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Interactions of a series of novel spiropyranocoumarin derivatives with reactive oxygen species

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Abstract

A series of new spiro-substituted pyranocoumarin derivatives have been synthesized starting from the commercially available 7-hydroxycoumarin and the conformation of the pyran ring was investigated. The antioxidant activity of the compounds was evaluated in-vitro, by means of three different tests: the interaction with the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), the competition with DMSO for hydroxyl radicals scavenging ability and the quenching of superoxide anions generated by the enzymic xanthine–xanthine oxidase system. In the DPPH test the spiroadamantane derivative **13** was the most active and possessed a 40% inhibition at a concentration of 400 μM. All compounds successfully compete with DMSO for hydroxyl radicals generated by the Fe^{3+}/ascorbic acid system. Compound **13** inhibited the oxidation of DMSO (3.125 mM) by 93% at 2 mM and by 71% at 0.25 mM. The corresponding second-order rate constants have been estimated and all compounds demonstrated higher rate constants compared with the reference compounds, 7-hydroxycoumarin and mannitol. Derivatives possessing extended conjugation showed the highest inhibitory activity for superoxide anions generated by the xanthine–xanthine oxidase system, although the results of this experiment possessed partial parallelism with the results observed in the other two tests. The overall obtained data indicate that the size of the different spiro- substituents influence the degree of free radical scavenging and demonstrate the importance of extended conjugation for the antioxidant activity. Due to its multiple mechanism of protective action, derivative **13** may serve as a lead for the development of analogues that could be useful for the treatment of pathophysiological processes dependent upon reactive oxygen species.

Introduction

A wide variety of pathological conditions, including cancer, inflammation, ischaemia, atherosclerosis and regressive changes in the ageing process, appear to have aetiological relation to the reactive oxygen species (ROS)-induced and free-radical-mediated oxidation of biomolecules, which takes place in states associated with inadequate antioxidant defence or with oxidative stress (Halliwell & Gutteridge 1986; Esterbauer & Cheesemann 1987; Ross & Glomset 1993). Prevention of the initial cellular damage caused by these species has been the subject of intense investigation and resulted in the discovery of several substances, naturally occurring or synthetic, which have been accredited as potent antioxidants (Halliwell 1990). These compounds could be useful as drug candidates, for combating degenerative changes and retarding the progress of the aforementioned diseases. In this respect a number of compounds widely distributed in the plant kingdom have drawn considerable attention and among them some flavone, xanthone and coumarin derivatives (Barclay et al 1990; Sato et al 1992; Hoult & Paya 1996; Rice-Evans et al 1996) are reported to possess beneficial radical-scavenging effects and can serve as lead compounds for the design of novel pharmacologically related analogues.

It has been shown that coumarins affect the formation and scavenging of ROS and influence processes involving free-radical-mediated injury, such as inflammation (Paya et al 1992). Coumarin itself (1,2-benzopyrone) inhibits the formation of superoxide radicals to a certain extent, while 4-hydroxycoumarin and its derivatives exhibit

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reducing activity against the stable 1,1-diphenyl 2-picrylhydrazyl (DPPH) free radical (Kirkiacharian et al 1999). It should also be noted that coumarin and 7-hydroxycoumarin have been patented in Germany for the scavenging of free radicals (Elstner et al 1988). Simple coumarins, such as fraxetin and esculetin, inhibit lipid peroxidation and scavenge superoxide anion radicals, but they may exert potentially damaging pro-oxidant actions in the presence of free ferric anions (Paya et al 1994; Martŝn-Aragón et al 1997, 1998). It has been reported that the antioxidative action of coumarin derivatives may include trapping of the initiating radicals, trapping the propagating lipid peroxyl radicals, recycling α-tocopherol or deactivating the excited photosensitizer (Raj et al 1998; Yu et al 1999). Nevertheless, the exact mechanism of the tissue-protective antioxidant activity of these compounds has not yet been established.

Prompted by the interesting free-radical scavenging activity of several coumarin derivatives we have accomplished the synthesis and evaluated the radical-scavenging activity of some new lipophilic pyranocoumarins, in an effort to elucidate the structural requirements and thus contribute in the structure–activity studies of this class of compounds.

Materials and Methods

All chemical were purchased from Aldrich Chemical Co. (Brussels, Belgium). The xanthine oxidase was purchased from Sigma Chemical Co. (St Louis, MO). Melting points were determined on a Büchi apparatus and are uncorrected. ^{1}H NMR spectra and 2-D spectra were recorded on a Bruker Avanche DRX-400 instrument, whereas ^{13}C NMR spectra were recorded on a Bruker AC 200 spectrometer in deuterated solvents and were referenced to TMS (δ scale). The signals of ^{1}H and ^{13}C spectra were unambiguously assigned by using 2D NMR techniques: COSY, NOESY HMQC and HMBC. Flash chromatography was performed on Merck silica gel 60 (0.040–0.063 mm). Analytical thin-layer chromatography (TLC) was carried out on precoated (0.25 mm) Merck silica gel F-254 plates. Elemental analyses were within $\pm$0.4% of the theoretical values.

9′,10′-Dihydrospiro[cyclopentane-1,8′(8′*H*)-pyran[2,3-*h*]benzo[*b*]pyran]-2′(2′*H*),10′-dione (5a)

A solution of 8-acetyl-7-hydroxycoumarin (3, Figure 1) (Prashant et al 2001) (1.7 g, 8.33 mmol), cyclopentanone (0.91 g, 10.87 mmol) and pyrrolidine (0.18 mL, 2.17 mmol) in anhydrous toluene was refluxed for 1 h using a Dean-Stark apparatus. The reaction mixture was extracted with HCl 9% to remove the bulk of pyrrolidine, the organic phase was dried (Na_2SO_4) and evaporated to dryness. The residue was purified by column chromatography (silica gel) using cyclohexane–ethyl acetate (5:1 to 2:1) as the eluent to give 5a (1.76 g, 78%). mp: 184–185 °C (Et_2O). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.6–2.2 (m, 8H, cyclopentane H), 2.85 (s, 2H, H-9′), 6.29 (d, 1H, J = 9.5 Hz, H-3′), 6.83 (d, 1H, J = 8.4 Hz, H-6′), 7.48 (d, 1H, J = 8.4 Hz, H-5′), 7.58 (d, 1H, J = 9.5 Hz, H-4′). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 23.79, 37.34 (cyclopentane C), 47.81 (C-9′), 91.00 (C-8′), 109.48 (C-10′a), 112.38 (C-4′a), 113.52 (C-3′), 115.25 (C-6′), 133.92 (C-5′), 143.20 (C-4′), 153.77 (C-10′b), 159.98 (C-2′), 163.49 (C-6′a), 188.89 (C-10′). Anal. Calcd for $C_{16}H_{14}O_4$: C: 71.10, H: 5.22. Found: C: 70.82, H: 5.13.

9′,10′-Dihydrospiro[cyclohexane-1,8′(8′*H*)-pyran [2,3-*h*]benzo[*b*]pyran]-2′(2′*H*),10′-dione (5b)

Compound 5b was prepared according to the procedure described for 5a.

Yield: 81%. mp: 167–168 °C (Et_2O).^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.2–2.0 (m, 10H, cyclohexane H), 2.70 (s, 2H, H-9′), 6.23 (d, 1H, J = 9.5 Hz, H-3′), 6.85 (d, 1H, J = 8.8 Hz, H-6′), 7.50 (d, 1H, J = 8.8 Hz, H-5′), 7.60

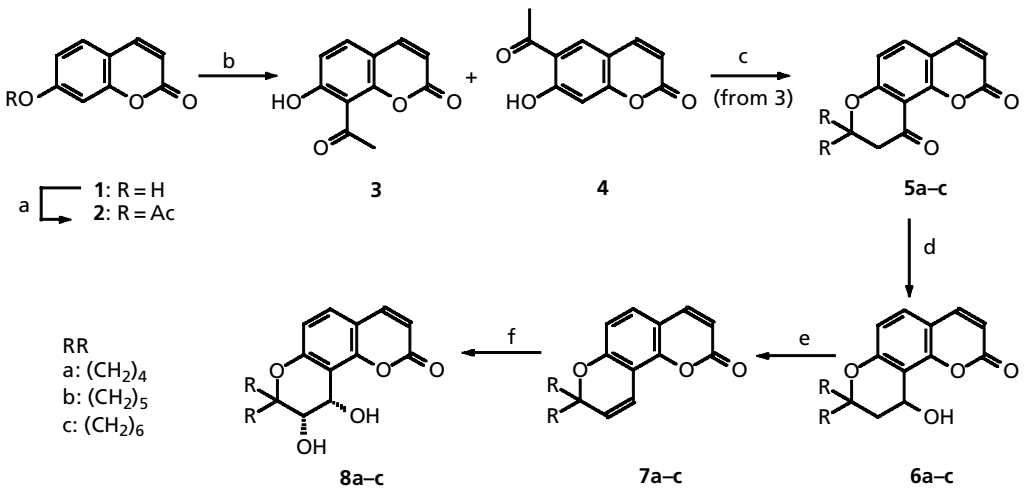

Figure 1 Synthesis of the spiropyranocoumarins. Reagents: a, Ac_2O, 140 °C; b, $AlCl_3$, 100 °C; c, carbocyclic ketone, pyrrolidine, toluene, 110 °C; d, $NaBH_4$, MeOH, room temperature; e, p-TsOH, toluene, reflux; f, OsO_4, N-methylmorpholine-N-oxide, room temperature.

(d, 1H, J = 9.5 Hz, H-4′). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 21.21, 24.81, 34.40 (cyclohexane C), 48.74 (C-9′), 81.23 (C-8′), 109.12 (C-10′a), 112.25 (C-4′a), 113.24 (C-3′), 115.04 (C-6′), 134.08 (C-5′), 143.27 (C-4′), 153.52 (C-10′b), 159.95 (C-2′), 162.78 (C-6′a), 188.84 (C-10′). Anal. Calcd for $C_{17}H_{16}O_4$: C: 71.82, H: 5.67. Found: C: 71.68, H: 5.44.

9′,10′-Dihydrospiro[cycloheptane-1,8′(8′*H*)-pyran [2,3-*h*]benzo[*b*]pyran]-2′(2′*H*),10′-dione (5c)

Compound 5c was prepared according to the procedure described for 5a.

Yield: 83% . mp: 154–156 °C (Et_2O). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.3–2.2 (m, 12H, cycloheptane H), 2.74 (s, 2H, H-9′), 6.24 (d, 1H, J = 9.5 Hz, H-3′), 6.82 (d, 1H, J = 8.8 Hz, H-6′), 7.48 (d, 1H, J = 8.8 Hz, H-5′), 7.58 (d, 1H, J = 9.5 Hz, H-4′). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 21.80, 26.81, 37.99 (cycloheptane C), 49.62 (C-9′), 85.64 (C-8′), 109.21 (C-10′a), 112.21 (C-4′a), 113.33 (C-3′), 115.13 (C-6′), 134.00 (C-5′), 143.19 (C-4′), 153.59 (C-10′b), 159.97 (C-2′), 163.02 (C-6′a), 188.93 (C-10′). Anal. Calcd for $C_{18}H_{18}O_4$: C: 72.47, H: 6.08. Found: C: 72.66, H: 5.92.

(±)-9′,10′-Dihydro-10′-hydroxyspiro [cyclopentane-1,8′(8′*H*)-pyran [2,3-*h*]benzo[*b*]pyran]-2′(2′*H*)-one (6a)

$NaBH_4$ (136 mg, 3.59 mmol) was added to a solution of (5a) (880 mg, 3.26 mmol) in methanol (20 mL) at 0 °C and the reaction mixture was stirred at room temperature for 24 h. The solvent was vacuum-evaporated and the residue was acidified with 2 M HCl, extracted with dichloromethane, dried (Na_2SO_4) and concentrated to dryness. The residue was purified by column chromatography (silica gel) using cyclohexane–ethyl acetate (1:1) as the eluent to provide 6a (825 mg, 93%). mp: 128–130 °C (EtOAc–n-hexane). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.5–2.0 (m, 8H, cyclopentane H), 2.13 (m, 2H, H-9′), 3.92 (d, 1H, J = 3.7 Hz, OH, D_2O exchangeable), 5.17 (ddd, 1H, J = 5.1 Hz, J = 4.3 Hz, J = 3.7 Hz, H-10′), 6.12 (d, 1H, J = 9.5 Hz, H-3′), 6.65 (d, 1H, J = 8.8 Hz, H-6′), 7.19 (d, 1H, J = 8.8 Hz, H-5′), 7.55 (d, 1H, J = 9.5 Hz, H-4′). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 23.05, 23.98 (cyclopentane C), 36.86 (C-9′), 38.02, 38.37 (cyclopentane C), 59.01 (C-10′), 86.87 (C-8′), 111.64 (C-3′, C-4′a), 112.47 (C-10′a) 114.84 (C-6′), 127.88 (C-5′), 144.06 (C-4′), 153.94 (C-10′b), 157.07 (C-6′a), 160.94 (C-2′). Anal. Calcd for $C_{16}H_{16}O_4$: C: 70.58, H: 5.92. Found: C: 70.34, H: 6.01.

(±)-9′,10′-Dihydro-10′-hydroxyspiro [cyclohexane-1,8′(8′*H*)-pyran[2,3-*h*] benzo[*b*]pyran]-2′(2′*H*)-one (6b)

Compound 6b was prepared according to the procedure described for 6a.

Yield: 95% . mp: 138–139 °C (EtOAc–n-hexane).^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.2–2.1 (m, 10H, cyclohexane H), 2.09 (m, 2H, H-9′), 3.38 (brs, 1H, OH, D_2O exchangeable), 5.21 (m, 1H, H-10′), 6.20 (d, 1H, J = 9.5 Hz, H-3′), 6.79 (d, 1H, J = 8.8 Hz, H-6′), 7.28 (d, 1H, J = 8.8 Hz, H-5′), 7.62 (d, 1H, J = 9.5 Hz, H-4′). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 21.65, 25.42, 34.74, 35.66 (cyclohexane C), 39.12 (C-9′), 59.12 (C-10′), 77.23 (C-8′), 111.90 (C-3′, C-4′a), 112.63 (C-10′a), 114.97 (C-6′), 128.18 (C-5′), 144.22 (C-4′), 154.22 (C-10′b), 156.77 (C-6′a), 160.80 (C-2′). Anal. Calcd for $C_{17}H_{18}O_4$: C: 71.31, H: 6.34. Found: C: 71.53, H: 6.39.

(±)-9′10′-Dihydro-10′-hydroxyspiro [cycloheptane-1,8′(8′*H*)-pyran[2,3-*h*] benzo[*b*]pyran]-2′(2′*H*)-one (6c)

Compound 6c was prepared according to the procedure described for 6a.

Yield: 91% . mp: 171–173 °C (EtOAc–n-hexane). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.4–2.1 (m, 12H, cycloheptane H), 2.12 (m, 2H, H-9′), 3.62 (brs, 1H, OH, D_2O exchangeable), 5.18 (m, 1H, H-10′), 6.18 (d, 1H, J = 9.5 Hz, H-3′), 6.74 (d, 1H, J = 8.8 Hz, H-6′), 7.25 (d, 1H, J = 8.8 Hz, H-5′), 7.60 (d, 1H, J = 9.5 Hz, H-4′). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 22.05, 29.58 (cycloheptane C), 37.93 (C-9′), 38.96, 40.06 (cycloheptane C,), 59.17 (C-10′), 81.67 (C-8′), 111.73 (C-3′), 111.97 (C-4′a), 112.58 (C-10′a), 115.08 (C-6′), 128.12 (C-5′), 144.26 (C-4′), 154.14 (C-10′b), 156.94 (C-6′a), 160.91 (C-2′). Anal. Calcd for $C_{18}H_{20}O_4$: C: 71.98, H: 6.71. Found: C: 72.16, H: 6.59.

Spiro[cyclopentane-1,8′(8′*H*)-pyran [2,3-*h*]benzo[*b*]pyran]-2′(2′*H*)-one (7a)

A solution of 6a (887 mg, 3.26 mmol) and p-toluenesulfonic acid (62 mg, 0.33 mmol) was refluxed in anhydrous toluene for 12 h using a Dean-Stark apparatus. The reaction mixture was extracted with water; the organic layer was dried (Na_2SO_4) and evaporated to dryness. The residue was purified by column chromatography (silica gel) using a mixture of cyclohexane–ethyl acetate (2:1) as the eluent to give 7a (720 mg, 87%). mp: 119–121 °C (Et_2O). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.5–2.1 (m, 8H, cyclopentane H), 5.71 (d, 1H, J = 10.1 Hz, H-9′), 6.16 (d, 1H, J = 9.5 Hz, H-3′), 6.64 (d, 1H, J = 8.6 Hz, H-6′), 6.84 (d, 1H, J = 10.1 Hz, H-10′), 7.14 (d, 1H, J = 8.6 Hz, H-5′), 7.55 (d, 1H, J = 9.5 Hz, H-4′). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 23.50, 39.73 (cyclopentane C), 88.29 (C-8′), 109.85 (C-10′a), 112.44 (C-3′), 112.50 (C-4′a), 113.44 (C-6′), 115.60 (C-10′), 127.48 (C-5′), 129.66 (C-9′), 143.81 (C-4′), 149.91 (C-10′b), 156.22 (C-6′a), 160.92 (C-2′). Anal. Calcd for $C_{16}H_{14}O_3$: C: 75.58, H: 5.55. Found: C: 75.43, H: 5.48.

Spiro[cyclohexane-1,8′(8′*H*)-pyran[2,3-*h*] benzo[*b*]pyran]-2′(2′*H*)-one (7b)

Compound 7b was prepared according to the procedure described for 7a.

Yield: 90% . mp: 142–143 °C (Et_2O). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.3–2.0 (m, 10H, cyclohexane H), 5.73 (d, 1H, J = 10.1 Hz, H-9′), 6.19 (d, 1H, J = 9.5 Hz, H-3′), 6.73 (d, 1H, J = 8.2 Hz, H-6′), 6.85 (d, 1H, J = 10.1 Hz,

H-10′), 7.18 (d, 1H, J = 8.2 Hz, H-5′), 7.56 (d, 1H, J = 9.5 Hz, H-4′). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 21.14, 25.06, 36.03 (cyclohexane C), 78.28 (C-8′), 110.06 (C-10′a), 112.40 (C-3′), 112.49 (C-4′a), 113.58 (C-6′), 115.42 (C-10′), 127.65 (C-5′), 130.49 (C-9′), 143.90 (C-4′), 150.01 (C-10′b), 156.27 (C-6′a), 161.05 (C-2′). Anal. Calcd for $C_{17}H_{16}O_3$: C: 76.10, H: 6.01. Found: C: 75.88, H: 5.93.

Spiro[cycloheptane-1,8′(8′*H*)-pyran[2,3-*h*] benzo[*b*]pyran]-2′(2′*H*)-one (7c)

Compound 7c was prepared according to the procedure described for 7a.

Yield: 95%. mp: 126 °C (Et_2O). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.4–2.1 (m, 12H, cycloheptane H), 5.76 (d, 1H, J = 10.0 Hz, H-9′), 6.19 (d, 1H, J = 9.3 Hz, H-3′), 6.71 (d, 1H, J = 8.3 Hz, H-6′), 6.81 (d, 1H, J = 10.0 Hz, H-10′), 7.17 (d, 1H, J = 8.3 Hz, H-5′), 7.56 (d, 1H, J = 9.3 Hz, H-4′). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 21.58, 29.55, 39.62 (cycloheptane C), 82.38 (C-8′), 109.89 (C-10′a), 112.52 (C-3′, C-4′a), 113.77 (C-6′), 114.01 (C-10′), 127.63 (C-5′), 131.41 (C-9′), 143.98 (C-4′), 150.05 (C-10′b), 156.31 (C-6′a), 161.15 (C-2′). Anal. Calcd for $C_{18}H_{18}O_3$: C: 76.57, H: 6.43. Found: C: 76.70, H: 6.38.

(±)-*cis*-9′,10′-Dihydro-9′,10′-dihydroxyspiro[cyclopentane-1,8′(8′*H*)-pyran [2,3-*h*]benzo [*b*]pyran]-2′(2′*H*)-one (8a)

Compound 7a (1 g, 3.73 mmol) was added to a solution of OsO_4 (2.5% in tert-butanol, 0.12 mL, 0.373 mmol) and 4-methylmorpholine-N-oxide (600 mg, 5.18 mmol) in tert-BuOH–tetrahydrofuran (THF)–H_2O (10:3:1, 10 mL). The reaction mixture was stirred at room temperature for 3 days. A saturated $NaHSO_3$ solution (5 mL) was then added and the mixture was stirred at room temperature for 2 h. The solvents were removed under vacuum and the residue was purified by column chromatography (silica gel) using cyclohexane–ethyl acetate (3:2) as the eluent to give 8a (840 mg, 74%). mp: 181 °C (EtOH). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.6–2.0 (m, 8H, cyclopentane H), 3.39 (d, 1H, J = 5.1 Hz, 9′-OH, D_2O exchangeable), 3.97 (dd, 1H, J = 5.1, 4.0 Hz, H-9′), 4.25 (d, 1H, J = 4.0 Hz, 10′-OH, D_2O exchangeable), 5.17 (t, 1H, J = 4.0 Hz, H-10′), 6.21 (d, 1H, J = 9.5Hz, H-3′), 6.76 (d, 1H, J = 8.8 Hz, H-6′), 7.28 (d, 1H, J = 8.8 Hz, H-5′), 7.62 (d, 1H, J = 9.5 Hz, H-4′). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 23.76, 24.36, 33.14, 34.97 (cyclopentane C), 61.40 (C-10′), 69.22 (C-9′), 90.05 (C-8′), 111.55 (C-10′a), 111.92 (C-4′a), 112.21 (C-3′), 115.06 (C-6′), 128.40 (C-5′), 144.45 (C-4′), 154.52 (C-10′b), 156.78 (C-6′a), 161.40 (C-2′). Anal. Calcd for $C_{16}H_{16}O_5$: C: 66.66, H: 5.59. Found: C: 66.43, H: 5.64.

(±)-*cis*-9′,10′-Dihydro-9′,10′-dihydroxyspiro [cyclohexane-1,8′(8′*H*)-pyran[2,3-*h*]benzo [*b*]pyran]-2′(2′*H*)-one (8b)

Compound 8b was prepared according to the procedure described for 8a.

Yield: 77%. mp: 183–185 °C (EtOH). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.4–2.2 (m, 10H, cyclohexane H), 3.16 (d, 1H, J = 4.4 Hz, 9′-OH, D_2O exchangeable), 3.85 (dd, 1H, J = 4.9 Hz, J = 4.4 Hz, H-9′), 3.99 (brs, 1H, 10′-OH, D_2O exchangeable), 5.18 (d, 1H, J = 4.9 Hz, H-10′), 6.22 (d, 1H, J = 9.5 Hz, H-3′), 6.84 (d, 1H, J = 8.8 Hz, H-6′), 7.31 (d, 1H, J = 8.8 Hz, H-5′), 7.64 (d, 1H, J = 9.5 Hz, H-4′). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 20.91, 21.04, 25.27, 29.84, 31.46 (cyclohexane C), 61.62 (C-10′), 70.46 (C-9′), 79.66 (C-8′), 110.81 (C-10′a), 112.10 (C-4′a), 112.23 (C-3′), 114.86 (C-6′), 128.58 (C-5′), 144.22 (C-4′), 154.71 (C-10′b), 156.20 (C-6′a), 160.52 (C-2′). Anal. Calcd for $C_{17}H_{18}O_5$: C: 67.54, H: 6.00. Found: C: 67.63, H: 6.17.

(±)-*cis*-9′10′-Dihydro-9′,10′-dihydroxyspiro [cycloheptane-1,8′(8′*H*)-pyran[2,3-*h*]benzo [*b*]pyran]-2′(2′*H*)-one (8c)

Compound 8c was prepared according to the procedure described for 8a.

Yield: 71%. mp: 201–202 °C (EtOH). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.2–2.31 (m, 12H, cycloheptane H), 3.22 (d, 1H, J = 3.9 Hz, 9′-OH, D_2O exchangeable), 3.92 (dd, 1H, J = 5.0 Hz, J = 3.9 Hz, H-9′), 4.21 (d, 1H, J = 5.0 Hz, 10′-OH, D_2O exchangeable), 5.18 (t, 1H, J = 5.0 Hz, H-10′), 6.21 (d, 1H, J = 9.6 Hz, H-3′), 6.79 (d, 1H, J = 8.3 Hz, H-6′), 7.29 (d, 1H, J = 8.3 Hz, H-5′), 7.62 (d, 1H, J = 9.6 Hz, H-4′). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 21.78, 22.07, 29.94, 32.25, 36.18 (cycloheptane C), 60.77 (C-10′), 70.92 (C-9′), 83.96 (C-8′), 111.53 (C-3′, C-10′a), 112.15 (C-4′a), 115.24 (C-6′), 128.40 (C-5′), 144.83 (C-4′), 154.73 (C-10′b), 156.66 (C-6′a), 161.99 (C-2′). Anal. Calcd for $C_{18}H_{20}O_5$: C: 68.34, H: 6.37. Found: C: 68.21, H: 6.29.

7-{[2-ethynyltricyclo[3,3,1,1^{3,7}]decan-2-yl]oxy} benzo[*b*]pyran-2(2*H*)-one (12)

2-Chloro-2-ethynyladamantane (11) (le Noble et al 1979; Eguchi & Arasaki 1988) (2.2 g, 11.3 mmol) was added to a mixture of 7-hydroxycoumarin (1) (1.783 g, 11.0 mmol), anhydrous K_2CO_3 (1.82 g, 13.2 mmol), KI (1.86 g, 11.2 mmol) and CuI (41.9 mg, 0.22 mmol) in dry acetone under argon and the reaction mixture was stirred at 70 °C for 4 h. The solvent was removed under vacuum, water was added to the residue and it was extracted with dichloromethane. The organic phase was dried (Na_2SO_4) and evaporated to dryness. The crude material was purified by column chromatography using cyclohexane–ethyl acetate (8:1) as the eluent to give pure 12 (2.6 g, 76%). mp: 192–193 °C (EtOAc). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.4–2.4 (m, 14H, adamantane H), 2.79 (s, 1H, C≡CH), 6.24 (d, 1H, J = 9.5 Hz, H-3), 7.08 (dd, 1H, J = 8.4 Hz, 2.2 Hz, H-6), 7.34 (d, 1H, J = 8.4 Hz, H-5), 7.37 (d, 1H, J = 2.2 Hz H-8), 7.60 (d, 1H, J = 9.5 Hz, H-4). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 26.30, 26.62, 31.50, 34.67, 36.01, 37.34 (adamantane C), 78.14 (C≡CH), 80.14 (C-2′), 83.14 (C≡C-H), 106.01 (C-8), 112.91 (C-4a),

113.29 (C-3), 116.26 (C-6), 128.18 (C-5), 143.41 (C-4), 155.09 (C-8a), 158.72 (C-6a), 161.29 (C-2). Anal. Calcd for $C_{21}H_{20}O_3$: C: 78.73, H: 6.29. Found: C: 78.85, H: 6.17.

Spiro[tricyclo[3,3,1,1^{3,7}]decane-2,8′(8′*H*)-pyran[2,3-*h*]benzo[*b*]pyran]-2′(2′*H*)-one (13)

A mixture of (12) (470 mg, 1.47 mmol) in N,N-diethylaniline (8 mL) was heated at 180 °C for 1 h. A 9% HCl solution was then added and the residue was extracted with dichloromethane. The organic phase was washed twice with HCl 9%, then dried (Na_2SO_4) and evaporated to dryness. The residue was purified by column chromatography using cyclohexane–ethyl acetate (8:1) as the eluent and the isomer 13 (348 mg, 74%) was eluted first. Data for 13: mp: 238–239 °C (EtOAc–n-hexane), ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.4–2.3 (m, 14H, adamantane), 6.20 (d, 1H, J = 10.2 Hz, H-9′), 6.25 (d, 1H, J = 9.5 Hz, H-3′), 6.81 (d, 1H, J = 8.4 Hz, H-6′), 6.91 (d, 1H, J = 10.2 Hz, H-10′), 7.21 (d, 1H, J = 8.4 Hz, H-5′), 7.58 (d, 1H, J = 9.5 Hz, H-4′). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 26.69, 27.00, 32.29, 33.46, 35.79, 37.75 (adamantane C), 81.49 (C-8′), 111.01 (C-10′a), 112.66 (C-4′a), 112.95 (C-6′), 113.75 (C-3′), 116.13 (C-10′), 127.72 (C-5′), 129.01 (C-9′), 143.95 (C-4′), 150.11 (C-10′b), 156.10 (C-6′a), 161.05 (C-2′). Anal. Calcd for $C_{21}H_{20}O_3$: C: 78.73, H: 6.29. Found: C: 78.57, H: 6.33. The linear isomer 14 (spiro[tricyclo[3,3,1,1^{3,7}]decane-2,8′(8′H)-pyran[3,2-g]benzo[b]pyran]-2′(2′H)-one) was eluted second (89 mg, 19%). Data for 14: mp: 215–218 °C (EtOAc–n-hexane). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.4–2.3 (m, 14H, adamantane H), 6.17 (d, 1H, J = 9.5 Hz, H-3′), 6.22 (d, 1H, J = 10.2 Hz, H-7′), 6.39 (d, 1H, J = 10.2 Hz, H-6′), 6.77 (s, 1H, H-10′), 7.04 (s, 1H, H-5′), 7.55 (d, 1H, J = 9.5 Hz, H-4′). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 26.57, 26.98, 32.20, 33.52, 36.13, 37.71 (adamantane C), 81.56 (C-8′), 104.60 (C-10′), 112.80 (C-3′), 112.94 (C-4′a), 120.11 (C-5′a), 121.62 (C-6′), 124.60 (C-5′), 129.41 (C-7′), 143.34 (C-4′), 155.25 (C-10′a), 156.57 (C-9′a), 161.09 (C-2′). Anal. Calcd for $C_{21}H_{20}O_3$: C: 78.73, H: 6.29. Found: C: 78.79, H: 6.14.

(±)-*cis*-9′,10′-Dihydro-9′,10′-dihydroxyspiro[tricyclo[3,3,1,1^{3,7}]decane-2,8′(8′*H*)-pyran[2,3-*h*]benzo[*b*]pyran]-2′(2′*H*)-one (15)

This compound was prepared according to the procedure described for 8a. Yield: 68%. mp: 208 °C (EtOH). ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.3–2.5 (m, 14H, adamantane H), 3.01 (brs, 1H, 9′-OH, D_2O exchangeable), 4.30 (brs, 1H, 10′-OH, D_2O exchangeable), 4.65 (d, 1H, J = 4.4 Hz, H-9′), 5.17 (d, 1H, J = 3.9 Hz, H-10′), 6.22 (d, 1H, J = 9.5 Hz, H-3′), 6.90 (d, 1H, J = 8.4 Hz, H-6′), 7.29 (d, 1H, J = 8.4 Hz, H-5′), 7.64 (d, 1H, J = 9.5 Hz, H-4′). ^{13}C NMR ($CDCl_3$, 50 MHz) δ (ppm): 26.98, 31.10, 31.68, 32.01, 32.52, 33.74, 37.86 (adamantane C), 62.15 (C-10′), 65.09 (C-9′), 83.95 (C-8′), 110.48 (C-10′a), 111.95 (C-3′), 112.43 (C-4′a), 114.82 (C-6′), 128.31 (C-5′), 144.30 (C-4′), 155.03 (C-10′b), 156.02 (C-6′a), 160.32 (C-2′). Anal. Calcd for $C_{21}H_{22}O_5$: C: 71.17, H: 6.26. Found: C: 70.89, H: 6.21.

NMR spectra and molecular calculations

COSY and NOESY spectra were acquired with 1024 complex points for 256 experiments with 2 s recycling delay, TPPI phase cycle and 1 s mixing time for NOESY. HSQC spectrum was obtained using B_0 gradient pulses, 128 FIDs in the t_1 domain and 1 K in the t_2 domain, 8 transients for each t_1 experiment and recycling delay 1.5 s. Molecular simulations were performed with Macromodel 6.5 (Mohamadi et al 1990) using MM2* as force field implemented in Macromodel.

Measurement of activity in reduction of DPPH

The method has been previously described in detail (Andreadou et al 2002). Briefly, to a solution of DPPH (final concentration 200 μM) in absolute ethanol, an equal volume of the compound dissolved in ethanol was added at various concentrations (5–400 μM). Ethanol was added to the control solution. Absorbance was recorded at 517 nm after 20, 30, 45 and 60 min of incubation at room temperature (Kirkiacharian et al 1999).

Competition with DMSO for hydroxyl radicals

The hydroxyl radicals generated by the Fe^{3+}/ascorbic acid system were detected by the determination of formaldehyde produced from the oxidation of dimethyl sulfoxide (DMSO). The reaction mixture contained EDTA (0.1 mM), Fe^{3+} (167 μM, as a 1:2 mixture with EDTA), ascorbic acid (20 μM) and DMSO (25, 12.50, 6.25, 3.125 mM), in phosphate buffer (50 mM, pH 7.4). The tested compounds were dissolved in the phosphate buffer (pH 7.4) and added to the reaction mixture (final volume 750 μL) at different concentrations (2–0.25 mM). The mixture was incubated at 37 °C for 30 min. The reaction was stopped by the addition of 250 μL trichloroacetic acid (17.5% w/v) and the formaldehyde formed was detected spectrophotometrically (Klein et al 1981; Andreadou et al 2002). The same experiments were repeated in the absence of DMSO. The second-order reaction rate constant, k_s, was determined using equation 1.

$$(1 + k_s[S])/(A_o/A) = k_{DMSO}[DMSO] \quad (1)$$

where A_o is the absorbance at 412 nm before the addition of the tested compound, A is the absorbance after the addition of the tested compound, [DMSO] is the concentration of DMSO (25, 12.5, 6.25, 3.125 mM), [S] is the concentration of the tested compound and $k_{DMSO} = 7 \times 10^9\ M^{-1}\ s^{-1}$.

Quenching of the superoxide anion radical

The $O_2^{\cdot-}$ quenching capacity of the synthesized compounds was tested by estimation of the reduction product of nitro blue tetrazolium (NBT), as described previously (Robak & Gryglewski 1988). The incubation system contained 200 μM xanthine and 600 μM NBT in 0.1 M phosphate buffer (pH 7.4). The tested substances were dissolved in 0.1% dimethylformamide (DMF) in buffer,

and added to the reaction mixture (300 μL, final concentration 1 mM). An equal volume of the solvent system was added to the control mixture. The reaction started with the addition of 0.07 U mL^{-1} of xanthine oxidase. After incubation (25 °C, 10 min), absorbance was recorded at 560 nm, against blank samples, which did not contain the enzyme. DMF was tested and found not to interfere with the assay at the concentration used (0.1% v/v).

Statistical analysis

All biological experiments were performed at least in triplicate and the results are presented as mean ± standard deviation (s.d.). Since the assumption of equal variance and normality were not fulfilled, non parametric statistical analysis was performed using Kruskal–Wallis one-way analysis of variance, followed by the post-hoc Tukey's test, to analyse: the comparative effect of the synthesized compounds on DPPH reduction, as evaluated by the decrease of absorbance at 517 nm as a function of a 200 μM concentration of the compounds on each time point (Figures 4 and 5); the effect of a 1 mM concentration of the different compounds on the percent inhibition of superoxide anion radical, generated by the xanthine–xanthine oxidase enzymic system (Figure 6) and the effect of compound type at 0.25 mM concentration on DMSO oxidation (Table 2).

On the other hand, the comparative effects of increasing concentrations of the synthesized compounds (5, 50, 400 μM) on their interaction with DPPH (Table 1) and their effects on the HO$^{\bullet}$-mediated oxidation of DMSO were evaluated as well, changing either the concentration (2, 1, 0.5 mM) of the compounds (Table 2), or the concentration (25, 12.5, 6.25, 3.125 mM) of DMSO (Table 3), using two-way analysis of variance, followed by the post-hoc Tukey's test.

All tests were performed with Sigma Stat for Windows (version 2.03, SPSS, Inc.) at the 0.05 level of significance.

Results and Discussion

Chemistry

The preparation of the target derivatives is depicted in Figures 1 and 2. We used 8-acetyl-7-hydroxycoumarin (3) as starting material, which was obtained together with its regioisomer 4, according to known procedures starting from commercial 7-hydroxycoumarin (1) through a Fries rearrangement of the intermediate acetate 2 (Figure 1) (Bender et al 1983; Prashant et al 2001). A solution of compound 3 in toluene was then treated at reflux with the appropriate carbocyclic ketone in the presence of pyrrolidine (Kabbe 1978) to provide the spiropyranobenzopyranodiones 5a–c in very good yield. Sodium borohydride reduction of the aforementioned diones followed by dehydration of the intermediate hydroxy analogues 6a–c with an acidic catalyst provided the coumarins 7a–c.

The adamantyl analogue 13 was prepared from commercially available 2-adamantanone (9) according to the reactions presented in Figure 2. Compound 9 was treated with potassium acetylide in toluene and the resulting ethynyl alcohol 10 was converted to the corresponding chloride, 11, after heating at reflux temperature in concentrated hydrochloric acid in the presence of calcium chloride and a catalytic amount of hydroquinone (le Noble et al 1979; Eguchi & Arasaki 1988). The chloride (11), after reaction with 7-hydroxycoumarin (1), provided the corresponding ether 12, which was subjected to thermal cyclization in boiling N,N-diethylaniline, resulting in a mixture of the spiroadamantyl derivatives 13 and 14, which were separated by column chromatography.

Catalytic syn-hydroxylation of compounds 7a–c and 13 with osmium tetroxide and N-methylmorpholine-N-oxide as oxidizing reagent (VanRheenen et al 1976) yielded the corresponding cis-diols 8a–c and 15.

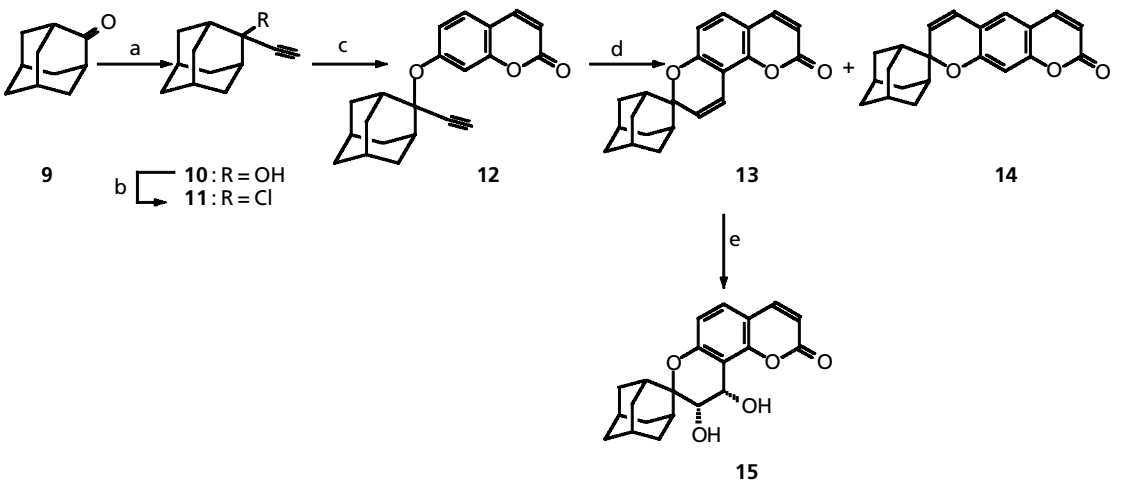

Figure 2 Synthesis of the spiroadamantylpyranocoumarins. Reagents: a, KC≡CH, THF, room temperature; b, HCl, $CaCl_2$, hydroquinone; c, 1, K_2CO_3, KI, CuI, acetone, 60 °C; d, N,N-diethylaniline 190 °C; e, OsO_4, N-methylmorpholine-N-oxide, room temperature.

Conformational analysis

The conformation of structurally analogous compounds bearing a dihydropyran ring, with hydroxyl substitutions, has been previously studied to a certain extent. The crystal structure of pyranocoumarin derivatives reveals that the pyran ring conformation depends on the presence or absence of substituents on the hydroxyl group adjacent to the benzylic position (Valencia-Islas et al 2002). The pyran ring of some derivatives of 1,2-dihydro-1,2-dihydroxyacronycine proved to be more rigid and adopted only one conformation, orienting the benzylic OH group in the pseudoequatorial position (Mikros et al 1999). On the other hand, the biological activity of some pyranothioxanthenone derivatives has been related to the possibility of dimer formation, via hydrogen bonds through the suitably oriented hydroxyl groups of the pyran ring (Kostakis et al 2001). Therefore, it was of interest to investigate the pyran ring conformation of the synthesized spirocoumarins reported herein, and for this purpose we have accomplished a study of the cyclohexyl-substituted derivative 8b.

The conformational analysis was performed using NOE spectral data (recorded in DMSO solution) and molecular mechanics calculations. The pyran ring is expected to adopt two half-chair conformations, with 8′-C and 9′-C on opposite sides of the ring plane. The stability of each conformer depends on the conformation of the cyclohexyl substituent, which can also adopt two chair conformations. Thus, four possible conformers were constructed using Macromodel software and geometry was optimized using MM2* force field. The resulting structures are shown in Figure 3 along with their relative energies. The hydroxyl group at 10′-C adopts either pseudoaxial (conformer I and conformer II) or pseudoequatorial orientation (conformers III and IV). NOEs detected between the proton of 10′-OH and the cyclohexyl ring proton resonating at 2.13 ppm indicate the existence of conformers I or II (OH pseudoaxial) and are also useful for the assignment of the cyclohexyl proton as $2''$-$H_{equatorial}$. Moreover, the observed NOESY cross-peak correlating the 10′-H with a proton of the cyclohexyl ring resonating at 1.54 ppm suggests that 10′-H adopts a pseudoaxial orientation (conformer III and conformer IV) and thus, this cyclohexyl proton was assigned as $6''$-H_{axial}. The above observations suggest the existence of an equilibrium between the two half-chair conformers, in solution.

Antioxidant activity

Coumarins have been proven to scavenge reactive oxygen species and influence processes involving free-radical-mediated injury (Paya et al 1992). 7-Hydroxycoumarin has been shown to be a potent free-radical scavenger, (Paya et al 1992), thus we have compared the effect of the synthesized coumarins on free-radical scavenging activity with that of 7-hydroxycoumarin.

The new compounds were tested for their ability to interact with DPPH. DPPH is a stable free radical, which, by accepting an electron or hydrogen radical, is converted into a stable diamagnetic molecule. Due to the existence of an odd electron, DPPH has a strong absorption band at 517 nm. When this electron becomes paired, the absorption decreases stoichiometrically with respect to the number of electrons taken up. The decrease of the absorbance produced by this reaction has been widely used to test the ability of several molecules to act as

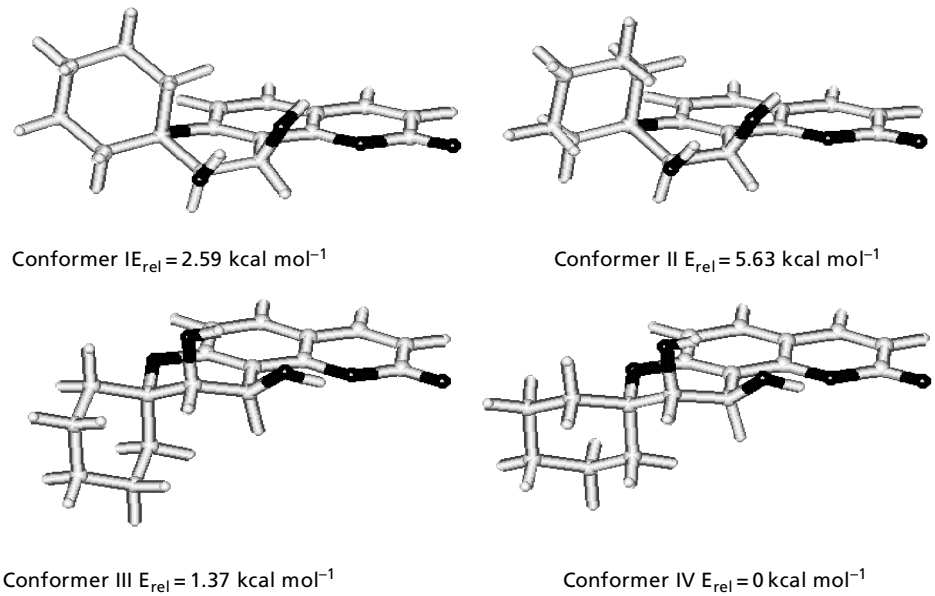

Figure 3 Representation of the low energy conformations for derivative 8b derived from molecular mechanics calculations.

free-radical scavengers (Dinis at al 1994). All the compounds tested were found to interact with DPPH and it is worth noting that they showed similar antioxidant activity in this test (Table 1). In the vast majority of cases, the interaction with DPPH depended upon the structure and the concentration of the compounds in a statistically significant way (Table 1, $P < 0.05$, two-way analysis of variance). The effect–time curves are presented in Figures 4 and 5. It is clear that the inhibition started in the first minutes after the addition of DPPH and levelled off after 60 min for all the synthesized compounds, while time did not influence the inhibition produced by 7-hydroxycoumarin. The spiroadamantane derivative 13 exhibited 40% inhibition at a concentration of 400 μM (Table 1), and its absorbance was statistically significantly decreased (Figure 4, $P < 0.05$ Kruskal–Wallis one-way analysis of variance, followed by post-hoc Tukey's test) at each time point when compared with the absorbance of DPPH, indicating this compound as the most potent radical scavenger among the compounds tested.

Table 1 Effect of different concentrations (5, 50, 400 μM) of the synthesized compounds on their interaction with DPPH (200 μM) at 20 min of incubation.

Compound	Percentage of interaction with DPPH[a]		
	5 μM	50 μM	400 μM
7a	10 ± 0.05	20 ± 0.04	27 ± 0.1
7b	19 ± 0.10	23 ± 0.18	31 ± 0.21
7c	8 ± 0.04	20 ± 0.07	23 ± 0.12
13	20 ± 0.09	36 ± 0.18	41 ± 0.22
8a	8 ± 0.06	21 ± 0.09	24 ± 0.11
8b	10 ± 0.04	29 ± 0.15	31 ± 0.21
8c	9 ± 0.07	26 ± 0.18	28 ± 0.19
15	11 ± 0.08	27 ± 0.12	38 ± 0.21
7-Hydroxycoumarin	1 ± 0.01	2 ± 0.01	14 ± 0.01
Ascorbic acid	1 ± 0.01	73 ± 0.06	98 ± 0.09

Data are mean ± s.d. [a]Based on absorbance values of samples with the tested compounds against controls containing an equal volume of the solvent (n = 3–5).

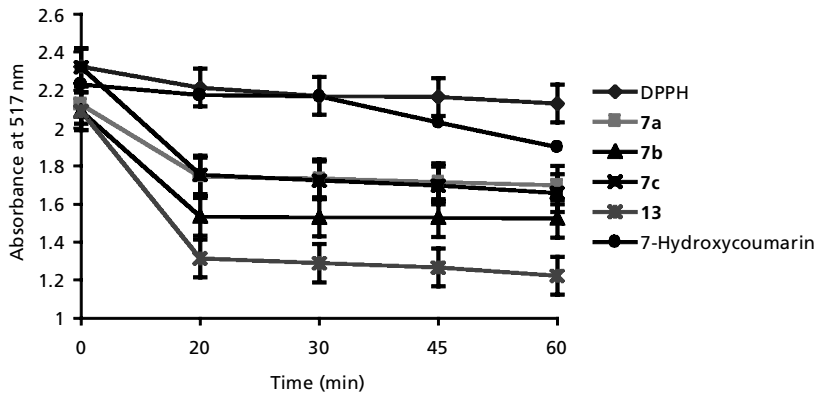

Figure 4 DPPH reduction, as evaluated by the decrease in absorbance, at 517 nm, as a function of a 200 μM concentration of compounds 7a–c, 13 on the time.

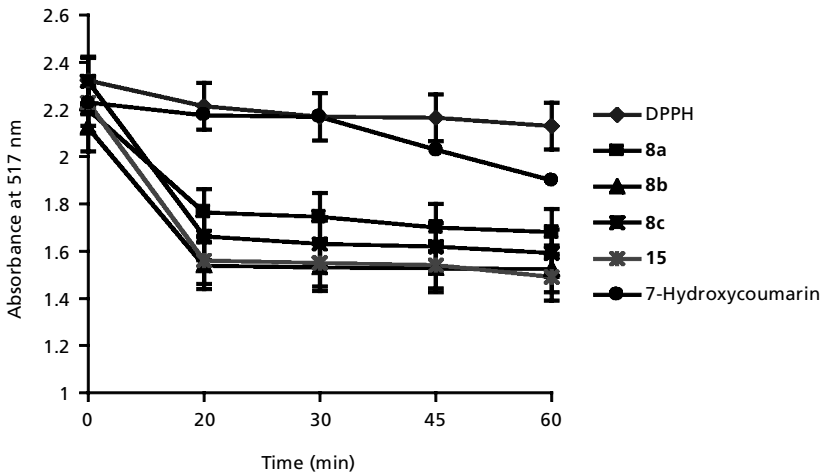

Figure 5 DPPH reduction, as evaluated by the decrease in absorbance, at 517 nm, as a function of a 200 μM concentration of compounds 8a–c, 15 on the time.

Table 2 Effect of different concentrations (2–0.25 mM) of the examined compounds on the HO•-mediated oxidation of DMSO (3.125 mM).

Compound	HO• Scavenging activity (% inhibition)[a]			
	2 mM	1 mM	0.5 mM	0.25 mM
7a	73 ± 1.1	54 ± 1.2	35 ± 0.8	15 ± 0.5
7b	67 ± 1.7	61 ± 2.1	6.5 ± 0.6	N/D
7c	93 ± 2.3	66 ± 0.9	53 ± 0.5	27 ± 1.1
13	93 ± 3.7	86 ± 2.8	71 ± 2.1	71 ± 3.1
8a	73 ± 1.2	45 ± 0.8	4 ± 0.01	N/D
8b	62 ± 2.0	47 ± 1.1	5 ± 0.05	N/D
8c	57 ± 0.9	50 ± 0.7	4 ± 0.02	N/D
15	49 ± 1.3	41 ± 1.2	2 ± 0.02	N/D
7-Hydroxycoumarin	60 ± 2.1	50 ± 2.5	49 ± 1.4	40 ± 2.1

Data are mean ± s.d. [a]Based on absorbance values of samples with the tested compounds, against controls containing equal volume of the solvent (n = 3–5). N/D, not determined.

Table 3 Effect of the examined compounds (2 mM) on the HO•-mediated oxidation of DMSO (25, 12.5, 6.25, 3.125 mM) and their k_s values.

Compound	Percentage of inhibition[a]				k_s($\times 10^{10}$ M^{-1} s^{-1})
	25 mM DMSO	12.5 mM DMSO	6.25 mM DMSO	3.125 mM DMSO	
7a	20 ± 0.02	41 ± 0.21	57 ± 0.41	73 ± 0.61	2.8 ± 0.04
7b	30 ± 0.02	36 ± 0.25	51 ± 0.34	67 ± 0.54	2.7 ± 0.07
7c	42 ± 0.40	49 ± 0.29	62 ± 0.51	93 ± 0.67	4.7 ± 0.15
13	61 ± 0.56	79 ± 0.45	87 ± 0.62	93 ± 0.71	18.9 ± 0.33
8a	4 ± 0.03	26 ± 0.18	35 ± 0.22	73 ± 0.49	1.9 ± 0.07
8b	17 ± 0.15	29 ± 0.20	44 ± 0.34	62 ± 0.51	1.7 ± 0.05
8c	14 ± 0.09	21 ± 0.16	36 ± 0.28	57 ± 0.90	1.3 ± 0.01
15	4 ± 0.01	8 ± 0.05	35 ± 0.22	50 ± 0.50	1.2 ± 0.01
7-Hydroxycoumarin	21 ± 0.12	37 ± 0.23	43 ± 0.34	60 ± 0.89	3.5 ± 0.31
Mannitol[b]					0.17
DMSO[b]					0.7

Data are mean ± s.d. [a]Based on absorbance values of samples with the tested compounds, against controls containing equal volume of the solvent (n = 3–5). [b]Andreadou et al (2000).

The HO• radical scavenging activity of the synthesized compounds was also investigated. As an HO• radical-generating system, the Fe^{3+}/EDTA-catalysed autoxidation of ascorbic acid was used. HO• radicals thus formed mediate the oxidation of DMSO to yield formaldehyde. The formaldehyde production from DMSO represents a convenient method to detect HO• (Klein et al 1981; Rekka & Kourounakis 1991; Andreadou et al 2002). The results presented in Table 2 show the effect of different concentrations of the tested compounds on the HO•-mediated oxidation of DMSO (3.125 mM). The effect of a standard concentration of the examined compounds (2 mM) on the HO•-mediated oxidation of different concentrations of DMSO was also studied and their corresponding k_s values were calculated (Table 3). In the vast majority of cases, the competition with DMSO for HO•-scavenging activity depended upon the structure and the concentration of the compounds and upon the concentration of DMSO in a statistically significant way (Tables 2 and 3, $P < 0.05$, two-way analysis of variance). Concerning the effect of the concentration of 0.25 mM on the HO•-mediated oxidation of DMSO, the activity of compounds 7a, 7c, 13 and 7-hydroxycoumarin was statistically different (Table 2, $P < 0.05$, one-way analysis of variance). All compounds exhibited significant HO•-scavenging activity, with reaction rate constants higher than those corresponding to DMSO and mannitol, both known as hydroxyl radical scavengers. This inhibition was principally due to their HO•-scavenging activity, since it is dependent upon the concentration of DMSO used (Table 3). Compound 13 proved to be the most potent of all other substances tested, including 7-hydroxycoumarin, demonstrating significant hydroxyl radical scavenging activity, in agreement with the data obtained with the DPPH protocol.

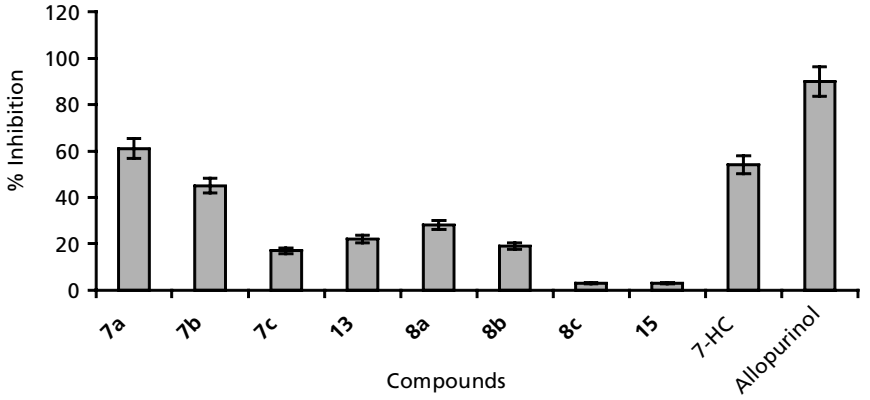

Figure 6 Percent inhibition of the synthesized compounds on xanthine–xanthine oxidase-generated superoxide anion radical. 7-HC, 7-hydroxycoumarin.

However, the apparent reaction rate constant, k_s, of this derivative ($18.9 \pm 0.33 \times 10^{10}$ M^{-1} s^{-1}, Table 3) was found to be much higher than expected under the experimental conditions applied. Nevertheless, compound 13 inhibited the oxidation of DMSO (3.125 mM) by 93% at 2 mM and by 71% at 0.25 mM using the same concentration of DMSO (Table 2). The above results may be attributed to a simultaneous inhibition of $HO^{\bullet}$ generation (Rekka & Kourounakis 1991) or interference from reaction intermediates or by-products.

We have also investigated the ability of the new derivatives to scavenge superoxide anions, generated by an enzymic (xanthine–xanthine oxidase) system, by measurement of the reduction product of NBT. The test showed that the order of activity for xanthine–xanthine oxidase inhibition was: 7a > 7-hydroxycoumarin > 7b > 8a > 13 > 8b > 7c > 8c > 15 (Figure 6). The inhibition by compound 7a was statistically significantly increased, when compared with the inhibition by compounds 8c and 15 (Figure 6, $P < 0.05$, one-way analysis of variance). Compound 7a, which bears a spirocyclopentyl substituent, showed the highest inhibitory activity for superoxide anions, better than that of 7-hydroxycoumarin, and we can observe that this activity is reduced as the size of the cycloalkyl substituent is increased. Allopurinol, used as a reference compound, showed strong inhibitory activity equal to 90% at 1 mM and it was found to be statistically different to that of compounds 7c, 8c and 15 (Figure 6, $P < 0.05$, one-way analysis of variance).

The partial parallelism of the DPPH radical, hydroxyl radical and superoxide anion scavenging activity could be due to the different characters between the radical species and some other factors involved in these reaction systems. The DPPH radical is a chemically induced free radical, which reacts with different radical scavengers in a simple manner, while the superoxide anion radical is generated by the xanthine–xanthine oxidase system, with the involvement of an enzymatic reaction (Xiong et al 1996).

The loss of the extended conjugation of compounds 7a–c and 13 by the insertion of the two hydroxyl groups resulted in a decrease of antioxidant activity, as demonstrated by the results concerning the cis-diols 8a–c and 15 in the three different in-vitro protocols. Moreover, derivative 13, due to its multiple mechanism of protective action, may serve as a lead for the development of analogues combining the adamantane moiety and a ring system with extended conjugation.

In conclusion, the synthesized coumarin derivatives can interact with DPPH, are potent hydroxyl radical scavengers, and most of them can scavenge superoxide anions generated in the xanthine–xanthine oxidase system. The reported novel compounds could find useful applications as protective agents against oxygen toxicity. Further experiments will focus on the investigation of the mechanism of free radical scavenging activity of the synthesized spiro-substituted coumarins as well as on their physicochemical properties, so as to identify their specific cellular localization.

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