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Retinylisoflavonoid as a Novel Membrane Antioxidant

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We report a novel molecular dyad as an antioxidant, retinylisoflavonoid, with a retinal analogue C_{22}-aldehyde and the isoflavonoid daidzein covalently linked. Its physicochemical properties, pK_a (pK_{a1} = 8.45, pK_{a2} = 11.42), oxidation potential (1.03 V vs NHE), and Log_{10} partition (Log P = 1.96), as well as the Trolox equivalent antioxidant capacity (TEAC = 0.4), have been characterized. Spectroscopic and quantum chemical investigations have revealed the following unique structural characters: (i) Either free in solution or included in liposomal membranes, the C_{22}-aldehyde moiety of retinylisoflavonoid is coplanar with the B-ring of daidzein owing to the strong intramolecular hydrogen bonding $C_{14'}$=O···HO−B4′. Accordingly, the C_{22}-aldehyde moiety extends its π-conjugation significantly to the B-ring. (ii) The inherent amphiphilicity of retinylisoflavonoid allows the C_{22}-aldehyde moiety embedded in the lipid phase of the liposomes, whereas the daidzein counterpart stays at the membrane surface, in effect facilitating interior-to-surface radical communication. As the result, the antilipooxidation activity of retinylisoflavonoid is improved significantly in protecting membrane lipids compared to the parent compounds alone or in combination, and importantly, the performance is more prominent under higher-level oxidative stress. This work provides an advanced case study of new antioxidant development based on optimized electronic and molecular structures.

1. Introduction

The intake of flavonoid or carotenoid from fruits and vegetables is known to be inversely correlated to the incidence of many lifestyle diseases,[1−4] and the benefit to human health has been ascribed to such exogenous radical scavengers and antioxidants.[5,6] As different kinds of antioxidants coexist in food or biological systems, it is important to examine the collective effect and the interactive mechanism of plural antioxidants. In this context, the regeneration of lipophilic α-tocopherol from its one-electron oxidized form by hydrophilic ascorbate is crucial for protecting membranal lipid or low density lipoprotein (LDL) from oxidative damage.[7−10] In addition, interaction between ascorbate and plant polyphenols[11] or carotenoid,[12] and that between tocopherol and polyphenol[13,14] or carotenoid,[15] had been investigated in either homogeneous solutions or structured biological media.

Despite the intensive research efforts with regards to the antioxidant interactions, only recently has the flavonoid−carotenoid interaction been examined, and a regeneration of β-carotene from its radical cation by the isoflavonoid puerarin at the water/lipid interface could explain the higher antilipooxidation activity observed for the puerarin/β-carotene combination than expected from their individual performance.[16] Such a synergistic antioxidation effect was also found for the combination of the flavonoid baicalin and β-carotene.[17] Most recently, we have carried out a systematic investigation on the interaction of six different (iso)flavonoids with β-carotene in liposomal systems and have indentified three critical physicochemical factors governing the regeneration reactions:[18] (Iso)flavonoid should locate appropriately at the lipid/water interface and be more reducing than carotenoid, and the regeneration should be fast. These prerequisites of corporative interaction are, respectively, relevant to the structural aspects, thermodynamics, and reaction kinetics. Indeed, antioxidant regeneration had been previously proposed for LDL systems, where the semiquinone radical of ubiquinol 10 ($Q_{10}H^{\bullet}$) or the α-tocopherol radical (α-$TO^{\bullet}$) export their radical character from LDL into the aqueous phase.[9] Taken together, the synergistic antioxidant interaction in complex systems may be generalized as *radical communication* between different antioxidants; e.g., (iso)flavonoid at the water/lipid surface regenerates carotenoid from its radical cation as the product of antilipooxidation in the lipid phase.

To validate the hypothetical mechanism of radical communication in antioxidation, a molecular dyad jointing (iso)flavonoid and carotenoid would be of interest. These types of compounds, i.e., carotenylflavonoid covalently linking carotenoid and flavonoid, have recently been reported by Beutner et al., and the high antioxidation capacity outperforming the activities of individual constituents has been demonstrated.[19] In the present work, we have designed a novel antioxidant dyad, retinylisoflavonoid (Scheme 1), covalently linking the retinal analogue C_{22}-aldehyde and the isoflavonoid daidzein with the phenolic hydroxyls fully preserved. Such a model compound allows the antioxidant interaction to be investigated as an intramolecular process. We have characterized the physicochemical properties and molecular structures of the novel molecular dyad in homogeneous solution and in a lipid membrane, evaluated its radical scavenging and antilipooxidation performance, and discussed its structure−activity relationship. It is found that the amphiphilic retinylisoflavonoid takes a planar conformation in the lipid bilayer as it does in solution and that the molecular dyad strides across the membrane interior to the water/lipid interface, facilitating the export of its radical

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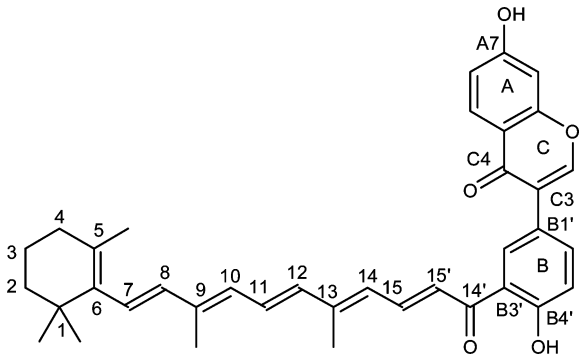
SCHEME 1: Molecular Structure of 3′(14′-Apo-β-carotene-14′-carbonyl)-daidzein (Referred to As Retinylisoflavonoid in This Paper)[a]



[a] The numbering system follows the convections of carotenoid and isoflavonoid.

character. In view of the recent report that certain flavonoids can protect retinal ganglion cells or retinal pigment epithelial cells from oxidative-stress-induced death with high potency,[20−22] our results may shed light on the mechanism behind (iso)flavonoid protection of retinal cells from oxidative stress and may be further beneficial for the design of new antioxidants of optimized activity for specific applications.

2. Materials and Methods

Chemicals and Synthetic Protocols. Daidzein was purchased from Huike Plant Exploitation Inc. (Shaanxi, China). 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), lithium aluminum hydride, potassium *tert*-butoxide, retinol acetate, 8-anilino-1-naphthalenesulfonic acid (ANS), and soybean L-α-phosphatidylcholine (PC) were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). 2,2′-Azobis(2,4-dimethylvaleronitrile) (AMVN) was from Wako Pure Chemical Industries (Osaka, Japan). All other reagents and solvents were supplied by Beijing Chemical Works (>99%, Beijing, China) and used as received without further purification except as follows. Diethyl ether, chlorobenzene, ethanol, and triethylamine were dried and distilled by standard procedures. Manganese dioxide was activated by washing with sulfuric acid and then dried at 120 °C for 2 h. 16-(9-Anthroyloxy) palmitic acid (16-AP) was synthesized from 16-hydroxyhexadecanoic acid and 9-anthracenecarboxylic acid.[23]

Synthetic routes are shown in Scheme 2. Detailed synthetic procedures are given in the Supporting Information S1. Briefly, retinol acetate (**1**) was reduced by $LiAlH_4$ to obtain retinol (**2**), which was oxidized by manganese dioxide to yield retinal (**3**). In parallel, daidzein (**4**) was acetylated by acetic anhydride to produce diacetyl daidzein (**5**), which underwent the aluminum trichloride catalyzed Fries rearrangement to form 3′-acetyl daidzein (**6**). Connection of retinal and 3′-acetyl daidzein was achieved by the aldol condensation under the catalysis of potassium *tert*-butoxide, which yielded the target 3′(14′-apo-β-carotene-14′-carbonyl)-daidzein (**7**, retinylisoflavonoid).

Spectroscopic Characterization. ^1H and ^{13}C NMR spectra were recorded on a Bruker AV 400 NMR (Bruker Daltonic Inc., Massachusetts, USA). High-resolution mass spectral analyses were performed with Bruker Apex IV FTMS (Bruker Daltonic Inc., Massachusetts, USA). UV−visible absorption spectroscopy was carried out on an absorption spectrometer (Cary 50, Varian, Australia). Fluorescence spectra were measured on a LS-55 luminescence spectrophotometer (Perkin-Elmer, Waltham, Massachusetts, USA). In resonance Raman scattering measurements, the continuous wave optical excitation at 488 nm was provided by an Ar^+ laser (GLG3050, NEC Corp., Tokyo, Japan), and the excitation power was 5 mW. A 90° geometry was used for collecting scattering light, which was sent to the entrance slit of a triple spectrograph (Trivista, SP2500i, Acton, Princeton, USA) and subsequently detected by an intensified CCD detector (ICCD PI-MAX, Princeton Instruments, Princeton, USA). The total exposure time was 7.5 min for each measurement. Spectral background from solvent was subtracted from the Raman spectra of samples.

Physicochemical Properties of Antioxidant and Membrane Fluidity Evaluation. For cyclic voltammetric measurements, anhydrous acetonitrile from Sigma-Aldrich Co. was used as received, and tetrahydrofuran was dried by refluxing over sodium metal for 24 h under nitrogen protection, which was then distilled into a flask under nitrogen atmosphere by using the vapor transfer method. Tetrahydrofuran solutions of samples, with the concentration of 5.4×10^{-4} M for retinylisoflavonoid and 6.7×10^{-4} M for daidzein, were prepared inside a nitrogen-charged glovebox, and the final concentration of supporting electrolyte, polarographic grade tetra-*n*-butylammonium perchlorate (TBAP), was made at 0.1 M. Each solution was prepared directly into an electrochemical cell equipped with the standard three electrodes, i.e., a glassy carbon working electrode, a Pt wire counter electrode, and a silver wire pseudoreference electrode calibrated against the ferrocene/ferrocenium couple. The electrochemical cell was sealed inside the glovebox to maintain nitrogen atmosphere and then subjected to cyclic voltammetric measurement on a CHI660B potentiostat (Chenghua, Shanghai, China). All of the measurements were done at room temperature.

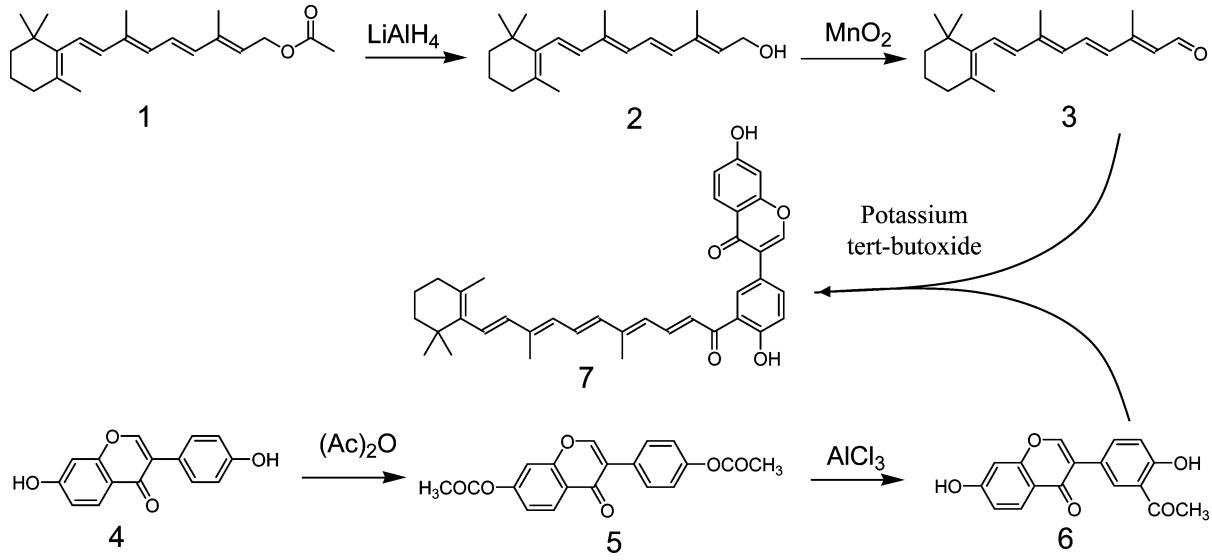
The Log_{10} partition (Log *P*), pK_a, and the Trolox equivalent antioxidant capacity (TEAC) of antioxidant were determined following the procedures described in refs 17 and 24. Liposome membrane fluidity was evaluated by the use of fluorescent probes ANS[25] and 16-AP[26] by measuring the fluorescence intensity and polarization (*P*), respectively. *P* was probed at 415 nm under the excitation wavelength of 380 nm according to the relation[27] $P = (I_{||} - G \cdot I_{\perp})/(I_{||} + G \cdot I_{\perp})$, where $I_{||}$ and $I_{\perp}$, respectively, represent the fluorescence intensities measured with fluorescence polarized parallel and vertical to excitation polarization and *G* stands for the instrumental polarization factor determined to be ∼1.

Quantum Chemical Calculations. The molecular geometries of retinylisoflavonoid and daidzein and their deprotonated and oxidized forms were optimized with the UB3LYP density functional theory in conjunction with the 6-31G(d,p) basis set by the use of the Gaussian 03 package.[28] The gas-phase deprotonation enthalpy (DE) and bond dissociation energy (BDE), respectively, were derived as the enthalpy differences of the reactions $ArOH \rightarrow ArO^- + H^+$ and $ArOH \rightarrow ArO^{\bullet} + H^{\bullet}$, respectively.[16] Raman spectra were calculated by the use of the B3LYP/6-31G(d,p) basis set, and the Raman activities and frequency shifts were corrected according to refs 29 and 30. The bandwidths of Raman lines were set to 5 cm^{-1} (fwhm, full width at half-maximum).

3. Results and Discussion

Synthesis of Retinylisoflavonoid. Since the phenolic hydroxyls are crucial for the bioactivities of (iso)flavonoid, we have intended to preserve the functionals of daidzein, A7-OH and B4′-OH, in synthesizing retinylisoflavonoid (Scheme 2). This was readily achieved via the Freis rearrangement. In

SCHEME 2: Synthetic Routes of Retinylisoflavonoid (7)



addition, the chain-like moiety of the dyad, 14′-apo-β-carotene-14′-carbonyl (hereafter abbreviated as C_{22}-carbonyl), is lipophilic and in length comparable to the fatty acid tail of PC. Such linear conjugation is expected to serve as a molecular wire linking covalently to the daidzein moiety to facilitate radical delocalization.

Molecular and Electronic Structures of Retinylisoflavonoid. As shown in Figure 1, daidzein in methanol shows UV absorption bands I and II, which are similar to those of daidzein in aqueous solution in acidic form (pH = 5.0).[31] Retinylisoflavonoid retains the two UV bands originating from the isoflavonoid backbone and exhibits an additional visual absorption band (III) that obviously stems from the C_{22}-carbonyl moiety. With reference to the absorption spectrum of C_{22}-aldehyde alone in ethanol maximized at 410 nm,[32] band III of retinylisoflavonoid shifts to the red for 32 nm. The extinction coefficients of bands I and II are notably larger than those of daidzein. Band III shows a rather small difference for retinylisoflavonoid in liposome and in solution, i.e., a 4 nm red shift along with a 7 nm decrease in bandwidth. Such spectral similarity suggests that retinylisoflavonoid molecules in lipid or in solution have little geometrical variation.

Two striking features of molecular and electronic structures of retinylisoflavonoid are drawn from Figure 2. (i) For all-*trans* C_{22}-aldehyde in organic solvent, it had been shown by resonance Raman spectroscopy that the $C_{15'}$ hydrogen and the $C_{14'}$ oxygen are in *cis* conformation,[32] which is also confirmed by our quantum chemical calculation (Supporting Information S2). However, for retinylisoflavonoid, the entire C_{22}-carbonyl backbone rotates about 180° around the $C_{14'}-C_{15'}$ bond, leaving the $C_{15'}$ hydrogen and the $C_{14'}$ oxygen in *trans* conformation (Figure 2a). As a result, the C_{22}-carbonyl backbone is almost perpendicular to the molecular long axis of daidzein (~90°), and the dyad takes a Γ-like geometry. (ii) Owing to the $C_{14'}=O\cdots HO-B4'$ hydrogen bonding, the $C_{14'}=O$ and $B3'-B4'-OH$ motifs form a six-membered ring, which stabilizes the nearly perfect coplanarity between the C_{22}-carbonyl and the B ring (dihedral angle, ~3°) and thereby allows the delocalization of the π-electron from the C_{22}-carbonyl to the B ring (Figure 2b, HOMO). Further extension of π-conjugation to AC rings is hampered by a significant twist between AC and B planes around the C3−B1′ bond (α_{AC-B} = 39.8°). On the other hand, the LUMO orbital shrinks in the β-ionone ring of C_{22}-aldehyde but extends more toward the B ring of daidzein, indicating appreciable intramolecular charge transfer (ICT) character upon the HOMO→LOMO transition. Such an extension of π-conjugation together with the ICT character is responsible for the large absorptivity of retinylisoflavonoid, as well as for the 32 nm red shift of band III with respect to C_{22}-aldehyde alone in methanol solution.

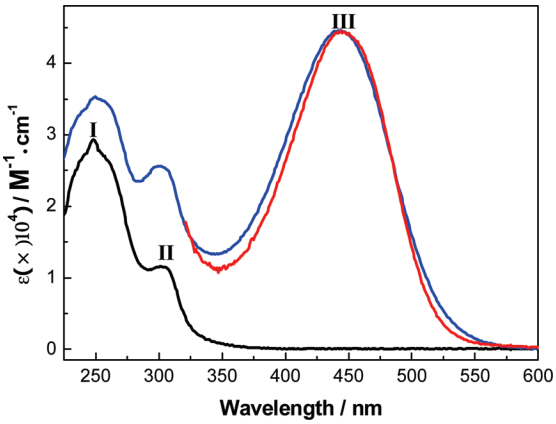


Figure 1. UV−visible electronic absorption spectra of daidzein in methanol (black) and retinylisoflavonoid in methanol (blue) or in liposome (red, normalized). Maximal extinction coefficients are ε_{248nm} = 2.93 × 10^4 $M^{-1}\cdot cm^{-1}$ for daidzein and ε_{442nm} = 4.46 × 10^4 $M^{-1}\cdot cm^{-1}$ for retinylisoflavonoid in methanol.

The Raman spectra of retinylisoflavonoid shown in Figure 3(a, b) originate essentially from the C_{22}-carbonyl substituent because the optical excitation is in rigorous resonance to its electronic absorption (band III). The Raman spectrum of retinylisoflavonoid in liposome is similar to that in dichloromethane except a 4 cm^{-1} downshift of the C=C stretching of C_{22}-carbonyl (1,543 cm^{-1} → 1,539 cm^{-1}). Therefore, the C_{22}-carbonyl of retinylisoflavonoid in liposome must hold a fully stretched conformation as it does in solvent, corroborating to the aforementioned similarity in electronic absorption spectra. With reference to the frequency of the $C_{14'}=O$ vibration for C_{22}-aldehyde free in carbon tetrachloride (1680 cm^{-1}),[32] that of retinylisoflavonoid shifts to 1579 cm^{-1} as a weak shoulder to the higher-wavenumber side of the intense C=C stretching at ~1539 cm^{-1} (Figure 3a) or 1543 cm^{-1} (Figure 3b). From C_{22}-aldehyde to retinylisoflavonoid, the ~100 cm^{-1} downshift

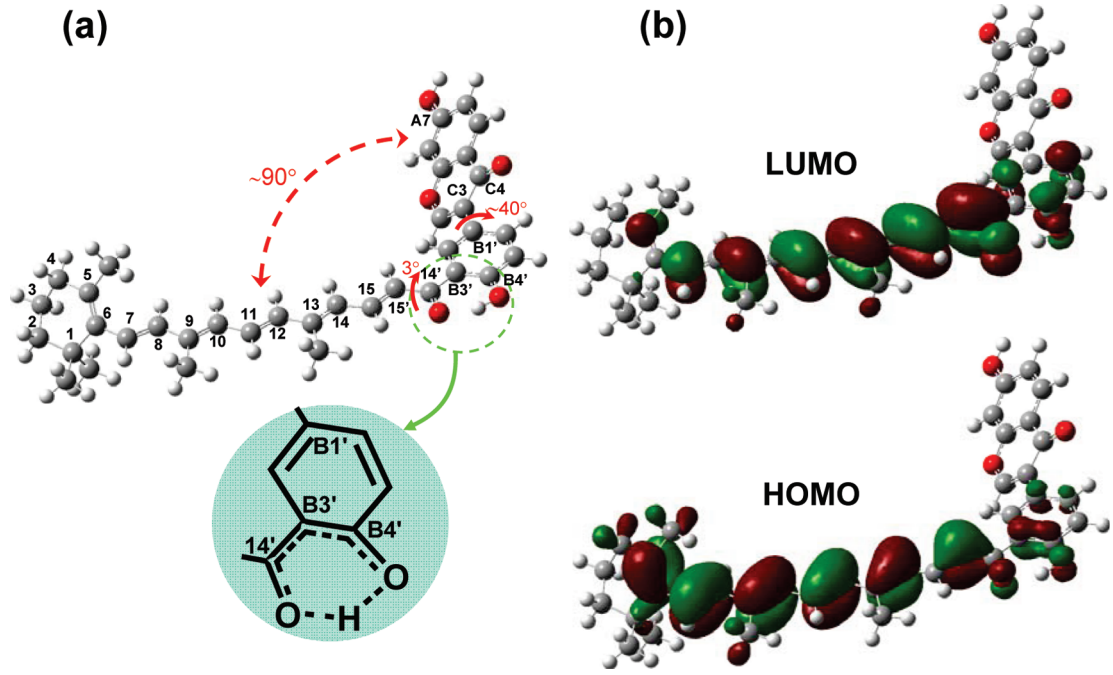


Figure 2. (a) Optimized molecular geometry and (b) frontier orbitals of retinylisoflavonoid. Dashed circle in (a) highlights the hydrogen bond between the B4′−OH and the 14′-carbonyl ($C_{14'}$═O···HO−B4′).

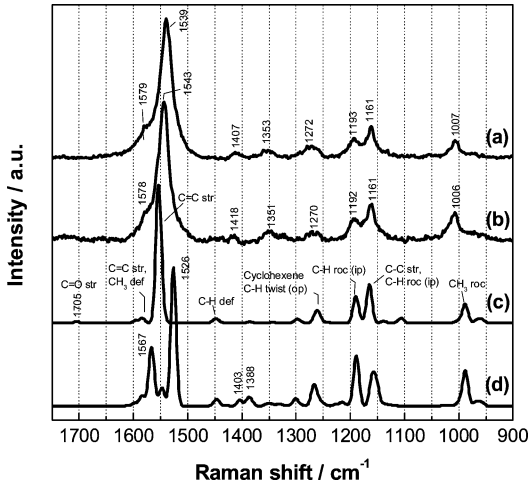


Figure 3. Resonance Raman spectra of retinylisoflavonoid (a) in dichloromethane (2.72×10^{-4} M) and (b) in liposome (1.5×10^{-5} M). Excitation wavelength was 488 nm. Calculated Raman spectra of (c) C22-aldehyde and (d) retinylisoflavonoid. Assignments of normal modes are based on the quantum chemical calculations and on ref 32.

of the $C_{14'}$═O stretching is reasonably ascribed to the presence of a strong intramolecular hydrogen bond, $C_{14'}$═O···HO−B4′ (Figure 2a).

Figures 3c and 3d, respectively, present the calculated Raman spectra of C_{22}-aldehyde and retinylisoflavonoid, with the assignments of the key Raman line indicated in Figure 3c. It is predicted that, from C_{22}-aldehyde to retinylisoflavonoid, the $C_{14'}$═O stretching shifts to the red for more than 100 cm^{-1}, in agreement with the experimental observation: For C_{22}-aldehyde, the $C_{14'}$═O stretching locates at 1706 cm^{-1}. However, the $C_{14'}$═O vibration (str) is no longer independent in retinylisoflavonoid owing to the strong coupling between the B-ring of daidzein and the C_{22}-carbonyl substituent. Specifically, the vibrational modes at 1567 or 1526 cm^{-1} are both contributed by the C═C stretching of the C_{22}-carbonyl, the C═O stretching, and the B-ring deformation. In addition, the mode active at 1567 cm^{-1} also couples to C−H wagging (ip). Similarly, the mode at 1403 cm^{-1} originates from the B-ring deformation coupled to the C═O stretching and that at 1388 cm^{-1} from the methyl deformation of the C_{22}-carbonyl substituent coupled to the C−H bending of the B-ring. Evidently, with respect to the theoretical Raman spectrum of retinylisoflavonoid (Figure 3d), that of C_{22}-aldehyde (Figure 3c) resembles more closely the experimental observations (Figures 3a and 3b). The discrepancy between the theoretical and the experimental retinylisoflavonoid spectra, besides the neglect of solvent effect in theoretical calculation, is mainly due to the resonance excitation of the C_{22}-carbonyl substituent.

Deposition of Retinylisoflavonoid in the Lipid Bilayer. Besides the physicochemical properties, the spatial deposition and molecular orientation of antioxidant in lipid membrane, as well as the change of membrane property owing to the adoption of antioxidant, may affect the antilipooxidation activity.[26,33,34] ANS and 16-AP with fluorophores locating in different regions of the lipid bilayer are used to probe the spatial deposition of antioxidants. Specifically, those bound to the membrane surface will boost ANS fluorescence because of the decrease in local fluidity,[35] whereas those deposited in the membrane interior will enhance the 16-AP fluorescence polarization owing to the increase in local stiffness.[27,36]

Figure 4a shows the sequence of ANS fluorescence intensity increase, blank < daidzein < retinylisoflavonoid < retinol acetate. The results indicate that retinol acetate accumulates preferentially at the membrane surface judging from the drastic enhancement of ANS fluorescence. Importantly, the fluorescence intensity of the liposome with retinylisoflavonoid incorporated is much stronger than that of the liposome with daidzein incorporated, implying that the surface accumulation of retinylisoflavonoid is relatively more significant. The level of surface accumulation of retinylisoflavonoid or daidzein may be correlated to their dipole moments (8.3 vs 5.3 D) and Log_{10} partition coefficients (1.96 vs 1.73) (Table 1). The relatively higher lipophilicity of retinylisoflavonoid is evidently due to the hydrophobic C_{22}-carbonyl substituent. Here, we note that retinyl-

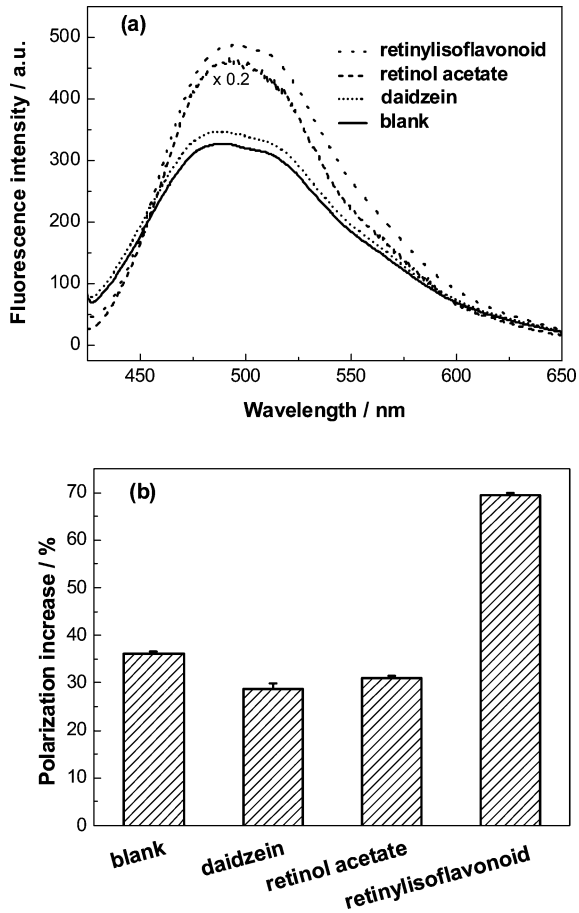


Figure 4. (a) ANS (25 μM) fluorescence spectra and (b) 16-AP (0.5 μM) fluorescence polarization increase of the aqueous dispersion of liposomes with the indicated antioxidants included (4.5 μM). Excitation wavelengths were 400 and 380 nm for the measurements in (a) and (b), respectively.

TABLE 1: Physicochemical Properties of Retinylisoflavonoid and Daidzein[a]

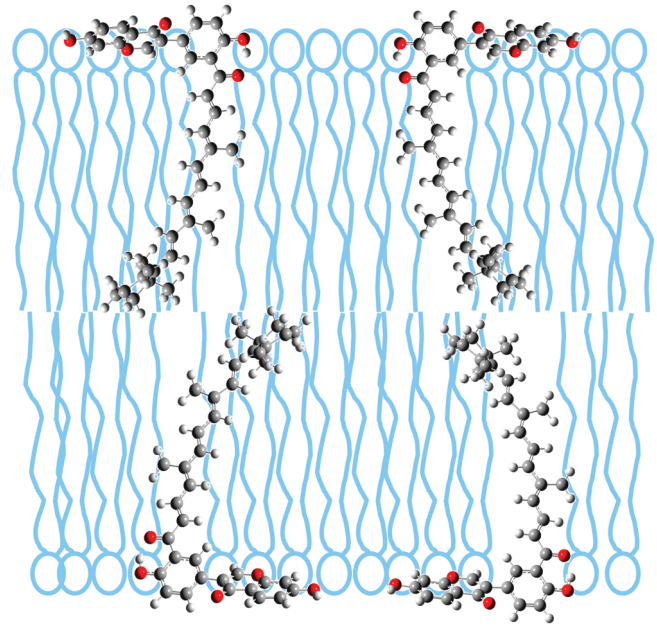
compound	pK_a	TEAC	E/V	Log P	μ/D	DE/kJ·mol^{-1}	BDE/kJ·mol^{-1}
retinylisoflavonoid		0.4	1.03	1.96	8.3		
A7-OH/A7-O$^-$	8.45					1439.3	
B4′-OH/B4′-O$^-$	11.42					1516.1	
A7-OH/A7-O$^\bullet$							387.2
B4′-OH/B4′-O$^\bullet$							457.1
daidzein		1.9	1.22	1.73	3.2		
A7-OH/A7-O$^-$	7.47					1448.7	
B4′-OH/B4′-O$^-$	9.65					1498.5	
A7-OH/A7-O$^\bullet$							386.9
B4′-OH/B4′-O$^\bullet$							368.4

[a] Experimental results: pK_a, TEAC, oxidative peak potential (E vs NHE) in tetrahydrofuran, Log P. Quantum chemical results: Dipole moment (μ), deprotonation enthalpy (DE), bond dissociation enthalpy (BDE).

isoflavonoid is somewhat amphipathic owing to its nonpolar C_{22}-carbonyl and polar daidzein moieties.

In Figure 4b, the polarization increase induced by retinylisoflavonoid is about twice that by daidzein, meaning that retinylisoflavonoid is relatively more abundant in the membrane interior. This together with the information reported by the ANS probe suggest that the Γ-like molecular dyad deposits in the lipid bilayer in an amphiphilic mode; i.e., the lipophilic C_{22}-carbonyl substituent is embedded in lipid, whereas the polar daidzein counterpart stays at the membrane surface as illustrated in Scheme 3. In this scheme, the B-ring hydroxyl B4′-OH as a less efficient radical scavenger points toward the lipid phase, while the more efficient A7-OH points toward the aqueous phase.

SCHEME 3: Schematic Model of Retinylisoflavonoid Deposition in the Lipid Bilayer



Radical Scavenging Capacity of Retinylisoflavonoid in Aqueous Solution. For daidzein, the pK_a's of A7-OH and B4′-OH are 7.47 and 9.5, respectively, a tendency which agrees with the ~50 kJ·mol^{-1} smaller DE of A7-OH (Table 1). A similar trend was found for the C-glycoside of daidzein, puerarin, whose A7-OH and B4′-OH have pK_a's of 7.2 and 9.8, respectively.[16] For retinylisoflavonoid, the considerably high pK_a of B4′-OH (11.42) is understandable in view of the $C_{14'}$=O···HO−B4′ hydrogen bonding that impedes B4′-OH deprotonation. It is known that an (iso)flavonoid with more phenolic hydroxyls bears higher TEAC.[37] However, despite the same number of phenolic hydroxyls, the TEAC of retinylisoflavonoid (0.4) is less than one fourth of that of daidzein (1.9). This is again due to the $C_{14'}$=O···HO−B4′ hydrogen bonding that hampers the hydrogen subtraction from B4′-OH as indeed confirmed by the ~89 kJ·mol^{-1} higher BDE compared to daidzein (Table 1). Compared to daidzein, the low deprotonation tendency and radical scavenging capacity do not favor its radical scavenging activities in the aqueous phase.

Oxidation potential may also be important for the radical scavenging capacity of an antioxidant.[38] Retinylisoflavonoid in tetrahydrofuran shows an oxidation potential of 1.03 V vs NHE as determined from the irreversible oxidation spike of the voltammogram. This value is lower than those of daidzein (1.22 V vs NHE) and retinal (1.40 V vs NHE) (Supporting Information S3), and retinylisoflavonoid is thus more reducing than daidzein or retinal, which is understandable in view of the rigorous coplanarity of its molecular structure along with the extended π-conjugation system. However, despite the higher electron donation tendency of neutral retinylisoflavonoid, this molecular dyad in the aqueous phase shows rather low TEAC capacity.

Antilipooxidation Activity of Retinylisoflavonoid. To initiate lipooxidation, the lipophilic azo compound AMVN incorporated in liposomes was thermolized to generate the AMVN-derived peroxyl radical ROO$^\bullet$.[9] The antilipooxidation activity

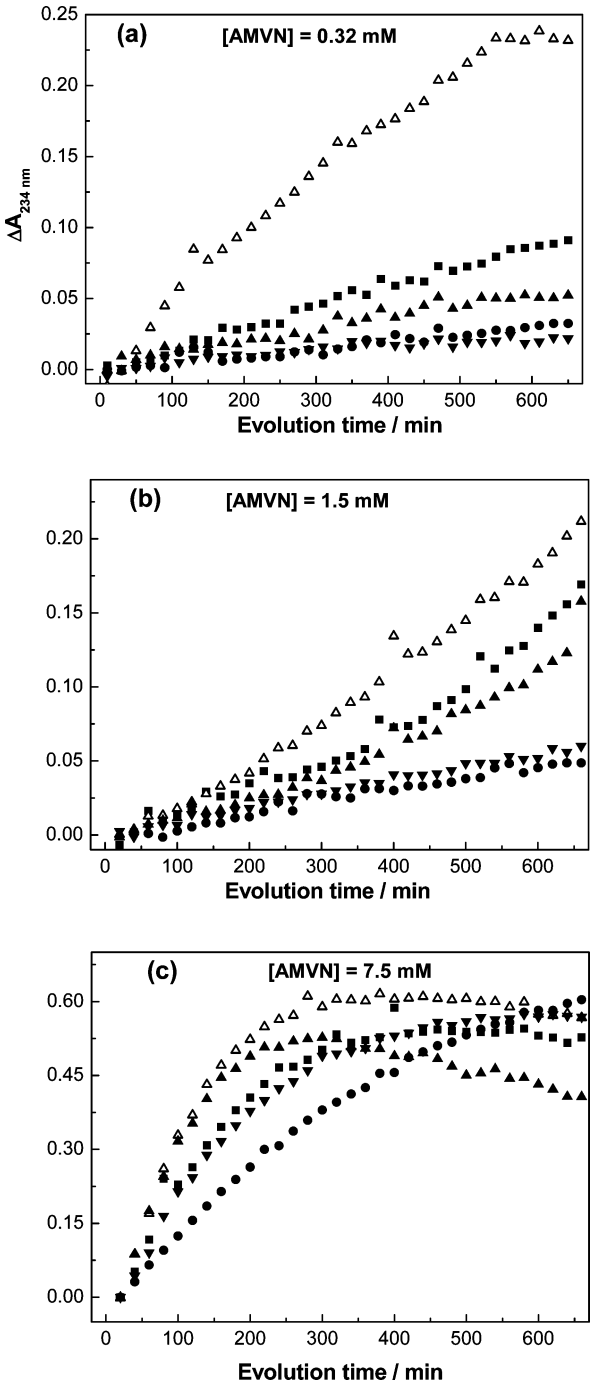


Figure 5. A_{234nm} kinetics for aqueous dispersion of liposomes with lipooxidation initiated by thermolysis of AMVN at the indicated concentrations. Each panel shows liposomes in blank (■) and those incorporated with retinylisoflavonoid (●), daidzein (△), retinol acetate (▲), and a daidzein/retinol acetate mixture (▼). Antioxidant concentration was 1.5 μM, and for the mixture, a daidzein-to-retinol acetate molar ratio of 1:1 was used.

was then measured by using the initial rate of the ΔA_{234nm} kinetics, i.e., the time evolution profile of the absorption increase at 234 nm indicative of formation of hydroperoxides of polyunsaturated lipids as primary oxidation products due to the shift in their double bonds, forming conjugated dienes. Thus, a smaller initial rate implies a higher activity of antilipooxidation. Retinol acetate and its physical mixture with daidzein were examined for comparison. Figure 5 displays the ΔA_{234nm} kinetics, and Table 2 lists the initial rates derived from the kinetics.

TABLE 2: Antilipooxidation Activity Characterized by the Initial Rate (min^{-1}) of ΔA_{234nm} Kinetics (cf. Figure 5)[a]

	AMVN concentration		
antioxidant	0.32 mM	1.5 mM	7.5 mM
retinylisoflavonoid	4.37×10^{-5}	8.77×10^{-5}	1.99×10^{-3}
retinol acetate/daidzein	3.42×10^{-5}	9.19×10^{-5}	3.97×10^{-3}
daidzein	6.92×10^{-4}	2.49×10^{-4}	8.78×10^{-3}
retinol acetate	7.88×10^{-5}	9.30×10^{-5}	7.31×10^{-3}
blank	1.46×10^{-4}	9.94×10^{-5}	5.27×10^{-3}

[a] Lipid peroxidation was induced by AMVN-derived ROO• at 43 °C (pH = 7.4).

It is seen from Figure 5 that at different AMVN concentrations daidzein shows pro- rather than antilipooxidation, which is contrary to our previous report.[24] The discrepancy may be relevant to the composition of PC for liposome preparation,[39] which is a nontrivial issue to be further verified. Hereafter, we would focus on discussing the antilipooxidation activity of retinylisoflavonoid with reference to those of daidzein, retinol acetate, and their mixture.

As seen from Figure 5 and Table 2, both retinylisoflavonoid and the retinol acetate/daidzein mixture exhibit significant antilipooxidation activity, and the activity of retinylisoflavonoid is higher than the other antioxidants irrespective of the AMVN concentration. Importantly, with respect to the other antioxidants, the performance of retinylisoflavonoid becomes more prominent with increasing AMVN concentration; e.g., the initial rate of oxidation for retinylisoflavonoid is lower than those for the other antioxidants for [AMVN] = 1.5 mM and especially for [AMVN] = 7.5 mM.

Retinol acetate at the membrane surface shows lower antilipooxidation activity than retinylisoflavonoid or the retinol acetate/daidzein mixture for low [AMVN] (Figures 5a and 5b); however, it behaves prolipooxidation for high [AMVN] (Figure 5c). It is intriguing to see that the retinol acetate/daidzein mixture shows antilipooxidation irrespective to the AMVN concentration, in contrast to the prolipooxidation behavior of individual daidzein or retinol acetate. Such s corporative effect is attributed to the interaction between lipophilic and hydrophilic antioxidants at the water−lipid interface as recently found for the combination of (iso)flavonoid and β-carotene.[16,17] Here, it is important to point out that, for the amphiphilic retinylisoflavonoid, such interaction is an intramolecular process and as such more efficient than bimolecular reactions.

4. Conclusion

We have newly synthesized a novel molecular dyad, retinylisoflavonoid, by covalently linking a retinal analogue C_{22}-aldehyde and an isoflavonoid daidzein. Compared to daidzein alone, the dyad shows considerable increase in pK_a, BDE of the isoflavonoid phenolic group, and Log P and a 4-fold decrease in TEAC and is accordingly a poorer antioxidant in homogeneous solution. Electronic absorption and resonance Raman spectroscopies of the dyad in homogeneous solution or in liposomes, as well as quantum chemical calculations, prove that retinylisoflavonoid possesses a π-electron system extending from the C_{22}-carbonyl to the B-ring of daidzein and that retinylisoflavonoid takes a coplanar and Γ-shape conformation in either solution or liposomes. The strong intramolecular hydrogen bonding, $C_{14'}$═O···HO−B4′, is crucial for the unique electronic and molecular structures as well as for the physicochemical properties of retinylisoflavonoid. It is demonstrated that retinylisoflavonoid exhibits excellent antilipooxidation activity in

liposomes with oxidation initiated in the lipid phase irrespective of the concentration of oxidative initiator and that the activity becomes more prominent under higher-level oxidative stress. On the basis of electronic and vibrational spectroscopic data, we have proposed an amphiphilic deposition scheme of retinylisoflavonoid in the lipid bilayer, where the C_{22}-carbonyl moiety is embedded in lipid and the daidzein counterpart stays at the membrane surface with the B4′-OH hydroxyl group pointing to the lipid phase (Scheme 3). The unique deposition and orientation of retinylisoflavonoid in the lipid membrane seems to facilitate its antioxidation activity observed for liposome because of the possible intramolecular, interior-to-surface radical communication across the lipid membrane. The present work provides an advanced case study for the development of a new antioxidant with optimized electronic and molecular structures for specific applications in biological or food systems.

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Supporting Information Available: Synthetic protocols of 3′(14′-apo-β-carotene-14′-carbonyl)-daidzein (retinylisoflavonoid), optimized molecular geometry of C22-aldehyde, and cyclic voltammetry. This material is available free of charge via the Internet at http://pubs.acs.org.

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