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Antioxidant Properties of Novel Lipophilic Ascorbic Acid Analogues

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Abstract

Structural modifications of ascorbic acid by the introduction of lipophilic moieties has led to derivatives with increased stability against thermal and oxidative degradation. Two series of new lipophilic ascorbic analogues were synthesized to obtain antioxidants devoid of autooxidant properties: 4-benzoyl-3-hydroxyfuran-2(5*H*)-ones (**3a**–**j**) and 4-acetyl-5-aryl-3,4-dihydrofuran-2(5*H*)ones (**5a**–**f**).

These compounds were submitted to three different tests: reduction of the stable free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH); superoxide-anion scavenging assay; and lipid-peroxidation assay. Most compounds interacted with DPPH: at a concentration of 5×10^{-3} M, the reducing activity of 4-benzoyl derivatives, **3c** and **3h**, was more than 50%; under the same conditions, the rate of inhibition for 4-acetylbutanolides, **5a** and **5f**, reached 60·6% and 87·3%, respectively; 93·3% inhibition was observed with ascorbic acid. In the superoxide-anion scavenging assay, at a concentration of $1\,\mathrm{mg\,mL^{-1}}$, 4-benzoyl derivatives, **3g** and **3i**, exhibited a good activity, with IC50 (dose resulting in 50% inhibition) values of 1·45 and $1{\cdot}35 \times 10^{-3}$ M, respectively. 4-Acetylbutanolide, **5f**, significantly inhibited the Fe^{2+}/ADP/ascorbate-induced lipid peroxidation of rat liver microsomes with an IC50 of $4{\cdot}9 \times 10^{-4}$ M.

This study demonstrates that enol functions in the structure of ascorbic acid analogues are not absolutely essential to bring about antioxidant effects.

There is now increasing evidence that free radicals and active oxygen species are involved in a variety of pathological events, often associated with ageing (Bast et al 1991; Bulkley 1993; Halliwell 1994). Under normal conditions, cells and tissues are protected from oxidative damage by various enzymes such as superoxide dismutase, catalase and peroxidase, as well as by glutathione, α-tocopherol (vitamin E) and ascorbic acid (vitamin C) (Halliwell 1990; Diplock 1994).

Ascorbic acid is known to act as an antioxidant both in-vitro and in-vivo. However, it may act as a prooxidant in-vitro in the presence of metal, but this effect is unlikely to be important in-vivo where metal ions are sequestered and other reductants are present (Niki 1991). There is considerable evidence that ascorbic acid may prevent a large number of chronic diseases including cancer, myocardial infarction, stroke, asthma, diabetes, arthritis and AIDS, but in most well-controlled trials, no clinical benefit has been clearly demonstrated (Halliwell 1991).

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The characteristic biological activity of ascorbic acid is derived from its enediol structure which endows a strong electron-donating ability. However, its well-known susceptibility to thermal and oxidative degradation together with its hydrophilicity has led to interest in derivatives with increased stability and better lipophilicity. Lipophilicity seems to be an important determinant of antioxidant activity as it regulates mobility and distribution within the membrane lipid bilayer (Niki et al 1985).

Our previous studies on ascorbic acid analogues (Coudert et al 1996) have shown that introduction of aromatic moieties into 3-hydroxyfuran-2(5*H*)-ones leads to lipophilic derivatives which are in some cases endowed with antioxidant properties. To improve effects on free-radical processes, in this

study we have synthesized two series of new ascorbic analogues: 4-benzoyl-3-hydroxyfuran-2-(5*H*)-ones (**3a**–**j**) and 4-acetyl-5-aryl-3,4-dihydro-furan-2(5*H*)-ones (**5a**–**f**). The antioxidant activity of these new compounds has been evaluated in-vitro by means of three tests: measurement of the reducing activity on the stable radical DPPH, superoxide-anion scavenging assay and lipid-peroxidation assay.

Materials and Methods

Materials

All starting materials for synthesis were reagent-grade from Janssen Chimica, Noisy-le-Grand, France. Reagents for biochemical assays were purchased from Sigma, Montluçon, France.

Analytical methods

Melting points were determined on a Reichert apparatus and are uncorrected. IR spectra were recorded with a FTIR-Nicolet Impact 410 spectrophotometer. NMR spectra were recorded on a Brüker 400 MSL apparatus. Chemical shifts were reported in parts per million (δppm) relative to tetramethylsilane used as an internal standard. Abbreviations used for signal patterns are: s, singlet; d, doublet; t, triplet; m, multiplet. Elemental analyses were performed at the Service Central d'Analyses, CNRS (Vernaison, France).

*General procedure for the synthesis of 4-benzoyl-3-hydroxyfuran-2(5*H*)-ones (**3a**–*j*)*

A solution of 0·022 mol of the appropriate acetophenone derivative and 0·022 mol of diethyl oxalate in 40 mL of anhydrous diethylether was added dropwise to fresh sodium methoxide suspended in diethylether (1 equiv for acetophenone or 2 equiv for hydroxy-substituted derivatives). The resulting mixture was vigorously stirred at room temperature for 1 h, and then at reflux for 2 h. The reaction mixture was cooled and 30–50 mL of water was then added, followed by a solution of 38% aqueous formaldehyde or a solution of the appropriate benzaldehyde (0·022 mol) in 10 mL of acetone. Stirring was continued for 2 h at room temperature.

The aqueous layer was removed and the organic layer was extracted three times with water. The combined aqueous extracts were cooled to 10°C and acidified to pH 1 with 50% aqueous hydrochloric acid. After stirring for 1 h, the resulting solid was filtered off, washed with ice-water, recrystallized from ethanol-water and dried to give compounds **3a**–**j**.

*General procedure for the synthesis of 4-acetyl-5-aryl-3,4-dihydrofuran-2(5*H*)-ones (**5a**–*f*)*

To a solution of α-angelicalactone (0·05 mol, 4·9 g) and the appropriate aldehyde (0·05 mol) in 200 mL methylene chloride, trifluoroborate etherate (0·05 mol) was added dropwise over a period of about 30 min at room temperature. After the addition was completed, the reaction mixture was vigorously stirred for about 70 h. Saturated sodium chloride solution (300 mL) was added and vigorous stirring was maintained for 15 min. The organic layer was removed and the aqueous layer extracted with methylene chloride.

The combined organic extracts were dried over magnesium sulphate and evaporated in-vacuo. The oily residue was subjected to silica-gel column chromatography (eluent: ethyl acetate/hexane 5 : 5) to furnish compounds (**5a**–**j**).

Lipophilicity measurements

Lipophilicity was determined by reversed-phase high-performance liquid chromatography using literature procedures (El Tayar et al 1985; Bechalany et al 1989). A Varian 5000 liquid chromatograph equipped with a detector operating at 254 nm was used. A Varian CDS 111L integrator was used for peak registration and calculation of retention times. An ODP column (15 cm × 6 mm i.d.) prepacked with octadecyl copolymer gel, particle size 5 μm, was used as a non-polar stationary phase. Mobile phases were prepared volumetrically from 65 : 35 to 95 : 5 combinations of methanol/acetic acid (0·085 N, pH 3). The flow rate was 1 mL min^{-1}. Isocratic capacity factors (k_i) were defined as $k_i = (t_r - t_o)/t_o$ where t_r is solute retention time and t_o is column dead time determined using methanol as the non-retained compound. Log k′ was used as the lipophilic index, obtained by linear extrapolation of log k_i to 50% water.

Measurement of activity in reduction of DPPH

The method of Andreadou et al (1997) was used to measure reduction of the stable radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH). Briefly, a solution of 4 mL DPPH (10^{-4} M, 2 mg) in ethanol (50 mL) was added to 1 mL of a solution of the test compound in DMF (dimethylformide), to prepare a 5×10^{-3} M sample solution. The absorbance at 517 nm was measured every 5 min over a 3-h period. The absorbance of a control sample without test

compound was measured simultaneously. The difference in absorbance between the control and the test compound was taken as the reducing activity.

Superoxide anion scavenging assay

The technique of Slater & Eakins (1974), utilizing the interactions of NADPH, phenazine methosulphate (PMS), molecular oxygen and nitro blue tetrazolium (NBT), was used for evaluating superoxide-anion scavenging. The NADP/PMS/O_2/NBT system involves the intermediate formation of the superoxide anion radical ($O_2^{\cdot -}$) from the interaction of reduced PMS with O_2; the superoxide-anion radical then reduces NBT to the highly coloured formazan. The reaction can be followed by measuring the absorbance of formazan at 578 nm. The incubation mixture contained disodium hydrogen phosphate buffer (200 μL, 76 mM, pH 7·4), PMS (200 μL, 10·8 μM), NBT (200 μL, 172 μM) and NADH + H^+ (200 μL, 360 μM). Each assay was performed after a 5-min incubation with several effector concentrations.

Lipid peroxidation assay

Iron-dependent peroxidation of male rat liver microsomes was assayed as previously described (Meunier et al 1989). Liver microsomes were prepared according to the technique of May & McCay (1968). Lipid peroxidation was initiated by the ADP-Fe^{2+}/ascorbate system (Müller et al 1984) which produced hydroxyl radicals ($HO^{\cdot}$). Incubations of 0·2-mL microsomal fractions (1 mg protein mL^{-1}) were carried out at 37°C with 0·2 mL potassium phosphate buffer (0·1M, pH 7·4), 0·2 mL of ADP solution in buffer (2 mM) and 0·2 mL of aqueous $FeSO_4$ solution (16 μM). Lipid peroxidation was started upon the addition of 0·2 mL of aqueous ascorbate solution (0·5 mM) to the above incubation mixture. The extent of polyunsaturated fatty-acid peroxidation was measured spectrophotometrically at 535 nm by the rate of malondialdehyde formed after 90 min microsomal incubation.

Acute toxicity in mice

The compounds were administered intraperitoneally in saline (0·9% NaCl) at doses of 200, 400, 600 and 800 mg kg^{-1}. Swiss male mice purchased from Depre (Saint-Doulchard, France), weighing 18–22 g, were used. Mice were kept in groups of ten in a temperature-controlled room with a 12-h light/dark cycle. Food and water were freely available during the time of the experiment. The mice were observed for 8 days to detect any sign of toxicity. LD50 was determined as the dose causing 50% death in animals.

Results and Discussion

Chemistry

4-Benzoyl-3-hydroxyfuran-2(5 *H*)-ones (**3a–j**) were prepared according to a previously described

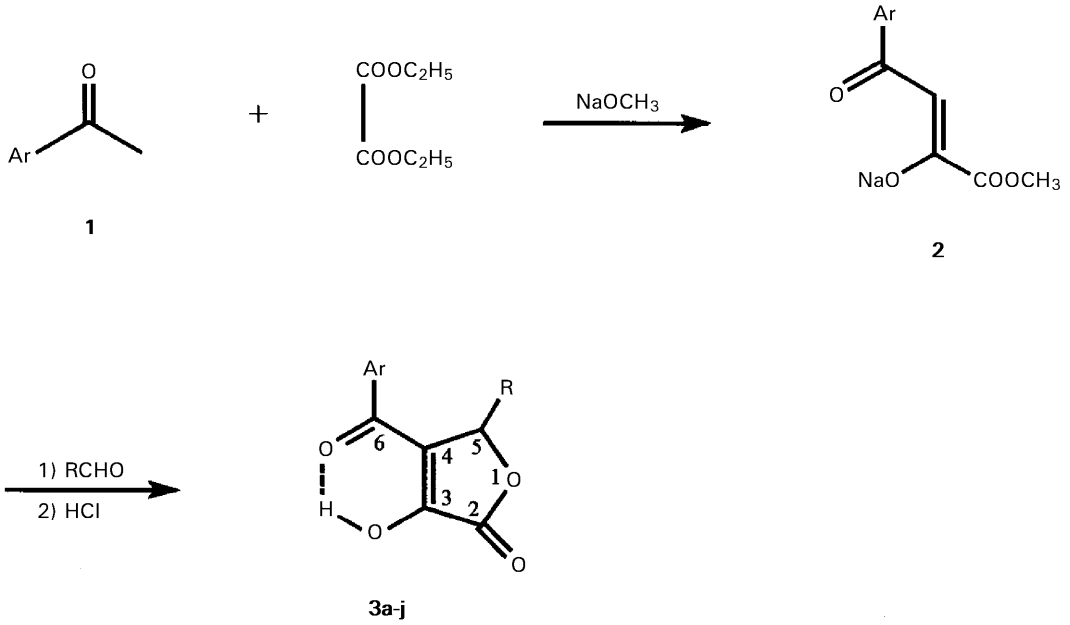


Figure 1. Preparation of 4-benzoyl-3-hydroxyfuran-2(5*H*)-ones **3a–j** from arylmethylketones.

procedure (Durantin et al 1972), as outlined in Figure 1. Claisen condensation between an arylmethylketone (**1**) and diethyl oxalate, in the presence of sodium methoxide, led to sodium enolates (**2**), resulting from the transesterification of methoxide with one of the ethyl ester groups of oxalate. Reaction of **2** with formaldehyde or aromatic aldehydes, followed by acidification with hydrochloric acid, provided α-hydroxylbutenolides, **3a–j**.

The enolic structure of compounds **3a–j** was stabilized by an intramolecular hydrogen bond between the hydroxyl group and the ketonic carbonyl function, which was confirmed by IR and NMR spectral data.

3-Acetyl-5-aryl-3,4-dihydrofuran-2(5*H*)-ones (**5a–f**) were synthesized from α-angelicalactone according to Figure 2 (Couquelet et al 1983). Aromatic aldehydes (**4**) were condensed with α-angelicalactone in the presence of a Lewis acid, boron trifluoride, to give a charged intermediate complex, which rearranged to *β*-acetylbutanolides, **5a–f**.

Table 1 shows the physical constants of compounds **3a–j** and **5a–f**. Tables 2 and 3 show the IR and NMR spectral data.

Biology

It is well known that the ascorbate radical/ascorbate thermodynamic couple is low, compared with the reduction potential of α-tocopherol free radical, glutathione radical, peroxyl radicals and hydroxyl free radical. Because of this, ascorbate will act as an antioxidant in each system, and also with superoxide and urate free radicals (Rose & Bode 1993). Furthermore, primary targets for attack by oxygen-derived free-radical species are the polyunsaturated fatty-acid moieties of membrane phospholipids. Attack on low-density lipoproteins (LDL) must also be considered and is of primary importance in the aetiology of atherosclerosis.

The main biological lipid antioxidant is vitamin E, especially α-tocopherol. There is a synergistic relationship by which ascorbic acid enhances the effectiveness of α-tocopherol by causing its regeneration from the radical form, that is the first consequence of its antioxidant action (Diplock 1994).

In practice, the propensity of ascorbic acid to thermal and oxidative degradation has led to interest in derivatives with increased stability in-vitro, while maintaining its inherent biological activity in-vivo. The chemical modification of hydroxyl groups of ascorbic acid is of particular interest, and numerous stable derivatives have been reported. Among these derivatives, 3-*O*-(dodecylcarbomethyl) ascorbic acid (Nihro et al 1991) and 2-*O*-octadecyl ascorbic acid (Kato et al 1988) have been developed as new lipophilic antioxidants, which exhibit vitamin C activity without enzymatic degradation.

Recently, novel hybrid L-ascorbic acid derivatives together with other biologically active substances such as kojic acid and α-tocopherol, linked at the C-2 or C-3 hydroxyl group, were described (Morisaki & Ozaki 1996). They exhibited good thermal stability and inhibitory activity against tyrosinase-catalysed melanin formation, active oxygen species and free radicals, compared with vitamin C and its conventional derivatives.

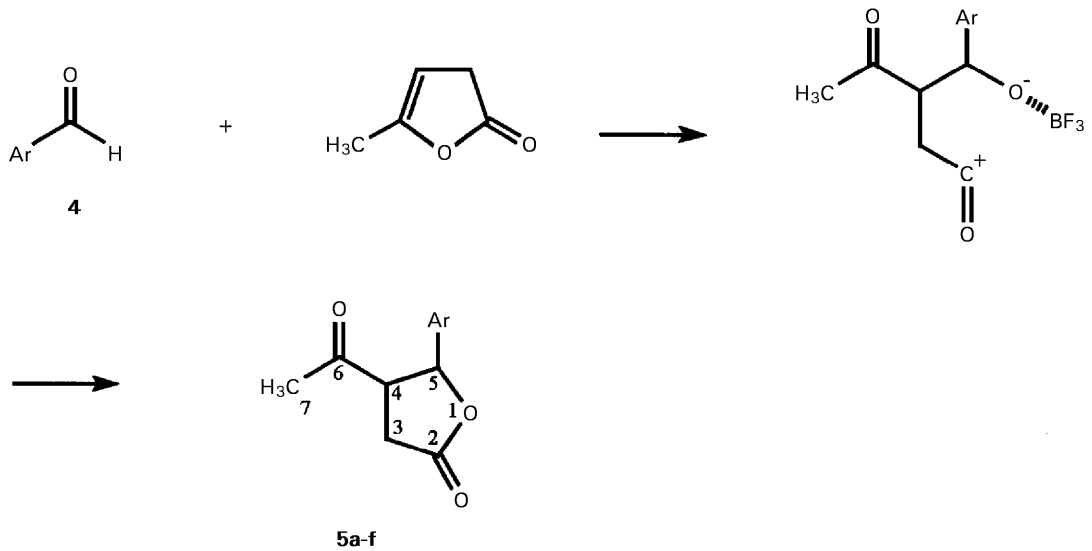
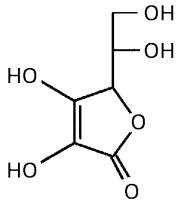
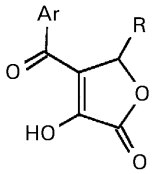


Figure 2. Preparation of 4-acetyl-5-aryl-3,4-dihydrofuran-2(5*H*)-ones **5a–f** from aromatic aldehydes.

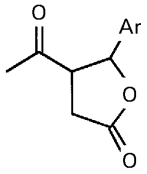
Table 1. Physical constants of compounds **3** and **5**.



Ascorbic acid



3a-j



5a-f

Compound	Ar	R	Formula	M.W.	Yield (%)	mp (°C)
3a	4-OCH_3 C_6H_4	H	$C_{12}H_{10}O_5$	234·20	52	155
3b	3-OCH_3 C_6H_4	H	$C_{12}H_{10}O_5$	234·20	29	149
3c	2-OCH_3 C_6H_4	H	$C_{12}H_{10}O_5$	234·20	36	111
3d	3,4-OCH_3 C_6H_3	H	$C_{13}H_{12}O_6$	264·23	49	179
3e	3,4,5-OCH_3 C_6H_2	H	$C_{14}H_{14}O_7$	294·26	38	174
3f	4-OH C_6H_4	H	$C_{11}H_8O_5$	220·18	13	226
3g	3-OH C_6H_4	H	$C_{11}H_8O_5$	220·18	51	167
3h	2-OH C_6H_4	H	$C_{11}H_8O_5$	220·18	24	228
3i	3-OH C_6H_4	2-OH C_6H_4	$C_{17}H_{12}O_6$	312·27	18	135 (dec)
3j	4-OCH_3 C_6H_4	2-OH C_6H_4	$C_{18}H_{14}O_6$	326·30	20	100
5a	4-OCH_3 C_6H_4	–	$C_{13}H_{14}O_4$	234·25	22	78
5b	3-OCH_3 C_6H_4	–	$C_{13}H_{14}O_4$	234·25	22	oil
5c	2-OCH_3 C_6H_4	–	$C_{13}H_{14}O_4$	234·25	71	126
5d	4-OH C_6H_4	–	$C_{12}H_{12}O_4$	220·22	15	114
5e	3-OH C_6H_4	–	$C_{12}H_{12}O_4$	220·22	24	oil
5f	4-OH-3,5-OCH_3 C_6H_2	–	$C_{14}H_{16}O_6$	280·27	21	125

Table 2. IR and ^{1}H NMR spectral data for compounds **3** and **5**.

Compound	IR (KBr; ν (cm^{-1}))	^{1}H NMR (δppm, in DMSO-d_6[a] or $CDCl_3$[b])
3a[a]	1773, 1647, 1599	3·89 (s, 3H, OCH_3), 5·08 (s, 2H, CH_2), 7·09 (d, 2H arom., J = 8·8 Hz), 7·90 (d, 2H arom., J = 8·8 Hz), 11·84 (br. s, 1H, OH)
3b[a]	1785, 1648, 1601	3·84 (s, 3H, OCH_3), 5·08 (s, 2H, CH_2), 7·24–7·48 (m, 4H, C_6H_4), 12·08 (br. s, 1H, OH)
3c[a]	1778, 1638, 1596	3·80 (s, 3H, OCH_3), 5·03 (s, 2H, CH_2), 7·04–7·52 (m, 4H, C_6H_4), 11·86 (br. s, 1H, OH)
3d[a]	1780, 1637, 1596	3·81 (s, 3H, OCH_3), 3·86 (s, 3H, OCH_3), 5·05 (s, 2H, CH_2), 7·09–7·57 (m, 3H, C_6H_3), 11·83 (br. s, 1H, OH)
3e[a]	1786, 1640, 1591	3·80 (s, 3H, OCH_3), 3·86 (s, 6H, 2OCH_3), 5·08 (s, 2H, CH_2), 7·26 (s, 2H, C_6H_2), 12·05 (br. s, 1H, OH)
3f[a]	3293, 1746, 1647, 1601	5·04 (s, 2H, CH_2), 6·87 (d, 2H arom., J = 8·9Hz), 7·79 (d, 2H arom., J = 8·9 Hz), 10·60 (br. s, 1H, OH)
3g[a]	3318, 1755, 1651, 1605	5·08 (s, 2H, CH_2), 7·06–7·36 (m, 4H, C_6H_4), 9·82 (br. s, 1H, OH), 11·99 (br. s, 1H, OH)
3h[a]	3237, 1775, 1636, 1580	5·05 (s, 2H, CH_2), 6·93–7·57 (m, 4H, C_6H_4) 11·07 (br. s, 1H, OH)
3i[a]	3467, 3357, 1740, 1628, 1589	7·09–7·46 (m, 9H, 2 C_6H_4 + CH), 9·85 (br. s, 1H, OH)
3j[a]	3486, 1736, 1626, 1601	3·86 (s, 3H, OCH_3), 7·43 (s, 1H, CH), 7·03–7·72 (m, 8H, 2 C_6H_4)
5a[b]	1790, 1708, 1614	2·10 (s, 3H, $COCH_3$), 2·83 (dd, 1H, J_1 = 17·6 Hz, J_2 = 9·2 Hz, H-3a), 2·93 (dd, 1H, J_1 = 17·6 Hz, J_2 = 9·9 Hz, H-3b), 3·52 (m, 1H, H-4), 3·80 (s, 3H, OCH_3), 5·43 (d, 1H, J = 8·2 Hz, H-5), 6·90–7·26 (m, 4H, C_6H_4)
5b[b]	1778, 1713, 1592	2·15 (s, 3H, $COCH_3$), 2·89 (m, 2H, H-3a and H-3b), 3·49 (m, 1H, H-4), 3·80 (s, 3H, OCH_3), 5·51 (d, 1H, J = 7·7 Hz, H-5), 6·87–7·30 (m, 4H, C_6H_4)
5c[b]	1785, 1704, 1602	2·25 (s, 3H, $COCH_3$), 2·70 (dd, 1H, J_1 = 17·7 Hz, J_2 = 9·4 Hz, H-3a), 2·86 (dd, 1H, J_1 = 17·7 Hz, J_2 = 6·1 Hz, H-3b), 3·49 (m, 1H, H-4), 3·85 (s, 3H, OCH_3), 5·71 (d, 1H, J = 5·1 Hz, H-5), 6·93–7·35 (m, 4H, C_6H_4)
5d[a]	3206, 1739, 1707, 1617	2·10 (s, 3H, $COCH_3$), 2·92 (m, 2H, H-3a and H-3b), 3·70 (m, 1H, H-4), 5·49 (d, 1H, J = 7·6 Hz, H-5), 6·82 (d, 2H arom., J = 8·4 Hz), 7·29 (d, 2H arom., J = 8·4 Hz), 9·67 (s, 1H, OH)
5e[b]	3541, 1782, 1715, 1604	2·13 (s, 3H, $COCH_3$), 2·86 (m, 2H, H-3a and H-3b), 3·49 (m, 1H, H-4), 5·47 (d, 1H, J = 7·5 Hz, H-5), 6·83–7·20 (m, 4H, C_6H_4)
5f[b]	3439, 1789, 1713, 1617	2·15 (s, 3H, $COCH_3$), 2·92 (m, 2H, H-3a and H-3b), 3·54 (m, 1H, H-4), 3·89 (s, 6H, 2 OCH_3), 5·46 (d, 1H, J = 7·6 Hz, H-5), 5·72 (br s, 1H, OH), 6·57 (s, 2H, C_6H_2)

Table 3. ^{13}C NMR chemical shifts of compounds **3** and **5** (δppm in DMSO-d_6[a] or $CDCl_3$[b]).

Compound	C-2	C-3	C-4	C-5	C-6	C-7	Ar, C_{ipso}	C_{ar}-OCH_3 or C_{ar}-OH	OCH_3
3a[a]	169·78	143·39	122·43	68·11	187·74	–	113·68–131·53 (3)	163·41	55·58
3b[a]	169·83	144·64	121·53	68·06	189·20	–	113·75–138·48 (5)	159·05	55·35
3c[a]	169·85	145·81	122·51	67·49	189·09	–	111·74–132·45 (5)	157·23	55·75
3d[a]	169·81	143·28	122·49	68·16	187·68	–	110·74–129·44 (4)	148·34	55·52
								153·38	55·78
3e[a]	169·89	144·07	121·75	68·16	188·06	–	106·76–131·97 (2)	141·91	56·04
								152·55	60·21
3f[a]	169·85	143·09	122·77	68·19	187·54	–	115·09–131·91 (3)	162·53	–
3g[a]	169·75	144·22	121·86	68·08	189·46	–	115·22–138·40 (5)	157·36	–
3h[a]	169·60	144·88	122·30	67·68	191·30	–	116·81–134·48 (5)	158·19	–
3i[a]	169·16	130·11	118·67	113·83	192·91	–	94·70–138·79 (10)	151·38	–
								157·39	
3j[a]	169·21	130·19	118·81	129·62	191·64	–	94·93–135·28 (8)	151·34	55·58
								162·66	–
5a[b]	174·07	32·09	56·00	81·98	204·34	30·19	114·37–129·72 (3)	160·22	55·34
5b[b]	174·01	31·90	55·85	81·60	204·37	30·17	111·19–139·69 (5)	160·03	55·32
5c[b]	175·31	30·59	55·26	78·70	205·23	29·24	110·82–130·02 (5)	155·93	53·49
5d[a]	174·76	31·36	54·40	81·14	205·70	29·27	115·40–128·64 (3)	157·92	–
5e[b]	175·40	31·83	55·60	81·97	205·13	30·04	112·48–139·46 (5)	156·75	–
5f[b]	173·99	32·42	82·39	102·62	204·51	30·44	128·89	147·38	56·44
								135·33	56·32

Table 4. Antioxidant activity of compounds **3** and **5**.

Compound	Reducing activity on DPPH (% inhibition at 5×10^{-3} M)	Superoxide-anion scavenging activity (% inhibition at 1 mg mL^{-1})	Lipid-peroxidation inhibitory activity (% inhibition at 1 mg mL^{-1})	log k′
3a	26·4 ± 0·8	NT	NT	1·14
3b	29·9 ± 1·0	NT	NT	1·25
3c	55·0 ± 0·5	58 ± 4	9 ± 2 (NS)	0·75
		(IC50 = $3·63 \times 10^{-3}$ M)		
3d	29·5 ± 0·5	NT	NT	0·91
3e	31·2 ± 0·7	NT	NT	0·90
3f	36·1 ± 3·1	NT	NT	0·83
3g	33·2 ± 3·8	91 ± 8	0	0·80
		(IC50 = $1·45 \times 10^{-3}$ M)		
3h	51·1 ± 1·9	NT	NT	1·05
3i	47·7 ± 9·1	89 ± 4	7 ± 1 (NS)	0·84
		(IC50 = $1·35 \times 10^{-3}$ M)		
3j	6·0 ± 2·0	NT	NT	1·70
5a	60·6 ± 12·9	NT	NT	0·81
5b	10·0 ± 0·5	55 ± 24	18 ± 2 (NS)	0·84
		(IC50 = $3·93 \times 10^{-3}$ M)		
5c	35·8 ± 1·0	NT	NT	0·72
5d	16·4 ± 3·9	20 ± 3 (NS)	11 ± 3 (NS)	0·39
5e	12·7 ± 2·4	26 ± 4 (NS)	13 ± 4 (NS)	0·48
5f	87·3 ± 0·4	29 ± 2 (NS)[a]	98 ± 1	1·19
			(IC50 = $4·85 \times 10^{-4}$ M)[a]	
Vitamin C	93·3 ± 1·8	24·0 ± 1·5	Pro-oxidant	
Vitamin E	IC50 = $1·96 \times 10^{-5}$ M	NT	NT	

NT: not tested; NS: not significant; [a]% inhibition at 0·5 mg mL^{-1}. IC5O: dose resulting in 50% inhibition.

Taking these data into account, we decided to synthesize new ascorbic acid analogues, including lipophilic aromatic moieties and without the enediol structure, which would retain antioxidative properties while having no autooxidative potential.

As antioxidant action is considered to be a complex process which may include prevention of formation or scavenging of free radicals, it was of interest to investigate the interaction of the synthesized compounds with the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). This interaction expressed the reducing activity of test compounds and indicated their ability to scavenge free radicals (Ratty et al 1988). Most of the tested compounds were found to interact with DPPH, but the order of this activity did not proceed in parallel

with that in the other two series of experiments (Table 4). 4-Benzoyl derivatives **3c** and **3h** exhibited more than 50% inhibition at a concentration of 5×10^{-3} M, while 93·3% inhibition was observed with ascorbic acid. It is noteworthy that in this test, the antioxidant activity of compounds of series **3** seemed to be increased by the electron-donating substituent OCH_3 or OH in the ortho position on the benzoyl aromatic nucleus.

In the series of 4-acetylbutanolides, **5**, two derivatives exhibited a good antioxidant activity, with 60·6% inhibition for **5a** and 87·3% for **5f**.

Contrary to the 4-benzoyl derivatives (series **3**), the activity of compounds of series **5** was increased by the presence of OCH_3 in the para position of the aryl nucleus (**5a**), or of OH in the same position, whereas two methoxy substituents occupied the neighbouring positions.

Surprisingly, compound **5d** was almost inactive. However, it was noted that the aromatic nucleus in question in each series was not the same one. In the 4-benzoylbutenolides (**3a–j**), the influence of the 5-aryl substituent on the olide ring appeared less important than in the 4-acetyl series (**5**).

Lipophilicity is a well known major factor controlling bio-activities, and the logarithm of the capacity factor (log k′) obtained from reversed-phase HPLC data has often been used to estimate hydrophobicity of compounds (Yamagami & Masaki 1995). Considering the importance of lipophilicity in the emergence of antioxidant properties of molecules (Ohkawa et al 1991; Yu et al 1993), we chose to determine capacity factors for an eluent containing 50% methanol. This mobile phase composition appeared to be the most reliable for predicting the conventionally hydrophobic parameter, log P value, for compounds possessing hydrogen-bonding substituents (Yamagami & Masaki 1995). Due to a lengthy retention time resulting in degradation of compounds, log k′ values (50% methanol) were obtained by linear extrapolation of log k_i determined from mobile phases containing 65–95% methanol.

From the data reported in Table 4, no clear correlation could be established between lipophilicity of the compounds and their activity in reducing DPPH.

Some of the synthesized compounds were evaluated as superoxide-anion scavengers as well as for their in-vitro lipid-peroxidation inhibitory effects. Others could not be tested due to their poor solubility in the biological reaction mixture.

In the superoxide anion scavenging assay, at a concentration of 1 mg mL^{-1}, compounds **3g** and **3i** exhibited a potent activity with IC50 (dose resulting in 50% inhibition) values of $1{\cdot}45 \times 10^{-3}$ M and $1{\cdot}35 \times 10^{-3}$ M, respectively, whereas ascorbic acid was almost inactive (Table 4). It was observed that a log k′ value of near 0·80 seemed to correlate with this activity.

Lipid peroxidation, the oxidative modification of polyunsaturated fatty acyl chains that can damage cell membranes, is considered to be a cause and effect of a range of conditions related to oxygen toxicity (Dix & Aikens 1993). Lipid peroxidation consists of a radical-initiated reaction and can serve as a suitable system for evaluating the antioxidative properties of synthesized derivatives. The autooxidation of Fe^{2+}/ADP/ascorbate causes massive lipid peroxidation in liver microsomes, and this can be monitored by the generation of malondialdehyde. When investigating the effects of the new ascorbic acid derivatives (series **3** and **5**) on lipid peroxidation, compound **5f** was found to significantly reduce malondialdehyde production with an IC50 value of $4{\cdot}85 \times 10^{-4}$ M. Similarly, Schmid et al (1993) reported the synthesis of 2-*O*-methyl-6-(alkylthio)ascorbic acid derivatives, one of which (6-*S*-*n*-hexadecylthio derivative) inhibited the Fe^{2+}/ADP/ascorbate-induced lipid peroxidation of rat liver microsomes with an IC50 value of 2 μM. The results of the lipid-peroxidation assay confirm the good activity of compound **5f** in the test for reducing activity of DPPH.

Due to the antioxidant activity of **5f** exhibited in the three in-vitro tests, its acute toxicity was investigated. It appeared that substitution of the furan nucleus by an acetyl group at the 4-position enhanced the toxicity of **5f** (LD50 $\approx$ 200 mg kg^{-1} i.p.) with regard to ascorbic acid (LD50 $>$ 800 mg kg^{-1} i.p.). At a dose of 500 mg kg^{-1} intraperitoneally, **5f** killed all mice. However, considered as a whole group, compounds **5a–f** (LD50 $>$ 150 mg kg^{-1} i.p.) were less toxic than the 4-benzoyl derivatives **3a–j** (LD50 $<$ 100 mg kg^{-1} i.p.), as has been observed previously (Point et al 1998).

It can be concluded from this work that 4-acetyl-5-(4-hydroxy-3,5-dimethoxyphenyl)-3,4-dihydrofuran-2(5*H*)-one (compound **5f**) seems to be the best candidate for the development of an efficient new ascorbic acid analogue, which may be useful in the treatment of diseases involving free radicals. Furthermore, although no clear structure–activity relationships could be established in these new series, it appears that, contrary to the currently assumed hypothesis, an enol function is not absolutely necessary to endow antioxidant properties.

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