing activity (RA) of the stable radical DPPH[8,9]

To an ethanolic solution of DPPH (0.05 mM) in absolute ethanol an equal volume of the compounds dissolved in DMSO was added. The mixture was shaken vigorously and allowed to stand for 20 min or 60 min; absorbance at 517 nm was determined spectrophotometrically and the percentage of activity was calculated. All tests were undertaken on three or four replicates and the results were averaged (Table 1).

#### 4.9.2. Soybean LOX inhibition study in vitro[8,9]

The tested compounds dissolved in DMSO were incubated at rt with sodium linoleate (0.1 mL) and 0.2 mL of enzyme solution ($1/9 \times 10^{-4}$ w/v in saline). The conversion of sodium linoleate to 13-hydroperoxylinoleic acid at 234 nm was recorded and compared with the appropriate standard inhibitor.

#### 4.9.3. Inhibition of linoleic acid lipid peroxidation[8,9]

Production of conjugated diene hydroperoxide by oxidation of linoleic acid in an aqueous dispersion is monitored at 234 nm. AAPH is used as a free radical initiator. This assay can be used to follow oxidative changes and to understand the contribution of each tested compound.

Azo compounds generating free radicals through spontaneous thermal decomposition are useful for in vitro studies of free radical production. The water-soluble azo compound AAPH has been extensively used as a clean and controllable source of thermally produced alkylperoxyl free radicals. Ten microlitres of the 16 mM linoleic acid sodium salt solution was added to the UV cuvette containing 0.93 mL of 0.05 M phosphate buffer, pH 7.4 prethermostated at 37 °C. The oxidation reaction was initiated at 37 °C under air by the addition of 50 μL of 40 mM AAPH solution. Oxidation was carried out in the presence of aliquots (10 μL) in the assay without antioxidant, lipid oxidation was measured in the presence of the same level of DMSO. The rate of oxidation at 37 °C was monitored by recording the increase in absorption at 234 nm caused by conjugated diene hydroperoxides.

#### 4.9.4. Evaluation of the cytocompatibility of conjugates

RAMEC rat endothelial cells were isolated as described previously.[41] The cells were routinely subcultured in Dulbecco's MEM full growth medium (Biochrom AG, Germany), containing 10% foetal calf serum, 200 mM L-glutamine, 2.5 μg/mL Amphotericin B, 100 μg/mL streptomycin, 100 U/mL penicillin and 0.003% endothelial cell growth supplement in a humidified atmosphere containing 5% $CO_2$ at 37 °C.

The cytocompatibility of the compounds was evaluated by the viability of RAMEC cells in the presence of different concentrations of the compounds (0.5–50 μM) using the MTT test.[42] The cells were seeded in 24-well plates at a density of 20,000 cells per well in 500 μL Dulbecco's MEM full growth medium containing 10% foetal calf serum, 200 mM L-glutamine, 2.5 μg/mL Amphotericin B, 100 μg/mL streptomycin, 100 U/mL penicillin and 0.003% endothelial cell growth supplement. Twenty-four hours after plating, different amounts of the compounds were added in the wells. After 24 h of incubation at 37 °C, 50 μL of MTT solution (5 mg/mL in

phosphate buffered saline, pH 7.4) were added into each well and the plates were incubated at 37 °C for 2 h. The medium was withdrawn and 200 μL of DMSO was added in each well and agitated thoroughly to dissolve the formazan crystals. The solution was transferred to 96-well plates and immediately read on a microplate reader, at a wavelength of 540 nm. The experiments were performed in hexaplicate. Cell viability was calculated from the ratio between the absorbance provided by the cells treated with the different compounds and the absorbance provided by the non-treated cells (control) and the 50% growth inhibition values ($IC_{50}$) were calculated. Viability data are subject to ±5% error.

### 4.10. In vivo assays

All screening tests were conducted on Fisher-344 inbred rats of ca. 150–200 g body weight; each group was composed of six animals. Compounds were dispersed in water with few drops of Tween 80 and ground in a mortar before use (or diluted in water as salts) and administered ip. Controls received the liquid vehicle.

#### 4.10.1. Acute toxicity tests

The approximate lethal dose in 50% of the tested animal was determined. Mortality was recorded 24 h post-injection. In acute toxicity experiments, in vivo the compounds are examined in doses up to 0.1 mmol/kg body weight.

#### 4.10.2. Inhibition of the Carrageenan-induced oedema[8,9]

Oedema was induced in the right hind paw of Fisher 344 rats (150–200 g) by the intradermal injection of 0.1 mL 2% Carrageenan in water. Both sexes were used. Females pregnant were excluded. Each group was composed of six animals. The animals, which have been bred in our laboratory, were housed under standard conditions and received a diet of commercial food pellets and water ad libitum during the maintenance but they were entirely fasted during the experiment period. Our studies were in accordance with recognised guidelines on animal experimentation. The tested compounds 0.01 mmol/kg body weight, were dispersed in water with few drops of Tween 80 and ground in a mortar before use and they were given intraperitoneally simultaneously with the Carrageenan injection. The salts were dissolved in water only and followed the same administration. Carragenan was injected intradermally into right foot pad, the left paw serving as control. The rats were euthanized 3.5 h after Carrageenan administration. For each animal the swelling caused by the phlogistic is given as percentage of weight increase of right hind paw in comparison to uninjected left hind paw. The difference between the weight of the injected and uninjected paws was calculated for each animal. The change in paw weight was compared with that in control animals (treated with water/or Tween-water) and expressed as a percent inhibition of the oedema% ICPE values (Table 1). The experiment was repeated twice (six animals each time) to compare the mean value in compounds treated groups with that in groups of anials that were injected only with the liquid vehicle. Indomethacin was tested as a reference compound in 0.01 mmol//kg (47%). Values % ICPE are the mean from two different experiments ($n = 6$ animals each time) with a standard error of the mean less than 10%.

$$\%\text{CPE Inhibition} = (A_c - A_{treat}/A_c) \times 100$$

$A_c$ = mean% increase of the weight of the hind paw of the control group

$A_{treat}$ = mean% increase of the weight of the hind paw of the group of the treated animals

Differences between the controls and the treated groups of animals in these experiments were tested for statistical significance by Student's *t*-test for nonparametric data.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.10.012. These data include MOL files and InChiKeys of the most important compounds described in this article.

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