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Antioxidant Activity of a Combinatorial Library of Emulsifier−Antioxidant Bioconjugates

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A combinatorial chemistry approach was employed for the design and systematic synthesis of antioxidant−emulsifier bioconjugates to improve antioxidant activity in the interface between oil and water. A combinatorial library of 12 bioconjugates was synthesized from the phenolic antioxidants Trolox (a water-soluble α-tocopherol analogue), dihydroferulic acid, dihydrocaffeic acid, and gallic acid in combination with serine ethyl ester, serine lauryl ester, and lauroyl serine by esterification of the serine side chain or amidation, respectively. The bioconjugates were characterized by NMR and mass spectrometry, and each was tested for antioxidant activity by measuring the radical scavenging rate of 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) in methanol, the radical scavenging rate of DPPH$^{\bullet}$ in a heterogeneous solvent system, the rate of oxygen consumption in an oil-in-water emulsion with metmyoglobin initiated oxidation, and the lag phase for diene formation in unilamellar liposomes with free radical initiation in the aqueous phase; each case was compared to the antioxidant activity of the parent antioxidant. In general, the conjugates with longer chain derivatives exhibited improved antioxidative activity in heterogeneous systems, whereas no improvement was observed in homogeneous solution. The rate of oxygen consumption in oil-in-water emulsion was found to decrease with increasing octanol/water partition coefficient, which is suggested to correspond to a saturation of the water/oil interface with antioxidant bioconjugate to approach maximal protection.

KEYWORDS: Antioxidant; emulsifier; combinatorial chemistry; bioconjugates

INTRODUCTION

Deterioration of food due to lipid oxidation is an inevitable but natural process, which results in chemical and physical changes with consequences to both human health and production costs. Radical processes in biological systems such as food are mediated or may be controlled by vitamin antioxidants and various polyphenols such as anthocyanins and flavonoids occurring widespread in the plant kingdom (*1*−*3*). Lipids in most foods are dispersed in water as emulsions, and a major cause of quality deterioration in emulsion is the susceptibility of lipids to oxidation in the lipid−water interface. Emulsions are extremely sensitive toward oxidation as the total surface area of lipid droplets is large compared to the surface of bulk oils (*4*). The effectiveness of an antioxidant in bulk oils is mainly dependent on the air−oil interface affinity of the antioxidant, whereas the affinity for the oil−water interface is more important for emulsions. Nonpolar antioxidants are predominantly more effective in emulsions because they are retained in the oil droplets and/or accumulate at the oil−water interface, the location where interactions between lipid hydroperoxides and pro-oxidants such as transition metal ions originating in the aqueous phase occur. Likewise, the effectiveness of chain-breaking antioxidants in retarding lipid oxidation in oil-in-water emulsions increases as their polarity decreases or their surface activity increases, because they become more likely to be localized at the oil−water interface where oxidation occurs (*4*−*6*).

Incorporation of antioxidants into membranes or liposomes as models for cellular membranes is improved when the water-soluble antioxidant is conjugated with a longer chain ester derivative (*7*). Improvement of antioxidant activity by altering the polarity of the antioxidant (e.g., adding a lipophilic molecule to a hydrophilic antioxidant) has previously been observed only in a few studies (*8*). The antioxidant efficiency of retinyl ascorbate, which is a conjugate of the lipophilic retinoic acid and the water-soluble antioxidant ascorbic acid, is improved compared to that of the parent antioxidant (*9*). Conversely, conjugating a lipid-soluble antioxidant (α-tocopherol) with phosphatidylcholine increases the antioxidative activity in lard compared to the addition of

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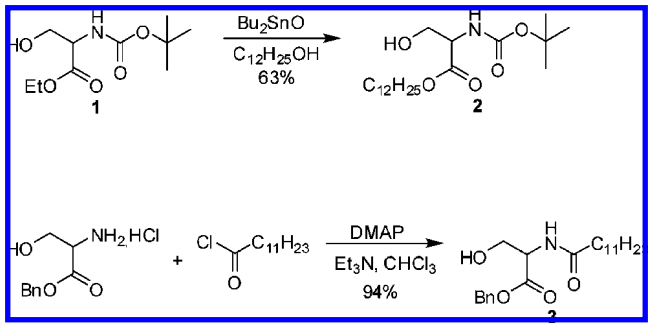
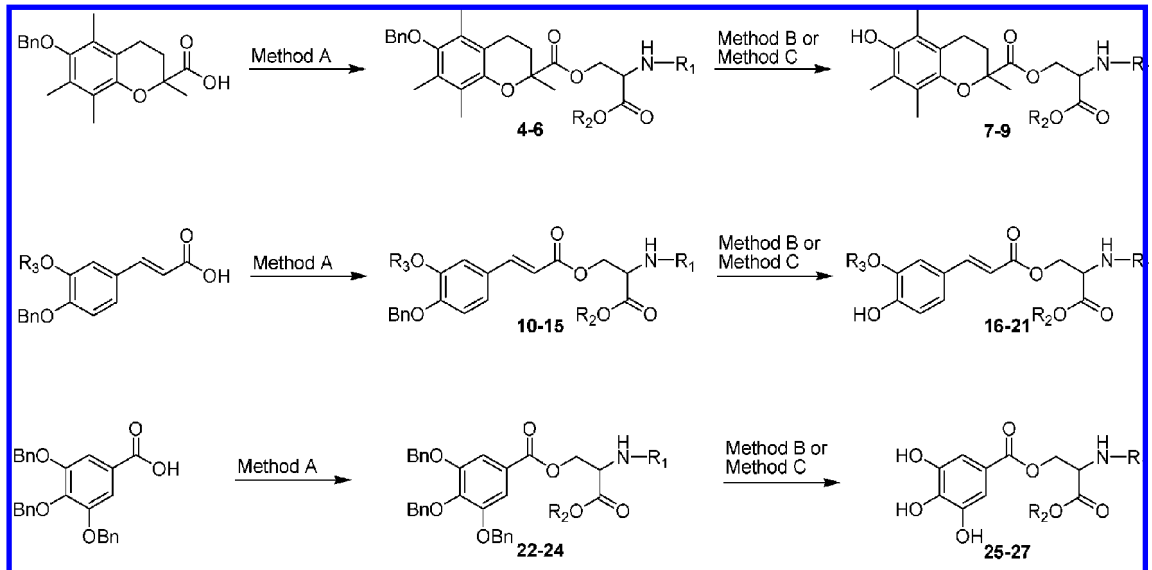


Figure 1. Synthesis of serine building blocks.

α-tocopherol and phosphatidylcholine alone or in combination. The increased antioxidative effect of α-tocopherol−phosphatidylcholine has been explained by its ability to form reverse micelles in oil with a trace amount of water, resulting in better accessibility of the radical scavenging chroman-6-ol group to the polar site, where iron-dependent initiation reactions take place (*10*). In addition, alteration of the lipid/water partition coefficient of the water-soluble antioxidants by conjugation of fatty acids of different chain lengths to form esters has been shown gradually to alter the antioxidant activity of the compound (*11, 12*). However, this effect is highly dependent on which antioxidant assay is used and in which system the antioxidant efficiency is investigated. Thus, in addition to the radical scavenging activity of an antioxidant, both its polarity and spatial interaction with lipid bilayers become important for the antioxidant activity during lipid oxidation in heterogeneous systems (*11*).

Hence, a systematic approach is required to clarify which structures for specific phenolic groups most efficiently postpone or terminate oxidation in heterogeneous systems. In the present study we have used combinatorial chemistry to establish a library of antioxidant−emulsifier bioconjugates from Trolox (a water-soluble vitamin E analogue), dihydroferulic acid, dihydrocaffeic acid, and gallic acid. Systematic variation in each of the four antioxidants was obtained by conjugation with serine ester of ethanol, serine ester of lauryl alcohol, and serine amide of lauric



4: R_1=Boc, R_2=Ethyl.

5: R_1=Boc, R_2=Lauryl.

6: R_1=Lauroyl, R_2=Benzyl.

7: R_1=H, R_2=Ethyl.

8: R_1=H, R_2=Lauryl.

9: R_1=Lauroyl, R_2=H.

10: R_1=Boc, R_2=Ethyl, R_3=Methyl.

11: R_1=Boc, R_2=Lauryl, R_3=Methyl.

12: R_1=Lauroyl, R_2=Benzyl, R_3=Methyl.

13: R_1=Boc, R_2=Ethyl, R_3=Benzyl.

14: R_1=Boc, R_2=Lauryl, R_3=Benzyl.

15: R_1=Lauroyl, R_2=Benzyl, R_3=Benzyl.

16: R_1=H, R_2=Ethyl, R_3=Methyl.

17: R_1=H, R_2=Lauryl, R_3=Methyl.

18: R_1=Lauroyl, R_2=H, R_3=Methyl.

19: R_1=H, R_2=Ethyl, R_3=H.

20: R_1=H, R_2=Lauryl, R_3=H.

21: R_1=Lauroyl, R_2=H, R_3=H.

22: R_1=Boc, R_2=Ethyl.

23: R_1=Boc, R_2=Lauryl.

24: R_1=Lauroyl, R_2=Benzyl.

25: R_1=H, R_2=Ethyl.

26: R_1=H, R_2=Lauryl.

27: R_1=Lauroyl, R_2=H.

Figure 2. Synthesis of conjugates: method A, **1**, **2**, or **3**, DCC, DMAP in CH_2Cl_2, 20 °C, 16 h; method B, (a) TFA, CH_2Cl_2, 20 °C, 1.5 h, (b) 10% Pd/C, 1 atm H_2, EtOAc, MeOH, 16 h; method C, 10% Pd/C, 1 atm H_2, EtOAc, MeOH, 16 h.

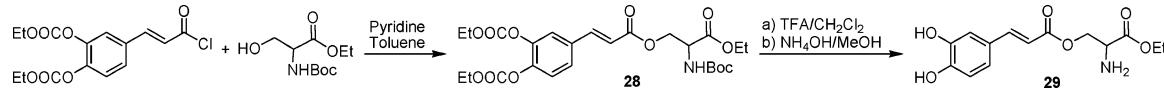


Figure 3. Alternative method resulting in a deprotected conjugate with the double bond intact.

acid to obtain both various alkyl chain lengths (ethyl vs lauryl) and different charge distributions for similar structures, that is, free amine versus free carboxylic acid for the lauryl derivatives. Serine was chosen as a versatile natural building block easily forming both ester and amide derivatives (**Figure 1**). Each of the 12 library members has been synthesized, as outlined in **Figure 2**, and the antioxidative activity evaluated by their ability to scavenging rate of the DPPH radical in methanol (MeOH) as a homogeneous solvent; increase the scavenging rate of the DPPH radical in a heterogeneous solvent system; decrease the rate of oxygen consumption in an oil-in-water emulsion; and reduce formation of conjugated dienes in liposomes and accordingly prolong the lag time for onset of oxidation. By synthesis of such emulsifier−antioxidant conjugates, which depending on structure and charge will concentrate in the lipid−water interface to various degrees, oxidation of lipids in heterogeneous systems such as emulsions, membranes, and liposomes can be investigated with respect to reaction mechanism. Throughout this study, our new bioconjugates were compared to the parent antioxidants from which they were derived. Notably, all of the bioconjugates synthesized and investigated are assembled from natural building blocks and would during digestion hydrolyze to naturally occurring compounds. This investigation represents to the best of our knowledge the first such example of the application of combinatorial chemistry and design to solve problems in food chemistry. Such combinatorial libraries should be useful for further systematic evaluation of the properties and effectiveness of antioxidants in a relevant series of assays rather than the more classical approaches based on more random selection of potential antioxidants.

MATERIALS AND METHODS

General Experimental Information. Commercially available reagents (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were used without further purification unless otherwise noted. Trolox was used as the racemic mixture. The reference compound dihydrocaffeic acid was obtained from a hydrogenation of caffeic acid. Solvents used for the synthesis were of analytical grade, dried over activated 4 Å molecular sieves when necessary (all solvents used under dry conditions had a water content of <25 ppm measured by coulometric Karl Fischer titration). Analytical TLC was performed using precoated silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) and visualized using either UV light or potassium permanganate stain. Flash chromatography was performed on silica gel 60 (0.040−0.063 mm) (Merck). HPLC was performed on a Waters 2525 system equipped with a Waters 2996 photodiode array detector and a Waters 2767 Sample Manager using a 100 mm × 19 mm i.d. XTerra prep MS C_{18} column (Waters) with a gradient of acetonitrile in Milli-Q water with a flow of 15 mL/min (Waters Corp., Milford, MA). Melting points were measured on a Reichert melting point microscope, model N254-1R (Vienna, Austria). ^{1}H and ^{13}C NMR spectroscopic data (see the Supporting Information for detailed data) were recorded on a Bruker Avance 300 (Bruker BioSpin MRI GmbH, Ettlingen, Germany) using deuterated solvents as a lock. Chemical shifts are reported in parts per million relative to the residual solvent peak (^{1}H NMR) or the solvent peak (^{13}C NMR) as the internal standard. Accurate mass determinations were performed on a Micromass LCT apparatus (Manchester, U.K.) equipped with an AP-ESI probe calibrated with Leu-Enkephalin (556.2771 g/mol). All spectrophotometric measurements were performed on a Shimadzu UV-2101PC UV−vis scanning spectrophotometer with automatic cell changer and a temperature-controlled water-jacket-regulated cell holder (Shimadzu Corp., Kyoto, Japan).

Synthesis. *N-tert-Butoxycarbonyl-L-serine Lauryl Ester,* ***2.*** *N-tert-*Butoxycarbonyl-L-serine ethyl ester, **1** (3.32 g, 14.2 mmol), lauryl alcohol (31 g, 166 mmol), and dibutyltin oxide (Bu_2SnO) (0.35 g, 1.42 mmol) were mixed and stirred under N_2 at 100 °C overnight. Lauryl alcohol was distilled off at reduced pressure (1 Torr). The residue was dissolved in CH_2Cl_2, washed with saturated $NaHCO_3$, dried (Na_2SO_4), and concentrated and purified by column chromatography [gradient of ethylacetate (EtOAc) in heptane] to give **2** as an oil: yield, 3.84 g, 72%; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{15}H_{32}NO_3$ 274.2382; found, 274.2387.

N-Lauroyl-L-serine Benzyl Ester, ***3.*** L-Serine benzyl ester hydrochloride (2.5 g, 10.8 mmol), 4-dimethylaminopyridine (DMAP) (1.32 g, 10.8 mmol), triethylamine (Et_3N) (1.5 mL, 10.8 mmol), and $CHCl_3$ (85 mL) were mixed under Ar, and lauroyl chloride (2.5 mL, 10.8 mmol) was added dropwise at room temperature. The reaction mixture was stirred overnight. Water (200 mL) was added, and the two phases were separated. The organic phase was dried (Na_2SO_4) and concentrated to a yellow solid. The solid was triturated with diethyl ether, resulting in a light yellow powder: yield, 3.86 g, 94%; mp, 74−75 °C; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{22}H_{36}NO_4$ 378.2644; found, 378.2679.

Method A. Serine building block (**1**, **2**, or **3**, 1.5 mmol), benzylated antioxidant (1.5 mmol), DMAP (0.18 g, 1.5 mmol), and dicyclohexylcarbodiimide (DCC) (0.31 g, 1.5 mmol) were dissolved in dry CH_2Cl_2 (20 mL).

The reaction mixture was stirred overnight and filtered. The filtrate was washed with 10% $KHSO_4$, dried, and concentrated. The residue was crystallized from ethanol (EtOH) to give white crystals.

Method B. Fully protected conjugate (0.76 mmol) and trifluoroacetic acid (TFA) (1 mL) were dissolved in dry CH_2Cl_2 (4 mL), and the reaction mixture was stirred for 1.5 h. The solvents were evaporated, and the residue was dissolved in EtOAc (40 mL) and MeOH (10 mL). Ten percent Pd/C was added, and the mixture was stirred under H_2 (1 atm) overnight. The mixture was filtered through silica gel, which was washed with MeOH. The filtrate was concentrated and the residue purified by HPLC.

Method C. Fully benzylated conjugate (0.24 mmol) was dissolved in EtOAc (20 mL) and MeOH (5 mL). Ten percent Pd/C was added, and the mixture was stirred under H_2 (1 atm) overnight. The mixture was filtered through silica gel, which was washed with MeOH. The filtrate was concentrated and the residue purified by HPLC.

O-(6-Benzyloxy-2,5,7,8-tetramethylchroman-2-carbonyl)-N-tert-butoxycarbonyl-L-serine Ethyl Ester, ***4****. Method A.* The residue was purified by flash chromatography (EtOAc/heptane) followed by HPLC to give **4** as a viscous oil: yield, 17%; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{26}H_{34}NO_6$ 456.2386; found, 456.2393.

O-(6-Benzyloxy-2,5,7,8-tetramethylchroman-2-carbonyl)-N-tert-butoxycarbonyl-L-serine Lauryl Ester, ***5****. Method A.* The residue was purified by flash chromatography (EtOAc/heptane) to give **5** as an oil: yield, 65%; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{36}H_{54}NO_6$ 596.3951; found, 596.3979.

O-(6-Benzyloxy-2,5,7,8-tetramethylchroman-2-carbonyl)-N-lauroyl-L-serine Benzyl Ester, ***6****. Method A.* The residue was purified by flash chromatography to give an oil: yield, 60%; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{43}H_{58}NO_7$ 700.4213; found, 700.4272.

O-(6-Hydroxy-2,5,7,8-tetramethylchroman-2-carbonyl)-L-serine Ethyl Ester, ***7****. Method B:* orange oil: yield, 25%; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{19}H_{28}NO_6$ 366.1917; found, 366.1906.

O-(6-Hydroxy-2,5,7,8-tetramethylchroman-2-carbonyl)-L-serine Lauryl Ester, ***8****. Method B:* yield, 100%; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{29}H_{48}NO_6$ 506.3476; found, 506.3473.

O-(6-Hydroxy-2,5,7,8-tetramethylchroman-2-carbonyl)-N-lauroyl-L-serine, ***9****. Method C:* yield, 70%; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{29}H_{46}NO_7$ 520.3274; found, 520.3252.

*O-(4-Benzyl-3-methylcaffeoyl)-N-tert-butoxycarbonyl-L-serine Ethyl Ester, **1**. Method A:* yield, 82%; mp, 99−100 °C; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{22}H_{26}NO_6$ 400.1760; found, 400.1784.

*O-(4-Benzyl-3-methylcaffeoyl)-N-tert-butoxycarbonyl-L-serine Lauryl Ester, **11**. Method A:* yield, 75%; mp, 81−82 °C; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{37}H_{54}NO_8Na$ 663.3747; found, 663.3705.

*O-(4-Benzyl-3-methylcaffeoyl)-N-lauroyl-L-serine Benzyl Ester, **12**. Method A:* yield, 100%; mp, 87−88 °C; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{39}H_{50}NO_7$ 644.3587; found, 644.3607.

*O-(3,4-Dibenzylcaffeoyl)-N-tert-butoxycarbonyl-L-serine Ethyl Ester, **13**. Method A:* yield, 75%; mp, 99−100 °C; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{33}H_{40}NO_8$ 578.2754; found, 578.2745.

*O-(3,4-Dibenzylcaffeoyl)-N-tert-butoxycarbonyl-L-serine Lauryl Ester, **14**. Method A.* The residue was purified by flash chromatography (EtOAc/heptane) to give **14** as an oil: yield, 27%; mp, 99−100 °C; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{38}H_{50}NO_6$ 616.3638; found, 616.3619.

*O-(3,4-Dibenzyloxycinnamoyl)-N-lauroyl-L-serine Benzyl Ester, **15**. Method A:* yield, 89%; mp, 56−60 °C; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{45}H_{54}NO_7$ 720.3900; found, 720.3970.

*O-(4-Hydroxy-4-methoxyphenyl-3-propanoyl)-L-serine Ethyl Ester, **16**. Method B.* The intermediate was isolated and characterized by NMR: yield, 83%; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{15}H_{22}NO_6$ 312.1447; found, 312.1477.

*O-(4-Hydroxy-4-methoxyphenyl-3-propanoyl)-L-serine Lauryl Ester, **17**. Method B:* overall yield, 21%; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{25}H_{42}NO_6$ 452.3012; found, 452.2987.

*O-(4-Hydroxy-4-methoxyphenyl-3-propanoyl)-N-lauroyl-L-serine, **18**. Method C:* yield, 98%; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{25}H_{40}NO_7$ 466.2805; found, 466.2780.

*O-(3,4-Dihydroxyphenyl-3-propanoyl)-L-serine Ethyl Ester, **19**. Method B:* overall yield, 100%; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{14}H_{20}NO_6$ 298.1291; found, 298.1283.

*O-(3,4-Dihydroxyphenyl-3-propanoyl)-L-serine Lauryl Ester, **20**. Method B:* overall yield, 34%; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{24}H_{40}NO_6$ 438.2856; found, 438.2852.

*O-(3,4-Dihydroxyphenyl-3-propanoyl)-N-lauroyl-L-serine, **21**. Method C:* yield, 31%; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{24}H_{38}NO_7$ 452.2648; found, 452.2618.

*O-Tribenzylgalloyl-N-tert-butoxycarbonyl-L-serine Ethyl Ester, **22**. Method A:* yield, 62%; mp, 98−100 °C; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{33}H_{34}NO_7$ 556.2335; found, 556.2284; calibrated with Fmoc-Cys(Acm)-OH (414.1249 g/mol).

*O-Tribenzylgalloyl-N-tert-butoxycarbonyl-L-serine Lauryl Ester, **23**. Method A:* yield, 52%; mp, 81−82 °C; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{43}H_{54}NO_7$ 696.3900; found, 696.3879.

*O-Tribenzylgalloyl-N-lauroyl-L-serine Benzyl Ester, **24**. Method A:* yield, 56%; mp, 106−108 °C; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{50}H_{58}NO_8$ 800.4162; found, 800.4159.

*O-Galloyl-L-serine Ethyl Ester, **25**. Method B:* overall yield, 75%; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{12}H_{16}NO_7$ 286.0927; found, 286.0955.

*O-Galloyl-L-serine Lauryl Ester, **26**. Method B:* overall yield, 60%; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{22}H_{36}NO_7$ 426.2492; found, 426.2456.

*O-Galloyl-N-lauroyl-L-serine, **27**. Method C:* yield, 86%; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{22}H_{34}NO_8$ 440.2284; found, 440.2256.

*O-(3,4-Diethoxycarbonylcaffeoyl)-N-tert-butoxycarbonyl-L-serine Ethyl Ester, **28**.* ***N-tert***-Butoxycarbonyl-L-serine ethyl ester (0.48 g, 2.04 mmol) was dissolved in pyridine (5 mL) under N_2 and cooled on ice. 3,4-Diethoxycarbonylcaffeoyl chloride (*28*)(0.70 g, 2.04 mmol) was dissolved in toluene (5 mL) and added. After 10 min at 0 °C, the reaction mixture was stirred at room temperature overnight. The solvents were evaporated and coevaporated with toluene twice. The residue was purified by flash chromatography (gradient of EtOAc in heptane) to give **28** as an oil: 0.41 g; yield, 37%; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{20}H_{26}NO_{10}$ 440.1557; found, 440.1533.

*O-Caffeoyl-L-serine Ethyl Ester, **29***(**Figure 3**). **28** (0.41 g, 0.76 mmol) was dissolved in CH_2Cl_2 (4 mL) and TFA (1 mL), and the reaction mixture was stirred for 3 h. The solvents were evaporated, and the residue was dissolved in MeOH (14.5 mL) and NH_3 (aq, 25%). The reaction mixture was stirred for 1 h. The solvents were evaporated, and the residue was freeze-dried overnight. The residue was purified by flash chromatography (EtOAc/heptane) and then by HPLC to give 44 mg of amorphous powder: overall yield, 20%; TOF ESMS (*m/z*) $[M + H]^+$ calcd for $C_{14}H_{18}NO_6$ 296.1134; found, 296.1115.

Antioxidant and Radical Scavenging Assays. *Chemicals.* Horse heart myoglobin (MMb, type III), methyl linoleate, and Tween 20 were obtained from Sigma (St. Louis, MO). 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH$^\bullet$) and L-α-phosphatidylcholine were from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). 2,2′-Azobis(2-amidinopropane) (AAPH) was from Wako Chemicals, Richmond, VA. Trolox (**A0**), ferulic acid (**B0′**), dihydroferulic acid (**B0**), caffeic acid (**C0′**), dihydrocaffeic acid (**C0**), and gallic acid (**D0**) were also tested as reference compounds. The emulsifier−antioxidant bioconjugates synthesized and examined are all shown in **Figure 4**.

Radical Scavenging of DPPH$^\bullet$ in MeOH. Scavenging of DPPH$^\bullet$ by the antioxidants was followed at 515 nm (25 °C) in MeOH (*13*). DPPH$^\bullet$ (0.1 mM) and antioxidant (0.98 mM) were mixed directly in disposable cuvettes, and absorbance at 515 nm was measured every 0.1 s for 60 s. The measurements were performed in triplicate. With large excess of antioxidant, the radical scavenging was expected to follow pseudo-first-order kinetics. However, as some deviation from first-order kinetics was seen for certain bioconjugates, probably due to secondary reactions following the initial scavenging reactions, the initial rate of reaction (v_i in mol/L/s) was determined from the slope of the initial linear part of the curve in the plot of [DPPH] versus time by division with the molar absorptivity of DPPH of $\varepsilon = 1.25 \times 10^4$ L/mol/cm.

Radical Scavenging of DPPH$^\bullet$ in a Heterogeneous Solvent System. All reactions were performed in sodium phosphate buffer (pH 7.0, 5 mM) with 4% (w/v) Tween 20 (*14*). DPPH$^\bullet$ was dissolved in EtOH and then diluted 40 times in 5 mM sodium phosphate buffer (pH 7.0) with 4% (w/v) Tween 20 to give a concentration of 0.125 mM. The antioxidants were dissolved directly in buffer with 4% (w/v) Tween 20 to give a concentration of 5 mM. DPPH (0.1 mM) and antioxidant (1.0 mM) were mixed carefully in disposable cuvettes, and absorbance at 515 nm was measured every 0.1 s for 60 s.

In the case of Trolox and its derivatives (group A compounds) it was necessary to dissolve the antioxidants in EtOH and then dilute the solution in buffer with 4% (w/v) Tween 20. The scavenging of DPPH$^\bullet$ by group A compounds was much faster than that shown by the rest of the investigated compounds, and the experiments were performed using a DX-17MV stopped-flow spectrofluorometer (Applied Photophysics, London, U.K.). Each syringe was filled with 0.2 mM DPPH$^\bullet$ in 5 mM sodium phosphate buffer (pH 7.0) with 4% (w/v) Tween 20 prepared from a 3 mM DPPH in EtOH solution and 2.0 mM antioxidant in 5 mM sodium phosphate buffer (pH 7.0) with 4% (w/v) Tween 20 prepared from a solution of 5 mM antioxidant in EtOH giving the same reaction concentrations as above (0.10 mM DPPH$^\bullet$ and 1.0 mM antioxidant). Absorbance at 515 nm was measured every 0.0025 s for 1.000 s. The measurements were performed in triplicate. The initial rate of reaction was calculated as for DPPH$^\bullet$ scavenging in MeOH.

Oxygen Consumption. Oxygen consumption was measured as described by Hu and Skibsted (*15*). Methyl linoleate was mixed with Tween 20 and air-saturated thermostated (25 °C) phosphate buffer (pH 6.8), and 20 μL of antioxidant solution (1.0 mM) in MeOH was added to give a final concentration of antioxidant of 7.9 μM. The oxidation was initiated by the addition of 25 μL of 0.20 mM MMb aqueous solution, and immediately thereafter measurements of the oxygen consumption were started. As a positive blank 20 μL of MeOH was used instead of antioxidant solution, and as a negative blank no antioxidant or no MMb was added. The relative oxygen consumption was measured with oxygen microsensors (Unisense, Aarhus N, Denmark) and recorded at time intervals of 10 s for 10 min. Profix Software v. 3.05 (Unisense, Denmark) was used for data handling. The initial rate of consumption [$V(O_2)$] was calculated from the slope of the oxygen consumption versus time curve in the linear region. All measurements were performed in duplicate. The influence of each of the antioxidants on the initial rate of oxygen consumption was expressed as an antioxidative index relative to the rate in the absence of antioxidant according to eq 1.

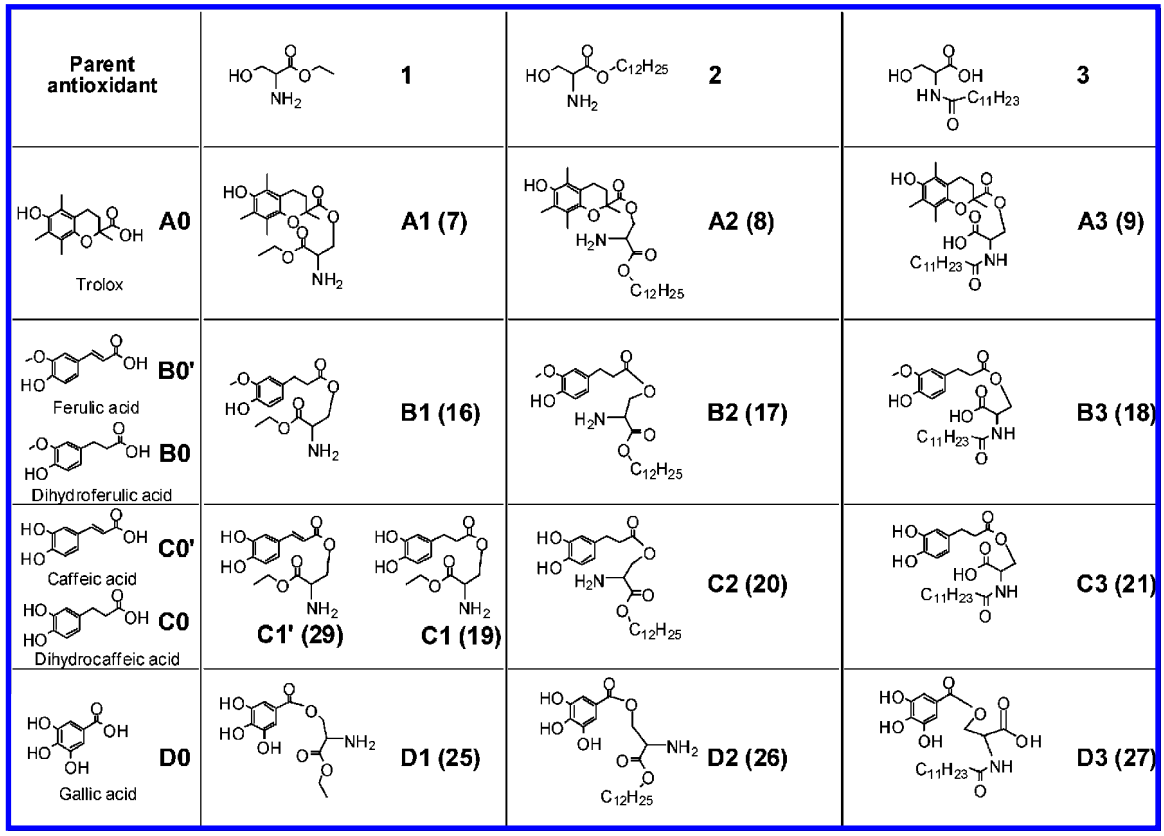


Figure 4. Emulsifier−antioxidant bioconjugate library: overview of all the compounds tested in the antioxidant assays (the serine building blocks were not tested). The numbers in parentheses refer to the compound numbering under Materials and Methods.

$$I_{\text{oxygen}} = 1 - \frac{V(O_2) \text{ with antioxidant}}{V(O_2) \text{ without antioxidant}} \quad (1)$$

Preparation of Liposomes. Liposomes were prepared as described by Roberts and Gordon (*16*). Briefly, 1.35 mg of L-α-phosphatidylcholine (PC) from soybean in 2 mL of $CHCl_3$ and 15 μL of antioxidant (1 mM) in MeOH (or 15 μL of MeOH for blank) were transferred to a round-bottom flask, which was covered with aluminum foil, and the solvents were evaporated at reduced pressure (water bath at 30 °C). Nitrogen was introduced when atmospheric pressure was established, and 10 mL of 10 mM sodium phosphate buffer (pH 7.4) was added. The content of the flask was vortexed for 10 min followed by 30 s in an ultrasonic bath. This produced a homogeneous white suspension. Large unilamellar liposomes were obtained by transferring the liposome suspension to a small volume extrusion device (Liposofast Basic, Aventin, Mannheim, Germany). The suspension was passed 20 times through a double layer of polycarbonate membranes (100 nm pore size).

Peroxidation of Liposomes. The liposome suspension (2.5 mL) was pipetted into a quartz cuvette with a stopper and incubated for 10 min at 37 °C in the spectrophotometer (Shimadzu UV-2101PC UV−vis scanning spectrophotometer). Lipid peroxidation was initiated by the addition of 25 μL of 75 mM AAPH in 10 mM sodium phosphate buffer (pH 7.4). The cuvettes were sealed with the stopper and inverted three times. The absorbance was recorded at 234 nm every 10 min against a blank of sodium phosphate buffer (pH 7.4). Each antioxidant was tested in duplicate. The contribution of AAPH in buffer to the absorbance at 234 nm was subtracted from the absorbance obtained for the antioxidants. The lag phase was measured as the time in minutes to the point where a tangent to the propagation phase intercepted the *x*-axis (*17*).

Statistical Analysis. Results were analyzed statistically by analysis of variance using the general linear models (GLM) procedure in the SAS 9.1 package, SAS Institute, Inc. Antioxidant was included as a fixed effect. In some cases it was necessary to transform data logarithmically to obtain homogeneous variance, and data were subsequently analyzed as described above. Means were used to compare differences, and least-squares differences (LSD) were applied to compare the mean values of the tested antioxidants.

RESULTS AND DISCUSSION

Synthesis. Three different amino acid building blocks were selected representing a short-chain and a long-chain serine ester as well as a long-chain serine amide. The first L-serine building block, *N-tert*-butoxycarbonyl-L-serine ethyl ester, **1** (*18*), was synthesized from L-serine ethyl ester and di-*tert*-butyl pyrocarbonate according to standard procedures (*19*). Transesterification of **1** to yield the corresponding lauryl ester **2** was done using dibutyltin oxide as catalyst using a method hitherto described for methanol, ethanol, and *n*-butanol as well as allyl, isopropyl, and benzyl alcohol (*18*). The third serine building block, **3**, was obtained by acylation of L-serine benzyl ester hydrochloride in the presence of triethylamine and DMAP using standard procedures (*20*).

Four different well-established antioxidants were converted into the appropriately protected building blocks required for the subsequent formation of the bioconjugates. Racemic 6-benzyloxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (*21*), benzylferulic acid (*22*), dibenzylcaffeic acid (*23*), and tribenzylgallic acid (*24*) were all synthesized using methods described by Moulin et al. (*21*). Then the benzyl ethers of the four antioxidants were coupled with the three serine building blocks (**1**−**3**) using DCC and DMAP in DCM, resulting in 12 different protected antioxidant−emulsifier conjugates. The yields varied from 17 to 100%.

Because a racemic mixture of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used and serine was used, as the L-isomer, the three conjugates containing the Trolox building block were obtained as a mixture of the two diaste-

Table 1. Overview of All Results in the Four Antioxidant or Radical Scavenging Assays[a]

antioxidant	v_i in MeOH[a] (10^{-6} L/mol/s)	v_i in micelles (10^{-6} L/mol/s)	I_{oxygen}	lag phase (min)	ClogP
A0	4.6 ± 0.5 b	490 ± 10 a	0.75 ± 0.08 b	67 ± 6 ns	3.0888
A1	4.2 ± 0.2 bc	220 ± 20 d	0.97 ± 0.03 a	67 ± 3 ns	3.1349
A2	18 ± 3 a	378 ± 8 b	0.96 ± 0.04 a	88 ± 24 ns	8.4249
A3	3.5 ± 0.1 c	340 ± 10 c	0.95 ± 0.01 a	81 ± 4 ns	7.8394
B0′	1.1 ± 0.2 a	2.1 ± 0.1 a	0.45 ± 0.15 b	172 ± 33 a	1.4212
B0	0.29 ± 0.04 c	1.86 ± 0.04 b	0.50 ± 0.03 b	79 ± 4 c	1.0852
B1	0.80 ± 0.07 a	1.51 ± 0.04 d	0.46 ± 0.05 b	28 ± 2 d	1.1523
B2	0.115 ± 0.009 d	1.8 ± 0.1 bc	0.96 ± 0.03 a	80 ± 3 c	6.4423
B3	0.55 ± 0.02 b	1.63 ± 0.07 cd	0.96 ± 0.02 a	130 ± 7 b	5.8568
C0′	4.1 ± 0.4 a	3.8 ± 0.2 c	0.49 ± 0.09 c	152 ± 9 b	0.975
C1′	3.1 ± 0.5 b	3.83 ± 0.09 c	0.71 ± 0.01 b	107 ± 4 e	0.7061
C0	4.9 ± 0.4 a	4.9 ± 0.6 b	0.49 ± 0.02 c	177 ± 3 a	0.639
C1	1.4 ± 0.7 c	3.5 ± 0.2 c	0.58 ± 0.04 c	66 ± 3 e	0.6475
C2	1.2 ± 0.2 c	10.0 ± 0.7 a	0.995 ± 0.002 a	131 ± 10 c	5.9961
C3	1.5 ± 0.1 c	9.2 ± 0.5 a	0.96 ± 0.01 a	144 ± 3 bc	5.4106
D0	3.9698 ± 0.0004 a	4.8 ± 0.4 c	0.34 ± 0.07 c	47 ± 1 ns	0.42549
D1	3.3 ± 0.2 b	4.0 ± 0.2 d	0.526 ± 0.005 b	75 ± 26 ns	0.33253
D2	4.01 ± 0.06 a	10.8 ± 0.6 a	0.95 ± 0.01 a	66 ± 9 ns	5.62253
D3	1.055 ± 0.007 c	6.7 ± 0.2 b	0.87 ± 0.04 a	74 ± 4 ns	5.03703

[a] The initial rates (v_i) in MeOH and micelles were determined for DPPH• scavenging, I_{oxygen} was determined from electrochemical oxygen consumption measurements, and lag phase was obtained from diene measurements in the liposome assay. The calculated 1-octanol/water partition coefficient (ClogP) is also given for all compounds. Values are given as mean ± standard deviation (n = minimum 2). Within the same antioxidant group, values bearing different letters are significantly different ($P < 0.05$) within the same column (ns, nonsignificant).

reoisomers. No attempts to separate the diastereoisomers were performed. This inherently complicates the NMR spectra, because the two isomers may give different chemical shift values.

Initially, we attempted to deprotect the compounds in one step using thioanisole and TFA (*25*), but these conditions resulted in several byproducts, and in the case of the caffeic acid and ferulic acid derivatives, the conditions led to a modification/reaction at the double bond (the structures of the byproduct were not determined). Instead, a number of different standard methods for the selective removal of benzyl groups in the presence of double bonds were attempted, but, unfortunately, none of these proved to be successful. Therefore, it was finally decided to remove the benzyl groups by hydrogenolysis, which concomitantly resulted in reduction of the double bonds in caffeic acid and ferulic acid. It has been shown that the antioxidative effects of caffeic acid and ferulic acid and their reduced analogues, respectively, are comparable (*26, 27*), and accordingly we expected that it would not have a significant impact on the results in the antioxidant assays.

In the case of compounds **A3**−**D3**, which contained only benzyl groups as protecting groups, the deprotection was done in a single step (method C) involving standard hydrogenation conditions at atmospheric pressure using 10% Pd/C in EtOAc and MeOH. The yields ranged from 31 to 98%. In the case of compounds **A1**−**D1** and **A2**−**D2**, containing both a Boc group and a benzyl group, the protecting groups were removed in two steps (method B): first, the Boc group was removed using TFA in CH_2Cl_2 (20% v/v). Normally, the Boc-deprotected compounds were used in the next deprotection step without further purification, but in one case (the deprotection of **10**) the intermediate was isolated and characterized by ^{1}H NMR and ^{13}C NMR to validate the method. Finally, the benzyl groups were removed using the same method as described under Method C. The overall yields ranged from 21 to 100% for the two deprotection steps.

The importance of the double bond in caffeic acid derivatives was assessed by synthesizing one caffeic acid conjugate using a method that would leave the double bond untouched. This included the use of ethyl carbonate as the phenol protecting group (*28*). The antioxidant and the serine building block (**1**) were coupled via the acid chloride (*28*) in toluene and pyridine to give the conjugate **28** in moderate yield (37%). Deprotection was done in two steps: first, a removal of the Boc group as described above followed by hydrolysis of the carbonate in aqueous ammonia and MeOH, resulting in **29** in moderate overall yield (20%).

Antioxidant and Radical Scavenging Activity of Bioconjugates. All results of the antioxidant or radical scavenging assays are summarized in **Table 1**. The octanol/water partition coefficient (ClogP) of each bioconjugate and parent antioxidant was calculated using ChemDraw 10.0 (CambridgeSoft Corp., Cambridge, MA), and these values are also included in **Table 1**.

Scavenging of DPPH• in MeOH. The concentration of DPPH was chosen to obtain an intermediate rate of scavenging of DPPH• for most of the antioxidants, with the ferulic acid derivatives being slowest (**Figures 5** and **6**) and Trolox derivatives being fastest (**Figure 6**). Ferulic acid scavenged DPPH• with the fastest rate compared to the other group **B** compounds including dihydroferulic acid. However, conjugating dihydroferulic acid with serine ethyl ester (**1**) and lauroyl serine (**3**) improved the scavenging rate from 2.9×10^{-7}/s for **B0** to 8.0×10^{-7} and 5.5×10^{-7}/s for **B1** and **B3**, respectively.

The correlation between the initial rate (v_i) for scavenging of DPPH• in MeOH and the type of conjugate is shown in **Figure 6**. For group **A** compounds, only **A2** exhibited a faster scavenging rate than Trolox. Negative or no improvement of conjugating the antioxidants on the rate of scavenging DPPH• was found for groups **C** and **D**. Improvement of the radical scavenging activity of the conjugates was expected in heterogeneous systems as emulsions containing interfaces. Hence, the lack of improvement of the radical scavenging activity in a homogeneous solution such as MeOH could be expected. The presence of the double bond in caffeic acid did not seem to have any significant positive effect on the scavenging rate of DPPH• as the scavenging rate by dihydrocaffeic acid was slightly higher than the scavenging rate by caffeic acid.

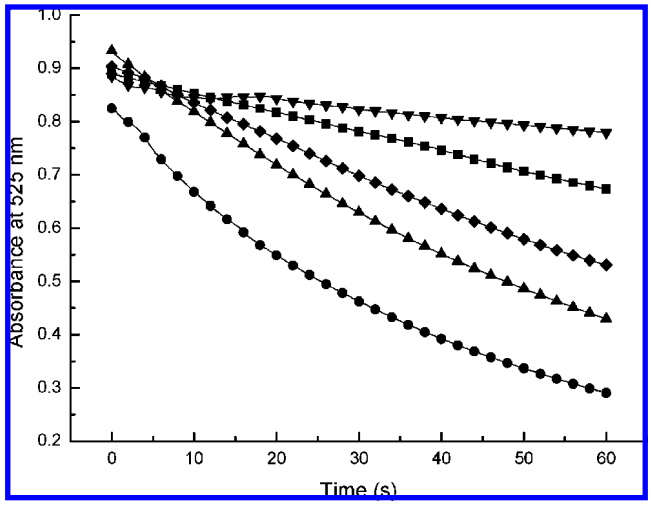


Figure 5. Scavenging of DPPH• (0.1 mM) in MeOH at 25 °C by 0.98 mM of ferulic acid (●), dihydroferulic acid (■), **B1** (▲), **B2** (▼), or **B3** (◆).

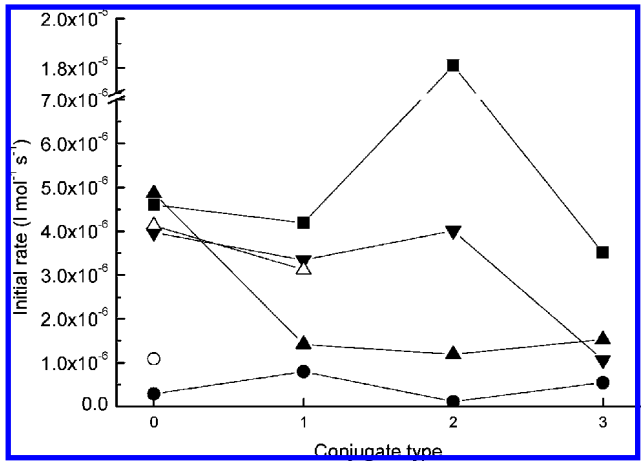


Figure 6. Correlation between the initial rate (v_i) for scavenging of DPPH• in MeOH and type of conjugate for each antioxidant and its derivatives. **A** group (■): **A0** (Trolox), **A1**, **A2**, and **A3**. **B** group (●): **B0** (dihydroferulic acid), **B1**, **B2**, and **B3**. **B0′** (○) (ferulic acid). **C** group (▲): **C0** (dihydrocaffeic acid), **C1**, **C2**, and **C3**. **C′** group (△): **C0′** (caffeic acid) and **C1′**. **D** group (▼): **D0** (gallic acid), **D1**, **D2**, and **D3**.

Scavenging of DPPH• in a Heterogeneous Solvent. The compounds were designed to improve the antioxidative effect in systems with interfaces, and therefore the DPPH assay was repeated in an aqueous solution, where the radicals are solublized in micelles. DPPH was dissolved in a small volume of EtOH and subsequently diluted in phosphate buffer (pH 7.0) with Tween 20 to avoid precipitation of DPPH. Similarly, Trolox and its derivatives were dissolved in EtOH and subsequently diluted in buffer as these compounds are more lipophilic than the rest of the conjugates. Furthermore, scavenging of DPPH• by Trolox and its derivatives was fast, and it was necessary to follow the reaction using a stopped-flow spectrofluorometer.

Comparison of the rate of DPPH• scavenging in this heterogeneous solvent in the presence of gallic acid and its derivatives showed that conjugating gallic acid with serine lauryl ester (**2**) or lauroyl serine (**3**) increased the scavenging rate (**Figure 7**). However, conjugation of gallic acid with serine ethyl ester (**1**) decreased the DPPH• scavenging rate, indicating an expected reduction of the antioxidative activity.

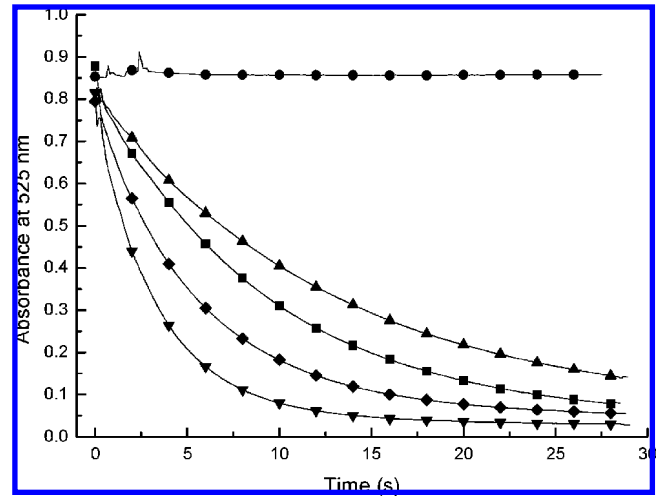


Figure 7. Scavenging of DPPH• (0.1 mM) in emulsion with 0.1 M sodium phosphate buffer (pH 7.0) and 4% (w/v) Tween 20 at 25 °C by 1.0 mM of gallic acid (■), **D1** (▲), **D2** (▼), and **D3** (◆) and in absence of antioxidant (●).

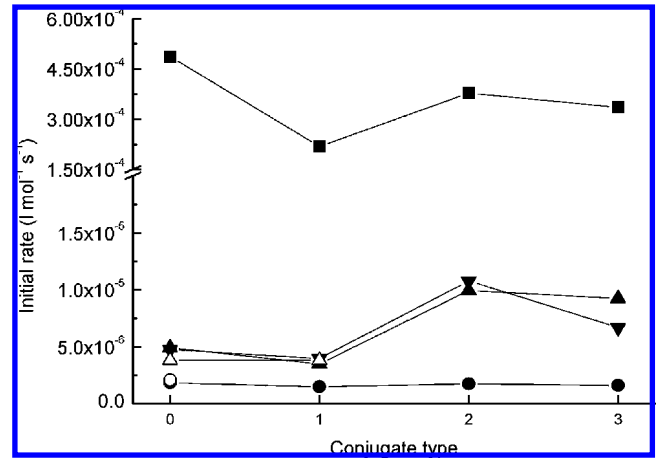


Figure 8. Correlation between the initial rate (v_i) for scavenging of DPPH• in aqueous micelles (pH 7.0) and type of conjugate for each antioxidant and its derivatives. **A** group (■): **A0** (Trolox), **A1**, **A2**, and **A3**. **B** group (●): **B0** (dihydroferulic acid), **B1**, **B2**, and **B3**. **B0′** (○) (ferulic acid). **C** group (▲): **C0** (dihydrocaffeic acid), **C1**, **C2**, and **C3**. **C′** group (△): **C0′** (caffeic acid) and **C1′**. **D** group (▼): **D0** (gallic acid), **D1**, **D2**, and **D3**.

The correlation between v_i in emulsion and the type of conjugate is illustrated in **Figure 8**. A similar effect of conjugating dihydrocaffeic acid (**C0**) as for gallic acid was observed. In contrast, no effect of conjugating the **B** group compounds on the scavenging rate of DPPH• was found, and the conjugates of Trolox (group **A** compounds) exhibited slower DPPH• scavenging than Trolox. The presence of the double bond in ferulic acid seems to have a slightly positive influence on the scavenging rate of DPPH• as a small, but significant, difference was observed in v_i between **B0** and **B0′**. In contrast, the DPPH• scavenging rate observed in the presence of dihydrocaffeic acid (**C0**) was higher than that in the presence of caffeic acid (**C0′**), indicating that removal of the double bond in caffeic acid improved the radical scavenging activity significantly. These observations are similar to the results obtained for DPPH• scavenging in homogeneous MeOH.

Oxygen Consumption. Initially, the oxygen consumption at different concentrations of antioxidant was measured. The final concentration of 7.9 μM antioxidant was chosen because it gave an intermediate inhibition of the oxidation of methyl linoleate.

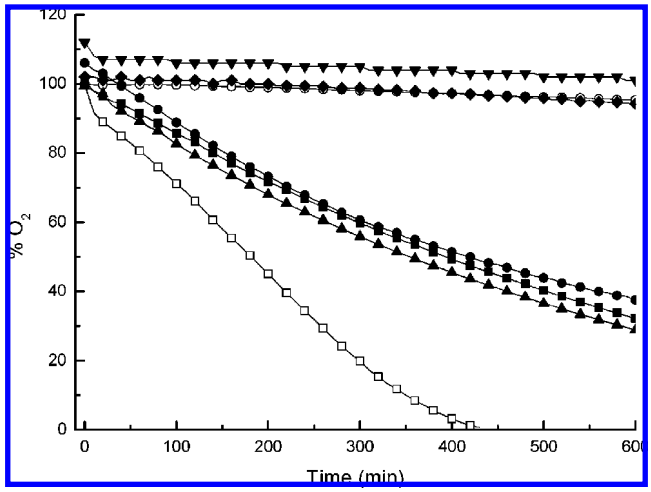


Figure 9. Oxygen consumption in an emulsion of methyl lineolate, Tween 20, and phosphate buffer (25 °C, pH 6.8) with initiation of oxidation by MMb in the presence of 7.9 μM of ferulic acid (●), dihydroferulic acid (■), **B1** (▲), **B2** (▼), and **B3** (◆) in the absence of antioxidant (□) or both antioxidant and MMb (○).

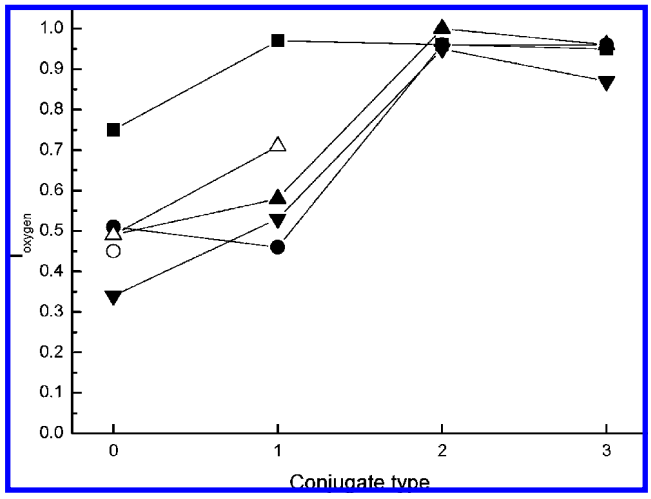


Figure 10. Correlation between I_{oxygen} and type of conjugate for each antioxidant and its derivatives. **A** group (■): **A0** (Trolox), **A1**, **A2**, and **A3**. **B** group (●): **B0** (dihydroferulic acid), **B1**, **B2**, and **B3**. **B0′** (○) (ferulic acid). **C** group (▲): **C0** (dihydrocaffeic acid), **C1**, **C2**, and **C3**. **C′** group (△): **C0′** (caffeic acid) and **C1′**. **D** group (▼): **D0** (gallic acid), **D1**, **D2**, and **D3**.

In the presence of the compounds **B0′** (ferulic acid), **B0** (dihydroferulic acid), and **B1**, an inhibition of the oxygen consumption was found compared to the measurement without antioxidant (**Figure 9**). The oxidative indices (I_{oxygen}, **Table 1**) for these compounds were similar (0.45, 0.50, and 0.46, respectively), which indicates that conjugation with serine ethyl ester (**1**) does not improve the antioxidative activity of dihydroferulic acid. In contrast, there was a remarkable reduction in oxygen consumption in the presence of the conjugates **B2** and **B3** (I_{oxygen} = 0.96 and 0.96, respectively), indicating a significant effect on the antioxidative activity of conjugation with longer chain building blocks.

A similar tendency was found for group **A**, **C**, and **D** conjugates as illustrated in **Figure 10** by correlation between I_{oxygen} and type of conjugate. In most cases (groups **A**, **C′**, **C**, and **D**) an improvement of antioxidative activity was found for all conjugates as compared to the parent antioxidants, and the most significant improvements were generally seen for the longer chain derivatives (**2** and **3**). No notable difference between the antioxidative activity of the parent antioxidants with double bonds (**B0′** and **C0′**) and the corresponding reduced compounds (**B0** and **C0**) was found.

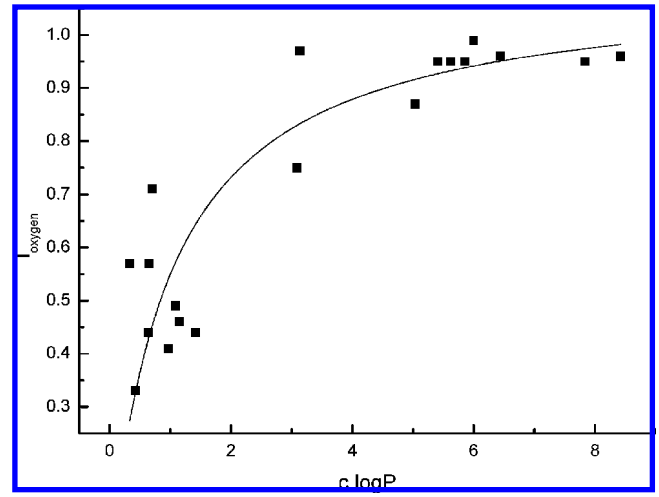


Figure 11. Oxygen consumption index plotted as a function of the calculated 1-octanol/water partition coefficient (ClogP). Curve is calculated by nonlinear regression analysis according to the empiric equation $I_{oxygen} = k(\text{ClogP})/[1 + (\text{ClogP})]$ to yield the parameter $k = 1.10 \pm 0.04$.

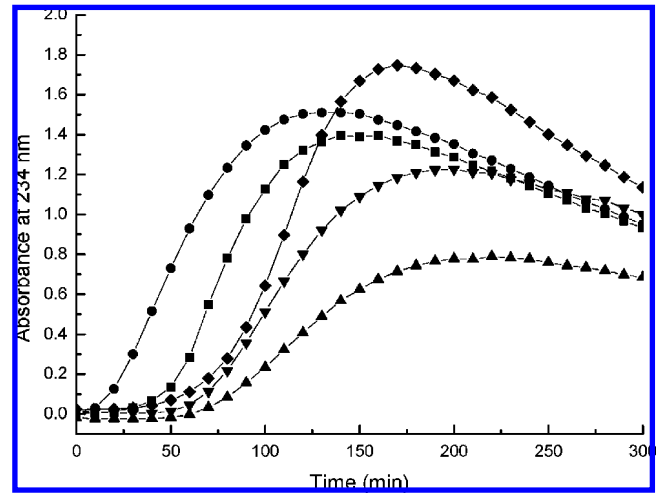


Figure 12. Formation of conjugated dienes in liposomes (37 °C, pH 7.4) in the presence of 1.5 μM gallic acid (■), **D1** (▲), **D2** (▼), or **D3** (◆) and in the absence of antioxidant (●) measured as absorbance at 234 nm after the addition of 0.75 mM AAPH as water-soluble radical initiator. The lag phase in minutes obtained by determining the intercept between the linear part of the curve with the *x*-axis is found in **Table 1**.

The oxygen consumption index (I_{oxygen}) was considered as a function of the calculated octanol/water partition coefficient (ClogP) to study the antioxidant efficiency in an emulsion depending on the hydrophobicity of the bioconjugates (**Figure 11**). It is suggested that I_{oxygen} depends on ClogP according to the empiric eq 2.

$$I_{oxygen} = \frac{k}{1 + (\text{ClogP})}(\text{ClogP}) \qquad (2)$$

The parameter k was determined by nonlinear regression. As may be seen from **Figure 11**, I_{oxygen} is approximating unity ($k = 1.10 \pm 0.04$) for the most hydrophobic bioconjugates, corresponding to a very efficient antioxidant. Other effects of the antioxidants on lipid oxidation could be possible through a direct interaction between antioxidants and myoglobin leading to protein denaturation or heme dissociation. Such effects would, however, enhance lipid oxidation.

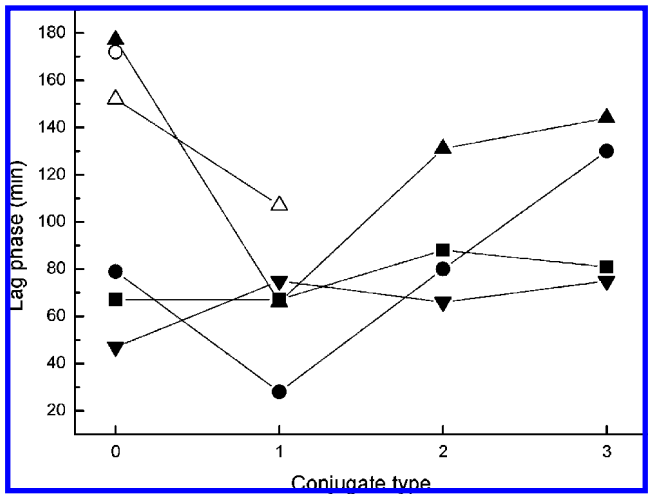


Figure 13. Correlation between the lag phase obtained from peroxidation of liposomes and the type of conjugate for each antioxidant and its derivatives. **A** group (■): **A0** (Trolox), **A1**, **A2**, and **A3**. B group (●): **B0** (dihydroferulic acid), **B1**, **B2**, and **B3**. **B0′** (○) (ferulic acid). **C** group (▲): **C0** (dihydrocaffeic acid), **C1**, **C2**, and **C3**. **C′** group (△): **C0′** (caffeic acid) and **C1′**. **D** group (▼): **D0** (gallic acid), **D1**, **D2**, and **D3**.

Peroxidation of Liposomes. The antioxidative effect of the bioconjugates on the formation of conjugated dienes in liposomes was investigated by measuring the absorbance at 234 nm. Results for gallic acid and its derivatives (group **D** compounds) are shown in **Figure 12**. In the absence of antioxidant the lag phase was 14 min, whereas the presence of gallic acid increased the lag phase (47 min), showing a small protection of gallic acid against oxidation of liposomes. The presence of the derivatives of gallic acid (**D1**, **D2**, and **D3**) further increased the lag phase (75, 66, and 74 min, respectively), indicating a better protection against oxidation than gallic acid and increased antioxidative effect of the bioconjugates compared to the parent antioxidant, although these observations were not statistically significant.

Correlation between results from the liposome assay and type of conjugation is summarized in **Figure 13**. Overall, the conjugates with serine ethyl ester as building block (**1**) exhibit lower antioxidative activity than the rest of the compounds. A slight improvement of the antioxidative activity was found for Trolox (group **A**) with the long-chain building blocks, although these results are not statistically significant. Improvement of antioxidative activity is found when dihydroferulic acid is conjugated with lauroyl serine (**B3**), compared to the compound without conjugation. However, the compounds with the best antioxidative effect are nonconjugated compounds: **B0′** (ferulic acid), **C0** (dihydrocaffeic acid), and **C0′** (caffeic acid). The effect of the double bond of the side chain on the antioxidative properties in the liposome assay is not clear. Removing the double bond from ferulic acid decreases the antioxidant capacity significantly (the lag phase is reduced from 172 to 79 min), whereas removing the double bond from caffeic acid increases the lag phase from 152 to 177 min.

In the present study, conjugation of water-soluble antioxidants with longer chain derivatives was found overall to improve the antioxidative effect in heterogeneous systems compared to the parent antioxidants, similar to what has been observed in other studies with retinyl ascorbate, ferulic acid esters, and gallic acid esters (*9, 11, 12*). However, the actual improvement of antioxidative activity is highly dependent on which assay is used for evaluation of the antioxidants.

Trolox and Its Derivatives (Group A). Improvement of the antioxidative activities of conjugates of Trolox was observed for oxygen consumption in a methyl linoleate emulsion and to a smaller extent on the formation of conjugated dienes in liposomes with the longer chain conjugates of Trolox (**A2** and **A3**). The scavenging rate of DPPH$^{\bullet}$ was highest for Trolox itself compared to the derivatives of Trolox.

Trolox is more lipophilic than ferulic acid, caffeic acid, and gallic acid, and Trolox was therefore expected to exhibit better antioxidative activities in emulsions compared to the other conjugates, as was also observed in the oxygen consumption assay and scavenging of DPPH$^{\bullet}$ in micelles. The effect of adding a lipophilic part to Trolox to align the antioxidant in the interface between oil and water is accordingly not as significant as observed for the other conjugates.

Ferulic Acid and Its Derivatives (Group B). Improvement of the antioxidative activity was found when dihydroferulic acid was conjugated with longer chain derivatives [serine lauryl ester (**2**) and lauroyl serine (**3**)] on the formation of conjugated dienes in liposomes and for oxygen consumption in oil-in-water emulsion. The results indicated that conjugation of a lipophilic molecule to the hydrophilic antioxidant reduces oxidation in the heterogeneous systems. No improvement on antioxidative activity was, however, observed when dihydroferulic acid was conjugated with serine ethyl ester (**1**), confirming that improvement depends on conjugation with longer hydrophobic chains. In general, the scavenging rate of DPPH$^{\bullet}$ was slow, and no marked difference was observed between the individual group **B** compounds, indicating that ferulic acid and derivatives are not good radical scavengers under the actual assay conditions. In the study by Kikuzaki et al. (*11*), the percentage of DPPH radical scavenging was determined for ferulic acid and related compounds in EtOH. Ferulic acid scavenged more DPPH radicals than various ferulate esters such as dodecyl ferulate, which is consistent with the results in the present study (**Figure 5**).

A marked difference in antioxidative activity between ferulic acid and dihydroferulic acid was found only for development of conjugated dienes in liposomes, where ferulic acid more efficiently protected the liposomes from oxidation than dihydroferulic acid. No obvious difference was found in the other antioxidant assays apart from DPPH$^{\bullet}$ scavenging in MeOH, but the rate of DPPH$^{\bullet}$ scavenging in presence of group **B** compounds was generally very low.

Caffeic Acid and Its Derivatives (Group C). Conjugation of dihydrocaffeic acid with the longer chain derivatives (**2** and **3**) significantly improved the antioxidative and radical scavenging activity as measured by oxygen consumption in an oil-in-water emulsion and as measured as scavenging rate of DPPH$^{\bullet}$ in the heterogeneous system. On the contrary, the scavenging rate of DPPH$^{\bullet}$ in MeOH, in micellular solution, and inhibition of conjugated diene formation in liposomes were reduced when dihydrocaffeic acid was conjugated with the tested emulsifier building blocks. In agreement with the present study, dihydrocaffeic acid has been found to scavenge DPPH$^{\bullet}$ in EtOH more effectively than its alkyl esters (methyl, ethyl, and propyl dihydrocaffeate) (*29*). In the same study, the caffeic acid alkyl esters were found to scavenge DPPH$^{\bullet}$ more effectively than caffeic acid itself, which is in contrast to the present study that found the scavenging rate of DPPH$^{\bullet}$ by caffeic acid (**C0′**) to be faster than that by **C1′**. Caffeic acid and dihydrocaffeic acid have, to our knowledge, not previously been synthesized with longer chain conjugates than the *n*-C_3 derivatives. The longer conjugation seems to improve the effect for the reduced caffeic

acid apart from the inability of the conjugates to inhibit the formation of conjugated dienes in liposomes and to scavenge DPPH• in methanol.

Interestingly, the reduction of the double bond in caffeic acid improves antioxidative activity to a small extent in all applied assays, which is consistent with the results of Silva et al. (*27*), who found that propyl dihydrocaffeate showed improved antioxidant activity compared to propyl caffeate. However, in another study it was concluded that both caffeic acid and dihydrocaffeic acid are efficient antioxidants for lard and human plasma low-density lipoprotein, but the presence of the double bond in the side chain of the catechol group affects the efficiency of antioxidant activity depending on the environment in which the oxidation takes place (*26*).

Gallic Acid and Its Derivatives (Group D). Conjugation of gallic acid showed the most significant improvement of the four groups of antioxidants as oxygen consumption in oil-in-water emulsions and formation of conjugated dienes clearly decreased. The rate of scavenging of DPPH• in the heterogeneous system was likewise increased as seen by a comparison of the results obtained for the derivatives of serine lauryl ester (**2**) and lauroyl serine (**3**) with the parent gallic acid. In the study of Kikuzaki et al. (*11*), gallic acid and derivatives inhibited PC hydroperoxide formation in liposomes in the order lauryl gallate > methyl gallate > gallic acid, also indicating improved antioxidant activity for longer chain conjugates. Furthermore, methyl gallate was found to scavenge a larger percentage of DPPH radicals compared to gallic acid and lauryl gallate in EtOH, showing that no effect of the longer chain conjugates was found in a homogeneous system (*11*). This observation is in agreement with the results obtained in the present study, where no effect of conjugating gallic acid was found on scavenging rate of DPPH• in MeOH. However, in a study where the DPPH radical scavenging efficiency of gallic acid and longer chain derivatives was compared by electron spin resonance spectroscopy in liposomes, gallic acid was found to exhibit the best antioxidative activity compared to the longer chain derivatives (*12*).

Effect of Charge of the Serine Building Blocks. At neutral pH, which was selected as the reaction condition in the antioxidant assays used in the present study, serine ethyl and lauryl esters (**1** and **2**, respectively) are positively charged, whereas lauroyl serine amides (**3**) are negatively charged. Group **2** compounds generally exhibited better DPPH• scavenging activity compared to group **3** compounds, whereas group **3** compounds generally more effectively inhibited the formation of conjugated dienes in liposomes than group **2** compounds. No notable difference was observed for the effect on oxygen consumption in the oil-in-water emulsions between group **2** and **3** compounds. The different assays evaluate effects of antioxidants at different stages in the overall oxidation process. The DPPH• scavenging assay relates to early stages in oxidative damage, and it is not surprising that positively charged antioxidant derivatives may scavenge radicals with a partial negative charge better than negatively charged antioxidants. In contrast, a negative charge seems to improve antioxidant activity toward the formation of conjugated dienes in liposomes. Notably, in the liposome assay, oxidation was initiated by AAPH, an azo initiator, which forms cationic radicals. In foods and other biological systems, Fe(III)/Fe(II) is a common catalyst for radical generation, and both in the present assay and in foods, negatively charged antioxidants seem to be more effective against lipid oxidation initiated by cations (*30*). It should further be noted that the use in the two assays, where lipids are becoming oxidized, of different initiators for experimental reasons does not lead to differences in the overall pattern in effects of bioconjugation.

Combinatorial chemistry principles have been used to design and synthesize an array of emulsifier−antioxidant bioconjugates. Such combinatorial libraries are useful for systematic evaluation of bioactive compounds such as antioxidants compared to a more classical trial and error approach. In homogeneous solution only minor improvement could be obtained for conjugation of the antioxidants. In contrast, prevention of oxidation in heterogeneous systems was clearly improved when a lipophilic appendage was added to a water-soluble polyphenolic antioxidant, indicating that oxidation takes place in the interface between oil and water in heterogeneous systems, and conjugation of polyphenols with emulsifier compounds provides better protection against oxidation as the antioxidant−emulsifier conjugates are aligned in the interface between oil and water. The improvement of protection was shown to depend on the octanol/water partition coefficient, and we suggest that optimal protection corresponds to saturation of the water−oil interface.

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Supporting Information Available: Detailed NMR data from compounds **2**−**29**. This material is available free of charge via the Internet at http://pubs.acs.org.

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