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Lipase-catalysed synthesis of esters of ferulic acid with natural compounds and evaluation of their antioxidant properties

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ABSTRACT

Lipases from *Candida antarctica* (Novozyme 435®), *Candida rugosa*, *Chromobacterium viscosum* and *Pseudomonas* sp. were used to perform transesterifications of vinyl ferulate with hydroxyl-steroids and *p*-arbutin. The antioxidant activity of the products was evaluated using the free radical 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) free radical quenching antioxidant assays, and inhibition of the oxidation of low-density lipoprotein, LDL. Arbutin ferulate was found to possess a 19% higher antiradical activity against the ABTS free radical than its precursor ferulic acid, and it also inhibited the oxidation of LDL more efficiently (by 10%) than its precursors. All of the biocatalytically synthesised products exhibited higher antioxidant activity than Trolox, the well known commercial benchmark antioxidant, and their precursor, ferulic acid.

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1. Introduction

Reactive oxygen species (ROS) have been identified as the responsible agents in the pathogenesis of various diseases [1] and normal physiological phenomena such as aging [2]. ROS, such as H_2O_2 and oxygen radicals (for example O_2^-, $ONOO^-$), are highly reactive chemical species which can result from oxidative metabolism and cause oxidation of cellular components including DNA, protein and lipids. An antioxidant may be defined as "any substance which, when present at low concentrations compared with those of the oxidisable substrate, significantly delays or inhibits oxidation of the substrate" [3,4]. Living systems have evolved endogenous antioxidant systems as a response to oxidative stress caused by ROS [5]. In addition, exogenous antioxidants, such as dietary antioxidants, have long been recognised for their health benefit in reducing physiological oxidative stress. Dietary antioxidants have been implicated in delaying aging processes which are due to oxidative damage, and amelioration or prevention of chronic degenerative diseases, through the prevention of endogenous and exogenous cellular oxidation reactions, and many antioxidants are marketed as nutraceuticals [6].

Ferulic acid is an antioxidant compound as a result of resonance stabilisation of its phenoxy radical by the conjugation of the aromatic nucleus and its extended side chain. This compound is readily isolated from maize waste, where it comprises up to 3% (w/w) [7]. Alkyl esters of ferulic acid, such as octyl ferulate, have been observed to have higher antioxidant activity than the acid itself and the activity of octyl ferulate was reported to be comparable to BHT [8]. Similarly, triterpene alcohol monoesters such as 24-methylenecycloartenyl ferulate and cycloartenyl ferulate display antioxidant activity [9] and were shown to inhibit oxidation more effectively than ferulic acid [8]. Moreover, γ-oryzanol, a rice bran extract primarily consisting of cycloartenyl ferulate and 24-methylenecycloartenyl ferulate, is widely used in the cosmetic industry as an antioxidant [10,11]. Finally, it has been shown that the sterol ferulates possess other pharmacological actions such as anti-inflammatory effects [12], modulation of pituitary secretion, and in the treatment of hyperlipoproteinanemia [11].

Biocatalysis has enabled the selective modification of various sugars and steroid functional derivatives [13–16]. As an extension of previous work referenced above, we describe here the regioselective esterification of the phenolic glycoside arbutin and of various steroids with ferulic acid, acylation catalysed by the lipases from *Candida antarctica* and *Candida rugosa*.

The biocatalytically produced compounds were assessed for antioxidant activity. The most common laboratory method for measuring antioxidant activity involves the use of the stable free radical DPPH•, which has an absorption band at 515 nm, and this which disappears upon reduction by an antioxidant. Thus, antioxidant activity can be determined by monitoring the decrease in

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absorbance and results are generally reported in terms of the amount (%) of the DPPH• quenched under standardised conditions. However, assessment of antioxidant activity generally requires the application of more than one assay method because antioxidants may exhibit their antioxidant activity via a range of different mechanisms, and single assay method may not provide a complete representation of the activity of any one compound. In addition, the activity of antioxidants is highly dependent on their chemical and physical environment [17]. The antioxidant activity of a putative antioxidant compound may occur as a result of its ability to prevent oxidation or to reverse the effects of oxidation, and furthermore, natural antioxidants are often multi-mechanistic, resulting in complex reaction kinetics. Thus, antioxidant activity may be inappropriately calculated under certain assay conditions. In this study, we have used a set of different standard antioxidant assays in order to minimise these effects. Free radical quenching assay protocols and the inhibition of oxidation of low-density lipoprotein (LDL) was tested, as an indicator of antioxidant activity, since the formation of artheromas (which are the major cause of coronary heart disease) is thought to result from the oxidation of LDLs [18].

2. Experimental

2.1. Materials and methods

2.1.1. Enzymes

Lipases from *C. antarctica* (Novozyme 435®), *C. rugosa*, *Chromobacterium. viscosum* and *Pseudomonas* sp. were purchased from Sigma–Aldrich. The enzymes were each used in quantities based on activities, as in previous investigations [14,15].

2.1.2. Chemicals

All chemicals including LDL were purchased from Sigma–Aldrich. Solvents were purchased from Merck South Africa and were HPLC grade.

2.2. Chemical synthesis

2.2.1. Esterification of ferulic acid to produce vinyl ferulate

The ferulic acid vinyl ester was prepared via the vinyl exchange reaction of vinyl acetate and ferulic acid (previously described by Gao et al. [19]). Ferulic acid (10 mmol) was placed in a three-necked-flask with 15 mL (0.16 mol) vinyl acetate. Mercury acetate (66 mg, 4% w/w) and THF (10 mL) were added. The mixture was stirred at 100 rpm under nitrogen for 30 min and 0.04 mmol (2 μL) sulphuric acid was added to start the reaction. The reaction mixture was heated to 40 °C with stirring for 12 h, and the progress was monitored regularly using thin layer chromatography (TLC), eluted with petroleum ether/ethyl acetate (4/1 v/v). The reaction was then terminated by the addition of excess sodium acetate (20 mg). The solvent was evaporated and the solid residue was subjected to silica gel chromatography using the same solvent as eluent.

2.2.2. Enzymatic transesterification of vinyl ferulate or trifluoroethyl cinnamate with octanol

Vinyl ferulate or trifluoroethyl cinnamate (50 mg/μL) and octanol (10 μL) were added to *tert*-butyl-methyl ether (1 mL), followed by *C. antarctica* B lipase (5 mg). The suspension was stirred at 45 °C, and samples were taken to monitor the conversion. The solution was filtered to remove the enzyme and products were identified using GC–MS.

2.2.3. Enzymic transesterification of vinyl ferulate with arbutin

74 mM arbutin and 213 mM vinyl ferulate were reacted in acetonitrile at 45 °C with shaking. 40 mg/mL immobilised *C. antarctica* B lipase (Novozyme 435®) was used as the biocatalyst, and the progress of the reaction was monitored by TLC. At the end of the reaction, the enzyme was filtered off and the solvent evaporated. The resulting solid residue was purified using silica gel chromatography. The mobile phase was ethyl acetate/methanol/water (10/0.5/0.1 v/v/v). Substrates and products were visualized at 254 nm and/or by plates treatment with phosphomolybdate reagent ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 42 g; $Ce(SO_4)_2$, 2 g; H_2SO_4 conc., 62 mL; made up to 1 L with deionized water). The isolated products were analysed by NMR spectroscopy. The isolated yield of the product was 50%.

*2.2.3.1. 6-O-Feruloyl arbutin (**3**).* ^{1}H NMR (300 MHz, CD_3OD) δ: 7.66 (1H, d, J = 15.9 Hz, *H*C=CH–C=O), 7.19 (1H, br s, ferulate Ar-H), 7.08 (1H, dd, J_1 = 8.0 Hz, J_2 = 1.2 Hz, ferulate Ar-H), 6.96 (2H, d, J = 8.9 Hz, arbutin Ar-H), 6.83 (1H, d, J = 8.3 Hz, ferulate Ar-H), 6.66 (2H, d, J = 9.0 Hz, arbutin Ar-H), 6.42 (1H, d, J = 15.9 Hz, HC=C*H*–C=O), 4.87 (3H, s, OCH_3), 4.73 (1H, d, J = 5.6 Hz, glucose H-1), 4.53 (1H, dd, J_1 = 11.8 Hz, J_2 = 1.2 Hz, glucose H-6_a), 4.36 (1H, dd, J_1 = 11.9 Hz, J_2 = 5.3 Hz, glucose H-6_b)

2.2.4. Enzymatic transesterification of steroids with vinyl acetate

A modification of the method described by Bertinotti et al. [14] was used. Dihydrocholesterol (5 mg, 0.012 mmol) was dissolved in 900 μL *tert*-butyl-methyl ether. Vinyl acetate (100 μL) and *C. antarctica* lipase B (5 mg) were added and the reactions were performed at 45 °C with shaking, monitoring the progress by TLC (eluent $CHCl_3$/MeOH (10/0.1 v/v) using an authentic sample of 3-*O*-acetyl dihydrocholesterol as a reference.

2.2.5. Synthesis of sterol ferulates

227 mM vinyl ferulate was reacted with 26 mM steroid (dihydrocholesterol or 5α-androstane-3β,17β-diol) in *tert*-butyl-methyl ether. The synthesis was attempted with four different lipases, namely *C. antarctica* B (10 mg/mL), *C. rugosa* (100 mg/mL), *Chromobacterium. viscosum* (100 mg/mL) and *Pseudomonas* sp. lipase (75 mg/mL) at 45 °C with shaking. The reactions were monitored by TLC at regular intervals, and Komarowsky's reagent was used for the visualisation of the steroids [20]. After enzyme filtration and solvent evaporation, the residue obtained from the reaction catalysed by *C. rugosa* lipase was purified by silica gel chromatography, eluted using petroleum ether/ethyl acetate (10/1.5 v/v), as the mobile phase. The isolated products were analysed by NMR spectroscopy. The isolated yield of dihydrocholesterol ferulate was 56% and of 3β-*O*-feruloyl-17β-hydroxy-5α-androstane was 44%.Dihydrocholesterol ferulate (**6**)^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.58 (1H, d, J = 15.9 Hz, *H*C=CH–C=O), 7.06 (1H, br d, J = 8.3 Hz, Ar-H), 7.03 (1H, br s, Ar-H), 6.90 (1H, d, J = 8.0 Hz, Ar-H), 6.26 (1H, d, J = 15.8 Hz, CH=C*H*–C=O), 4.82 (1H, m, H-3 sterol), 3.92 (3H, s, OCH_3), 2.0–1.2 (30H, m, 12 (CH_2) and 6 (CH)), 0.91 (3H, s, CH_3), 0.89 (3H, s, CH_3), 0.88–0.85 (9H, 2xd, unresolved).

*2.2.5.2. 3β-O-Feruloyl-17β-hydroxy-5α-androstane (**8**).* ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.59 (1H, d, J = 15.6 Hz, *H*C=CH–C=O), 7.06 (1H, dd, J_1 = 8.4 Hz, J_2 = 1.6 Hz, Ar-H), 7.03 (1H, d, J = 1.6 Hz, Ar-H), 6.91 (1H, *d*, J = 8.4 Hz, Ar-H), 6.26 (1H, d, J = 16.0 Hz, CH=C*H*–C=O), 4.82 (1H, sep, J = 5.2 Hz, H-3 sterol), 3.92 (3H, s, OCH_3), 3.64 (1H, t, J = 8.4 Hz, H-17 sterol), 1.54-1.26 (9 (CH_2) and 4 (CH)), 0.87 (3H, s, CH_3), 0.74 (3H, s, CH_3).

2.3. Antioxidant tests

2.3.1. DPPH• assay

The method used was as described by Bondet et al. [21]. A DPPH• standard curve was constructed in order to determine DPPH• quan-

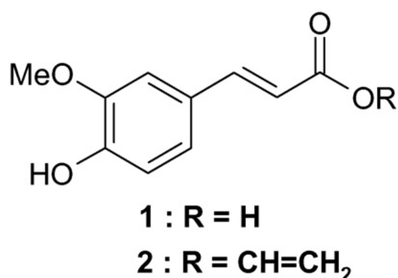

Fig. 1. Structure of ferulic acid (**1**) and vinyl ferulate (**2**).

tities used. The linear regression obtained was $y = 9973.8x - 0.0091$ where y was the absorbance at 515 nm and x the molarity of DPPH•. 3.5 mL of 6×10^{-5} M methanolic DPPH• was reacted with 0.5 mL (final concentration 15 μM) samples of the antioxidants to be tested. Absorbance at 515 nm was monitored until the reaction reached steady state.

2.3.2. Trolox equivalent antioxidant capacity (TEAC) assay

$ABTS^{\bullet+}$ was generated by reacting 7 mM ABTS and 2.45 mM potassium persulphate in water. The solution was stored in the dark for 12–16 h. The free radical solution was then diluted in 25 mM phosphate buffered saline (PBS), pH 7.4, to an absorbance of 0.70 ± 0.02. 10 μL of the test antioxidants (1.2–0.6 μM final concentration) dissolved in ethanol was added to 990 μL of the diluted $ABTS^{\bullet+}$ solution. The change in absorbance at 734 nm was measured. All tests were carried out at 30 ± 2 °C in triplicate. The TEAC of the antioxidant is calculated relative to the decrease in absorbance of Trolox a standard antioxidant, after 6 min, on a molar basis [22].

2.3.3. LDL oxidation

Oxidation of low-density lipoproteins (LDL) was performed according to the method developed by Nardini et al. [23]. The LDL was dialysed in a 200-fold volume of 1× phosphate buffered saline pH 7.4 at 4 °C in the dark. 100 μg/mL LDL was then oxidised using 5 μM $CuCl_2$ for 4 h at 37 °C in the presence or absence of 0.5 μM test antioxidant. Diene formation was measured spectroscopically at 234 nm.

3. Results and discussion

3.1. Synthesis of feruloyl esters

3.1.1. Preparation of the activated ester vinyl ferulate

In our approach to the synthesis of ferulic acid derivatives, the initial step was the production of an activated acyl donor of ferulic acid (**1**), vinyl ferulate (**2**) via a vinyl exchange reaction between vinyl acetate and ferulic acid in the presence of mercuric acetate [19]. The product **2** was obtained in 45% yield and the structure was confirmed by NMR spectroscopy (Fig. 1).

3.1.2. Acylation of dihydrocholesterol, octanol and arbutin catalysed by C. antarctica lipase B

The acetylation of dihydrocholesterol with *C. antarctica* lipase B was initially attempted in order to confirm that this lipase was capable of acetylating a steroid moiety [14], using *tert*-butyl-methyl ether as the solvent. The reaction was successful, as confirmed by TLC analysis, and the corresponding 3-*O*-acetate was isolated by flash chromatography and characterized. However, a similar acylation reaction performed with vinyl ferulate as the acylating agent was unsuccessful, and the sterol was recovered unaffected. This result was quite unexpected, as *C. antarctica* lipase B has been reported to accept phenylpropenoic esters as acyl donors (see, for instance, Ref. [24]). To obtain additional information on the substrate specificity of this enzyme, a much simpler substrate, *n*-octanol, was tested. With this compound, the biocatalysed transesterifications with vinyl ferulate or trifluoroethyl cinnamate were successful, as confirmed by GC–MS analysis which showed the formation of octyl cinnamate and octyl ferulate, respectively. Next, we attempted the same transesterification reactions using the glucoside arbutin (**3**) as an acceptor. In this case too, the expected products were isolated and characterized as 6-*O*-feruloyl-arbutin (**4**, 50% yields) by ^{1}H NMR analysis (Fig. 2).

3.1.3. Biocatalysed synthesis of a sterol ferulate

Based on the successful transesterifications with substrates other than dihydrocholesterol (above), it was concluded that, probably, the steric hindrances of the phenylpropenoic derivatives and of the tetracyclic steroid skeleton prevented the simultaneous access of these two moieties into the active site of *C. antarctica* B lipase, thus preventing the expected acylation reactions. Therefore, we investigated the use of alternative lipases, namely the enzymes from *C. rugosa*, *Chromobacterium. viscosum* and *Pseudomonas* sp., to catalyse the transesterification of dihydrocholesterol by vinyl ferulate. We found that *C. rugosa* lipase was able to give the expected acylation, thus allowing the isolation of dihydrocholesterol ferulate (**5**) in 56% yields. As an additional substrate we considered 5α-androstane-3β,17β-diol (**7**). This diol was also accepted as a substrate by the enzyme and, as expected [15], the acylation was regioselective and only the corresponding 3-*O*-feruloyl derivative (**8**) was isolated (44% yields) and characterized. A crude mixture of the substrate (**1**) with compound (**8)** was also tested for antioxidant activity. The composition of the mixture was confirmed by NMR (Fig. 3).

3.2. Measurement of antioxidant activity of products

3.2.1. DPPH• free radical quenching assay

In this study, the assay was conducted using 15 μM concentrations (in the final reaction mixture) of the candidate antioxidant compounds, and measuring the absorbance changes over a period of up to 3 h. The products arbutin ferulate (**4**), dihydrocholesterol ferulate (**6**), and 3β-*O*-feruloyl-17β-hydroxy-5α-androstane (**8**) all showed lower total DPPH• quenching activity than ferulic acid itself. However, when the crude reaction mixture, which contained a 4:1 mixture of **8** (3β-*O*-feruloyl-17β-hydroxy-5α-androstane) and ferulic acid (**1**) (composition determined by NMR analysis), was tested before purification, a significantly higher antioxidant activity was observed. These results are summarised in Table 1. The reaction rates for these DPPH• quenching reactions were also observed under the standard conditions described, and were found to differ widely. For example, the reaction of ferulic acid resulted in quenching of 40% of the DPPH• in a period of 50 min, after which the reaction stopped. Under the same conditions, arbutin ferulate (**4**) and dihydrocholesterol ferulate (**6**) quenched 11% of the DPPH• in 30 min and 35% in 80 min, respectively, and the steroid ferulate product, 3β-*O*-feruloyl-17β-hydroxy-5α-androstane (**8**), quenched 23% in 20 min. Interestingly, again, the unpurified crude reaction mixture from the reaction of ferulic acid with 5α-androstane-3β,17β-diol gave the highest observed DPPH• quenching activity (62.7%) and this reaction was observed to continue for approximately 150 min (Fig. 4). It is clear that testing of a mixture of arbutin and the steroids (**5**) and (**7**) is also necessary and this will pursued in further investigations.

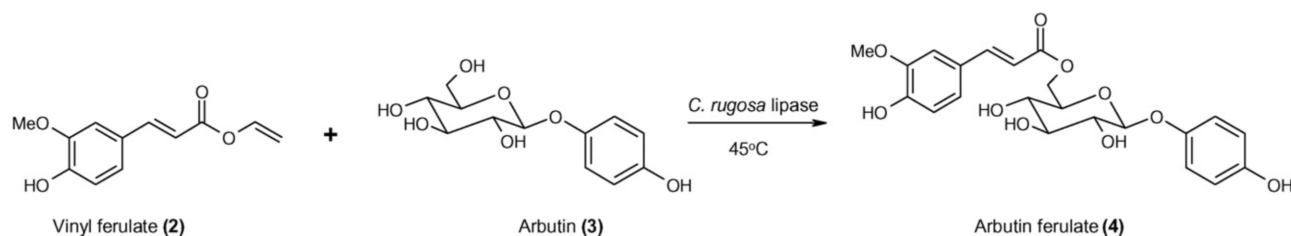

Fig. 2. *C. rugosa*-catalysed regioselective esterification of arbutin to give 6-*O*-feruloyl-arbutin (**4**).

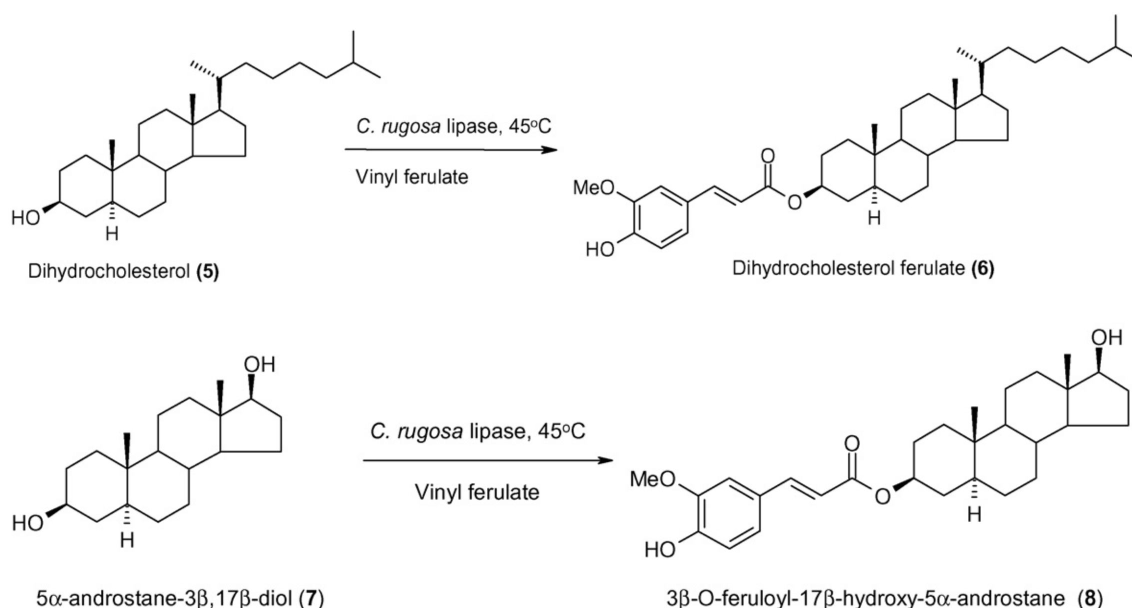

Fig. 3. *C. rugosa*-catalysed regioselective esterification of dihydrocholesterol (**5**) and 5α-androstantane-3β,17β-diol (**7**) with vinyl ferulate.

Thus, the presence of the residual ferulic acid together with the sterol ferulate product apparently prolonged and increased the antioxidant activity, suggesting antioxidant synergism between the compounds. Such synergy has been observed previously, where antioxidants act together to give a total antioxidant effect amounting to more than a simple additive level [25–27]. For example, Lu and Liu [27] found that certain antioxidants, including ferulic acid, interacted with the antioxidant lipoic acid (suggested to be due to donation of electrons to the antioxidant in a cyclic reaction), thereby increasing its antioxidant capacity; it is proposed that ferulic acid has a similar interaction with product (**8**).

3.2.2. Measurement of antioxidant activity using the TEAC assay

The TEAC assay was conducted as described by Re et al. [22]. The assay is based on the decolourisation of the $ABTS^{•+}$ radical due to the scavenging of the radical cation by the putative antioxidants, which is compared with that of Trolox, is a water soluble vitamin E analogue. The TEAC is calculated as the percentage decrease of absorbance at 734 nm [22], based on the gradient of the graph of the percentage decrease of absorbance vs. concentration (the “concentration response curve”) for the antioxidant in question, divided by the gradient of the plot for Trolox [22]. Fig. 5 and Table 2 show the percentage decreases in absorbance elicited by the test compounds. The concentration response curves for the antioxidant compounds **1**, **8** and Trolox were then plotted to obtain the TEAC values (Table 3). The TEAC of the mixture of **1** and **8** was again observed to be higher than that of ferulic acid, indicating stronger antioxidant activity (Table 3). Correlation was found between the measured DPPH and TEAC antioxidant activities for all the products, with the exception of arbutin ferulate (**4**), which was found to have higher $ABTS^{•+}$ free radical scavenging ability than all the test compounds other than the crude mixture of **1** and **8**, but a lower free DPPH radical quenching ability all of the compounds tested.

Table 1
Antioxidant capacities of compounds tested using the DPPH• assay

Compound	% DPPH• remaining after 20 min	Time to reach steady state (min)	% DPPH• quenched, at steady state
Ferulic acid (**1**)	62.9 ± 1.9	50	40.1 ± 2.1
Arbutin ferulate (**4**)	87.7 ± 1.2	20	11.1 ± 1.4
Dihydrocholesterol ferulate (**6**)	69.9 ± 1.9	80	35.1 ± 11.1
3β-*O*-Feruloyl-17β-hydroxy-5α-androstane (**8**)	76.9 ± 0.6	20	23.1 ± 0.6
8 and **1** mixture in a 4:1 ratio (crude reaction mixture)	56.7 ± 4.3	Not reached after 150 min	62.7[a] ± 2.7

15 μM final concentration tested.
[a] Measured after 150 min.

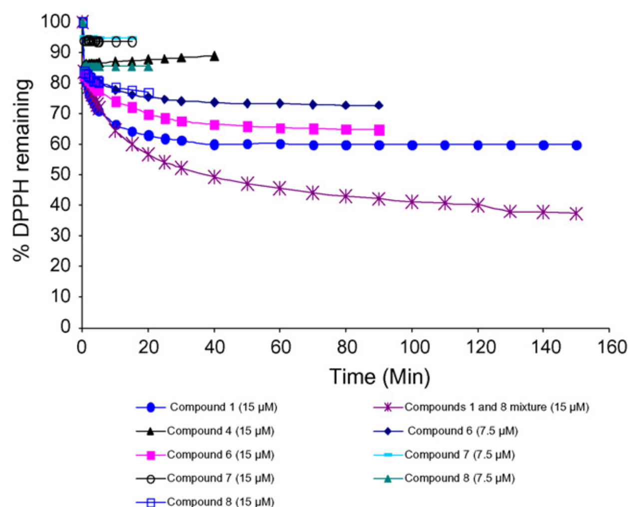

Fig. 4. DPPH quenching reaction of reaction products shown as % DPPH remaining vs. time. (The increase (1.25%) in the absorbance of the reaction mixture of DPPH• and **4** between 5 and 10 min was considered insignificant and not to represent significant pro-oxidant behaviour.)

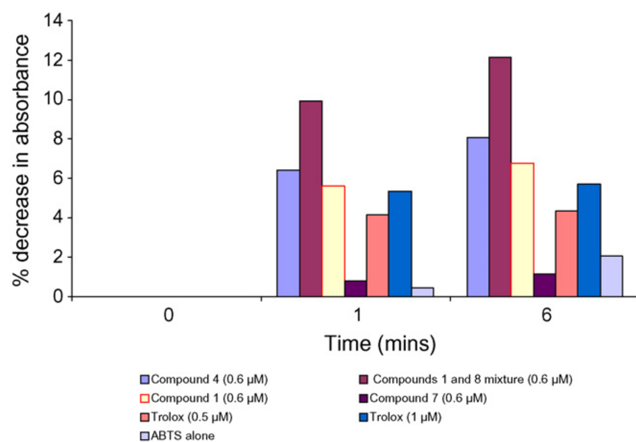

Fig. 5. Changes in absorbance of ABTS$^{\bullet+}$ at 714 nm with time elicited by addition of 0.6 μM antioxidants or Trolox.

Table 2
ABTS$^{\bullet+}$ radical cation scavenging activity of test compounds

Compound	Compound final concentration (μM)	% decrease in absorbance
1 and **8** (1:4 mixture)	0.6	12.0
4	0.6	8.1
1	0.6	6.8
Trolox	1.0	5.7
8	0.6	4.94[a]
Trolox	0.6	<3.4
7	0.6	1.1

[a] Calculated on the basis of the composition of the mixture determined to be a 1:4 ratio of compounds **1** and **8**, from NMR analysis.

Table 3
Quenching of the ABTS$^{\bullet+}$ free radical in the Trolox assay system and corresponding TEAC values for test compounds

Compound	Maximum % decrease of absorbance after 6 min	TEAC (μM)
1	6.76	1.49
4:1 **8** and **1** mixture	12.15	2.70
Trolox	4.15	1
7	1.13	ND

ND = not determined.

3.2.3. Inhibition of LDL oxidation by antioxidants

The ability of the biocatalytically synthesised compounds to inhibit LDL oxidation was assessed by adding them to a reaction mixture containing LDL and copper (II) ions (used to initiate oxidation), and then monitoring the effect on lipid peroxidation-associated diene conjugation after 4 h [23]. An increase in the absorbance at 234 nm indicated diene conjugation. Arbutin ferulate (**4**) was found to confer the highest inhibition of LDL oxidation (23%), higher than that of its precursors, ferulic acid (**1**, 16%) and arbutin (**3**, 13%) (Fig. 6). Thus, the reaction of arbutin with ferulic acid increased the antioxidative activity of arbutin towards copper-induced oxidation of LDL by 10%. The compound **8**, 3β-*O*-feruloyl-17β-hydroxy-5α-androstane, also had a strong antioxidative effect with respect to lipid peroxidation, as did the steroid 5α-androstane-3β,17β-diol (**7**) from which it was derived. Thus, all of the antioxidants tested showed prevention of diene conjugation due to lipid peroxidation except for the mixture of **1** and **8**, and dihydrocholesterol ferulate (**6**). It was unexpected that the mixture would have an absorbance-increasing effect (indicating accelerated diene conjugation) and further investigations will be conducted in order to provide an explanation.

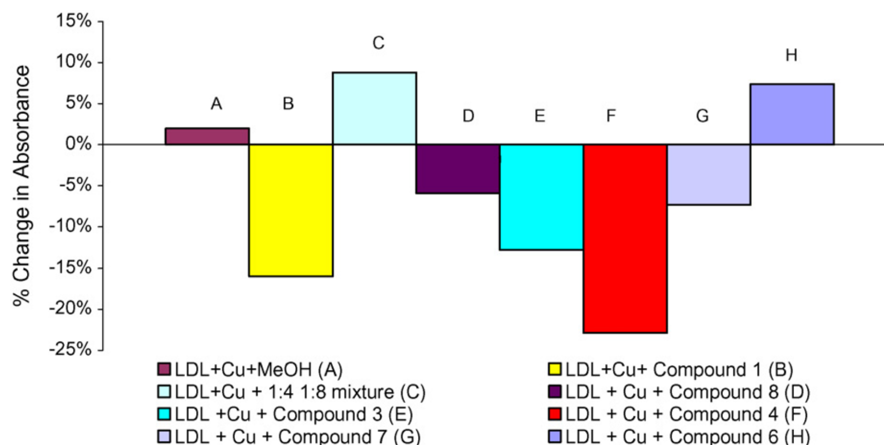

Fig. 6. Changes in absorbance due to the Cu^{2+}-induced formation of conjugated dienes in LDL samples. An increase in absorbance indicates the formation of conjugated dienes and a negative change indicates inhibition of the formation of conjugated dienes, representing antioxidant behaviour.

The low lipid-protecting activity of compound **6** may be explained in terms of the polar paradox theory, in that this is a very lipophilic compound and the LDL particles in which it would exert antioxidant activity are also lipophilic [28]. Thus, it is proposed that this product, due to its non-polarity, enters into the lipophilic LDL particle core, preventing it from exerting much antioxidant activity. Other triterpene androgens and progestins such as testosterone and progesterone were found to have pro-oxidant activity on LDL in that they accelerated the rate of LDL oxidation initiated by Cu^{2+} [28], as observed for compound **6**. Therefore, these results suggest that lipophilic compounds, such as compound **6**, are poor inhibitors of LDL oxidation, a finding which has significant therapeutic implications with respect to the action of *in vivo* LDL oxidation inhibitors. The LDL oxidation mechanism is not currently well understood [29,30], and further structural information on LDL particles for instance, would be required to provide more detailed interpretation of the results obtained for the products **6** and the 1:4 mixture of compounds **1** and **8**.

In considering the three antioxidant assay measurements, the results confirm the generally held consideration that different tests may provide different results, and it is necessary to consider the chemical conditions of each test in drawing conclusions. In general, the water-based $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical cation reactions give strong indication of the chemical antioxidant capacity of test compounds and could be considered useful in assessing the antioxidants activity of compounds to be used, for instance, as food preservatives. This is, in fact, a very common application of the TEAC assay method. However, many biological systems are more hydrophobic and the lipid peroxidation test may well provide more appropriate indication of the potential for an antioxidant, to be used, for example, in protecting physiological lipids or cell membranes.

4. Conclusion

This paper reports the first biocatalysed synthesis of sterol derivatives of ferulic acid, and demonstrates the feasibility of synthesis of other similar compounds. It was demonstrated that *C. rugosa* lipase was capable of performing transesterifications of the vinyl derivative of ferulic acid with steroids.

The antioxidant activities of some of the synthesised compounds were shown to be higher than that of the precursor ferulic acid, using standard antioxidant assay methods. These products are expected to have application in the pharmaceutical sector since sterol ferulates (such as oryzanol) have been found to have applications in the treatment of hyperlipoproteinanemias [11], and as anti-tumour promoters [12]. Inhibition of LDL oxidation could provide evidence of potential therapeutic use of product **4** in the treatment of coronary heart disease. Further, antioxidant synergy was observed between 3β-*O*-feruloyl-17β-hydroxy-5α-androstane (**8**) and its precursor ferulic acid (**1**). Synergistic interactions of antioxidants play an important role in the prevention of oxidative damage in cells for example in disease and ageing [26], where interaction can occur between endogenous and exogenous antioxidants in cells, dependent on their respective redox potentials [31]. Thus, the synergy between product **8** and compound **1** may have therapeutic significance. In addition, slower kinetics, as observed in the case of this mixture, may be beneficial *in vivo*, because a diverse array of free radicals is continually generated *in vivo*, and an antioxidant which sustains activity over a longer period of time would be superior to one that acts quickly and loses its activity; a longer period of activity would allow quenching of more free radicals as they are produced.

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