Bioorganic & Medicinal Chemistry 17 (2009) 6547–6559

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Toward establishing structure–activity relationships for oxygenated coumarins as differentiation inducers of promonocytic leukemic cells

María E. Riveiro [a,c,f], Dominick Maes [b], Ramiro Vázquez [a,f], Monica Vermeulen [e,f], Sven Mangelinckx [b], Jan Jacobs [b], Silvia Debenedetti [d], Carina Shayo [c,f], Norbert De Kimpe [b], Carlos Davio [a,f,*]

[a] Cátedra de Química Medicinal, Departamento de Farmacología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junin 956, 1113 Buenos Aires, Argentina
[b] Department of Organic Chemistry, Faculty of Bioscience Engineering, Ghent University, B-9000, Ghent, Belgium
[c] Laboratorio de Patología y Farmacología Molecular, Instituto de Biología y Medicina Experimental, Buenos Aires, Argentina
[d] Cátedra de Farmacognosia, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina
[e] Departamento de Inmunología, Instituto de Investigaciones Hematológicas, Academia Nacional de Medicina, Buenos Aires, Argentina
[f] Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 2 May 2009
Revised 31 July 2009
Accepted 4 August 2009
Available online 8 August 2009

Keywords:
Polyoxygenated coumarins
Differentiation
Leukemia

ABSTRACT

The presumption that some coumarins might be lead compounds in the search for new differentiation agents against leukemia is based on the fact that natural coumarins, 5-(3-methyl-2-butenyloxy)-6,7-methylenedioxycoumarin (**C-2**) and 5-methoxy-6,7-methylenedioxycoumarin (**C-1**) inhibit proliferation and induce differentiation in U-937 cells [Riveiro, M. E.; Shayo, C.; Monczor, F.; Fernandez, N.; Baldi, A.; De Kimpe, N.; Rossi, J.; Debenedetti, S.; Davio, C. *Cancer Lett.* **2004**, *210*, 179–188]. These promising findings prompted us to investigate the anti-leukemia activity of a broader range of related polyoxygenated coumarins. Twenty related natural or synthetically prepared coumarins, including a range of 5-substituted ayapin derivatives which have become easy accessible via newly developed synthesis methods, were evaluated, where treatments with 5-(2,3-dihydroxy-3-methylbutoxy)-6,7-methylenedioxycoumarin (**D-3**) and 5-(2-hydroxy-3-methoxy-3-methylbutoxy)-6,7-methylenedioxycoumarin (**D-2**) were able to inhibit the cell growth and induce the differentiation of U-937 cells after 48 h treatment. These results provide insight into the correlation between some structural properties of polyoxygenated coumarins and their in vitro leukemic differentiation activity.

1. Introduction

Malignancies are characterized by three major cellular disorders: arrest of cell differentiation, an inhibition of apoptosis, and an accelerated proliferation of clonal cells. Until recently, chemotherapy and hematopoietic stem-cell transplantation were the only therapeutic options in acute leukemia. A potential alternative to treat this prevalent disease is the engagement of malignant cells into the maturation pathway, known as 'differentiation therapy'. The induction of differentiation restores a natural cell death program and inhibits the excessive proliferation; the effect of all-*trans*-retinoic acid in acute promyelocytic leukemia represented one of the first examples of differentiation therapy in hematological malignancies.[2,3]

Development of anticancer drugs is loaded with challenges which go beyond the screening of potential molecules in vitro or in vivo and issues of pharmaceutical industry. Over the years, the identification of new effective differentiation inducers for the treatment of leukemia has remained a focus of intense interest.

Nowadays, coumarins represent an important group of organic compounds that are used as additives to food, cosmetics, and optical brightening agents.[4] In recent years, coumarin compounds have attracted research interest due to their broad pharmacological/biological activities. Coumarin compounds can display anticancer,[5–7] anti-HIV,[8] anticoagulant,[9] antimicrobial,[10] anti-inflammatory, and antioxidant[11] activities.

Plants continue to provide with new chemical entities for the development of drugs against various pharmacological targets, including cancer, HIV, pain, and Alzheimer's disease. Based on

Abbreviations: PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; IC_{50}, inhibitory proliferation concentration 50; CC_{50}, cytotoxic concentration 50; ATP, adenosine triphosphate; dbcAMP, dibutyryl cyclic adenosine monophosphate; rhC5a, recombinant human C5a; NBT, nitrobluetetrazolium.

* Corresponding author at present address: Cátedra de Química Medicinal, Departamento de Farmacología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junin 956, 1113 Buenos Aires, Argentina. Tel.: +54 114964 8233; fax: +54 114786 2564.
E-mail address: cardavio@ffyb.uba.ar (C. Davio).

0968-0896/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2009.08.002

the knowledge of new lead compounds with differentiation activity in leukemic cells we screened different extracts obtained from *Pterocaulon polystachyum*, from which petroleum ether extract exerted anti-proliferative and differentiation activity on U-937 cells with no substantial cytotoxic effect. Two trioxygenated coumarins isolated from this extract, 5-methoxy-6,7-methylenedioxycoumarin (**C-1**) and 5-(3-methyl-2-butenyloxy)-6,7-methylenedioxycoumarin (**C-2**) inhibited proliferation and induced differentiation in U-937 cells. These results were the first report indicating the differentiation and anti-proliferative activities of these novel coumarins in human leukemia U-937 cells.[1]

In order to gain insight into the differentiation properties on leukemic cells of trioxygenated coumarins, we performed a study to understand the relationship between the structure and the biological activity. Thus, we fully investigated the synthesis of natural and synthetic coumarin analogues, with a similar oxygenation pattern and evaluated these coumarins biologically with the aim of elucidating the key structural requirements to induce differentiation in leukemic cells. More specifically, to be able to study the influence of the substitution at position 5 on the biological activity of 6,7-methylenedioxycoumarins, an convenient access to a range of natural and synthetic ayapin derivatives was necessary (Table 1) while avoiding low-yielding and long extraction procedures from natural sources. While ayapin **4** and the natural derivatives **D-6** and **BA-4** have been synthesized previously, a synthetic access to other 6,7-methylenedioxycoumarins, that is, **BA-1**-**BA-3**, **D-2**-**D-5**, had to be developed. Furthermore, a synthetic access to the natural 7,8-methylenedioxycoumarin **D-7** would facilitate the study of the biological importance of the position of the methylenedioxy moiety. Alternative oxygenated coumarins **D-11**, **D-12**, **D-14**, **BA-5**, and **BA-7**-**BA-10** lacking a 6,7-methylenedioxy-substitution are also included in this biological study and are accessible as described in previous studies (Table 1).

2. Material and methods

2.1. Chemicals

RPMI medium 1640, antibiotics, bovine serum albumin (BSA), dibutyryl cyclic adenosine monophosphate (dbcAMP), 4β-phorbol 12-myristate 13-acetate (PMA), phosphate-buffered saline (PBS), Fura 2-AM, nitrobluetetrazolium (NBT), recombinant human C5a (rhC5a), ATP (adenosine triphosphate), and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, USA). Fetal calf serum was purchased from Natocor (Argentina). All commercial chemicals and solvents were of reagent grade and used without

Table 1
Structure of natural and synthetic coumarins used in this study

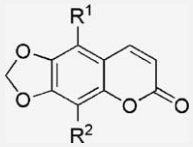
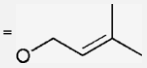
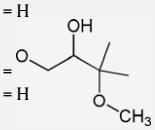
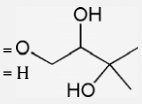
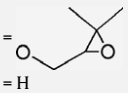
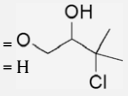
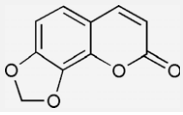
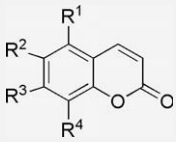
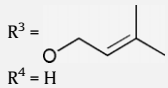
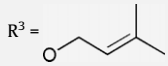
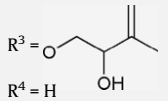
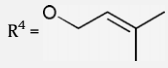
			Reference
			
R^1 = H, R^2 = H	**4** Ayapin	6,7-Methylenedioxycoumarin	14
R^1 = OH, R^2 = H	**BA-1**	5-Hydroxy-6,7-methylenedioxycoumarin	13
R^1 = F, R^2 = H	**BA-2**	5-Fluoro-6,7-methylenedioxycoumarin	–
R^1 = Br, R^2 = H	**BA-3**	5-Bromo-6,7-methylenedioxycoumarin	–
R^1 = MeO, R^2 = H	**C-1**	5-Methoxy-6,7-methylenedioxycoumarin	1
R^1 =  , R^2 = H	**C-2**	5-(3-Methyl-2-butenyloxy)-6,7-methylenedioxycoumarin	1
R^1 =  , R^2 = H	**D-2**	5-(2-Hydroxy-3-methoxy-3-methylbutoxy)-6,7-methylenedioxycoumarin	13
R^1 =  , R^2 = H	**D-3**	5-(2,3-Dihydroxy-3-methylbutoxy)-6,7-methylenedioxycoumarin	–
R^1 =  , R^2 = H	**D-4**	5-((3,3-Dimethyloxiran-2-yl)methoxy)-6,7-methylenedioxycoumarin	–
R^1 =  , R^2 = H	**D-5**	5-(2-Hydroxy-3-chloro-3-methylbutoxy)-6,7-methylenedioxycoumarin	–
R^1 = OCH_3, R^2 = OCH_3	**D-6**	5,8-Dimethoxy-6,7-methylenedioxycoumarin	14
R^1 = H, R^2 = OCH_3	**BA-4**	8-Methoxy-6,7-methylenedioxycoumarin	14
	**D-7**	7,8-Methylenedioxycoumarin	–

Table 1 (*continued*)

			Reference
	R^1 = H R^2 = H R^3 = H R^4 = H	Coumarin	
	R^1 = H R^2 = OH R^3 =  R^4 = H	**D-11** 6-Hydroxy-7-(3-methyl-2-butenyloxy)coumarin	22
	R^1 = H R^2 = OCH_3 R^3 =  R^4 = H	**D-12** 6-Methoxy-7-(3-methyl-2-butenyloxy)coumarin	23
	R^1 = H R^2 = OCH_3 R^3 =  R^4 = H	**D-14** 6-Methoxy-7-(2-hydroxy-3-methyl-3-butenyloxy)coumarin	23
	R^1 = OCH_3 R^2 = OCH_3 R^3 = OCH_3 R^4 = H	**BA-7** 5,6,7-Trimethoxycoumarin	24
	R^1 = OCH_3 R^2 = OH R^3 = OCH_3 R^4 = H	**BA-10** 6-Hydroxy-5,7-dimethoxycoumarin (fraxinol)	24
	R^1 = OCH_3 R^2 = H R^3 = OCH_3 R^4 = OH	**BA-9** 8-Hydroxy-5,7-dimethoxycoumarin (leptodactylone)	24
	R^1 = OCH_3 R^2 = H R^3 = OCH_3 R^4 = OCH_3	**BA-8** 5,7,8-Trimethoxycoumarin	24
	R^1 = OCH_3 R^2 = H R^3 = OCH_3 R^4 = 	**BA-5** 8-(3-Methyl-2-butenyloxy)-5,7-dimethoxycoumarin (artanin)	24

further purification unless otherwise specified. Diethyl ether and tetrahydrofuran were distilled from sodium and sodium benzophenone ketyl. Dichloromethane was distilled over calcium hydride. Methanol was dried by reaction with magnesium, and distilled.

2.2. Synthesis

2.2.1. General

^{1}H NMR spectra (270 or 300 MHz), ^{13}C NMR spectra (68 or 75 MHz) and ^{19}F NMR spectra (282 MHz) were recorded with a Jeol EX 270 NMR or Jeol Eclipse FT 300 NMR spectrometer. The compounds were diluted in deuterated solvents and peaks are relative to tetramethylsilane (TMS) as internal standard. Peak assignments were accomplished by using COSY, DEPT, HSQC, and HMBC spectra. Infrared spectra were measured with a Perkin–Elmer Spectrum One FT-IR spectrophotometer. For liquid samples, spectra were recorded by preparing a thin film of product between two sodium chloride plates. Solid compounds were mixed with potassium bromide and pressed at high pressure until a transparent disk was obtained. Only selected absorbances (ν_{max}/cm^{-1}) were reported. Low resolution mass spectra of pure compounds were recorded via direct injection on an Agilent 1100 Series LC/MSD type SL mass spectrometer with Electron Spray Ionisation geometry (ESI 70 eV) and using a Mass Selective Detector (quadrupole). High resolution mass spectra were obtained on a Finnigan MAT 95 XPAPI-GC-Trap tandem Mass spectrometer system. Melting points of crystalline products were measured with a Büchi B-540 apparatus. Flash chromatography was carried out using a glass column filled with silica gel (Acros, particle size 0.035–0.070 mm, pore diameter ca. 6 nm). Thin layer chromatography was performed on glass-backed silica plates (Merck Kieselgel 60 F_{254}, precoated 0.25 mm), which were developed using standard visualization techniques or agents: UV fluorescence (254 and 366 nm), coloring with iodine vapors and permanganate solution.

2.2.2. Synthetic procedures

2.2.2.1. Synthesis of 2-(*t*-butyldimethylsilanyloxy)-4,5-methylenedioxybenzaldehyde 14. A mixture of 2-hydroxy-4,5-methylenedioxybenzaldehyde **2** (3.32 g, 20 mmol) and *t*-butyl(chloro)dimethylsilane (3.02 g, 20 mmol) was dissolved in dichloromethane (50 mL). The reaction mixture was cooled to 0 °C and triethylamine (4.05 g, 40 mmol) was added. Subse-

quently, the reaction mixture was slowly warmed up to room temperature and stirred further at room temperature for 24 h. Then, dichloromethane (50 mL) was added and the organic phase was washed with 1 N aqueous HCl solution (3 × 50 mL), saturated sodium bicarbonate solution (50 mL), and water (50 mL). After drying ($MgSO_4$), filtration, and evaporation of the solvent in vacuo, 2-(*t*-butyldimethylsilanyloxy)-4,5-methylenedioxybenzaldehyde **14** was obtained in 96% yield. IR (cm^{-1}): 2956; 2931; 2860; 1673; 1621; 1504; 1477; 1441; 1367; 1255; 1189; 1078; 1038; 901; 842; 784. ^1H NMR (300 MHz, $CDCl_3$): δ 0.26 (s, 6H, 2× $SiCH_3$); 1.02 (s, 9H, $SiC(CH_3)_3$); 5.99 (s, 2H, OCH_2O); 6.36 (s, 1H, $COCH_{ar}CO$); 7.21 (s, 1H, $COCH_{ar}CCHO$); 10.24 (s, 1H, CHO). ^{13}C NMR (75 MHz, $CDCl_3$): δ −4.4 ($Si(CH_3)_2$); 18.3 ($SiC(CH_3)_3$); 25.7 ($SiC(CH_3)_3$); 101.3 ($COCH_{ar}CO$); 102.1 (OCH_2O); 105.5 ($COCH_{ar}CC$); 121.1 (C_qCHO); 142.9 ($OCH_2OC_qCHC_qCHO$); 153.9 and 156.9 (OC_qCHC_qO); 188.2 (CHO). MS (70 eV, ES^+, *m*/*z* (%)): 281 ($M+H^+$).

2.2.2.2. Synthesis of 2-[6-(*t*-butyldimethylsilanyloxy)benzo-1,3-dioxol-5-yl]-1,3-dimethylimidazolidine 15. A mixture of 2-(*t*-butyldimethylsilanyloxy)-4,5-methylenedioxybenzaldehyde **14** (2.80 g, 10 mmol) and *N,N′*-dimethylethylene-1,2-diamine (0.97 g, 11 mmol) was dissolved in toluene (50 mL). The flask was fitted with a Dean–Stark apparatus and refluxed for 6 h. Toluene and excess amine were evaporated in vacuo from the reaction mixture and 2-[6-(*t*-butyldimethylsilanyloxy)benzo-1,3-dioxol-5-yl]-1,3-dimethylimidazolidine **15** (3.50 g) was obtained quantitatively as a brown oil. IR (cm^{-1}): 2951; 2885; 2777; 1630; 1501; 1479; 1429; 1254; 1174; 1154; 1039; 909; 857; 841. ^1H NMR (300 MHz, $CDCl_3$): δ 0.22 (s, 6H, $Si(CH_3)_2$); 1.02 (s, 9H, $SiC(CH_3)_3$); 2.19 (s, 6H, 2× NCH_3); 2.53 (m, 2H, $NCH_aH_bCH_aH_bN$); 3.35 (m, 2H, $NCH_aH_bCH_aH_bN$); 3.89 (s, 1H, $CH(N(CH_3)CH_2)_2$); 5.89 (s, 2H, OCH_2O); 6.35 (s, 1H, $COCH_{ar}CO$); 7.11 (s, 1H, $COCH_{ar}CCH$). ^{13}C NMR (75 MHz, $CDCl_3$): δ −3.8 ($Si(CH_3)_2$); 18.5 ($SiC(CH_3)_3$); 26.1 ($SiC(CH_3)_3$); 39.7 (2× NCH_3); 53.4 (NCH_2CH_2N); 83.4 (NCHN); 100.1 ($COCH_{ar}CO$); 101.2 (OCH_2O); 108.5 ($COCH_{ar}CC$); 121.9 (C_qCHN_2); 142.4 ($OCH_2OC_qCHC_qCHN_2$); 147.5 and 149.9 (OC_qCHC_qO). MS (70 eV, ES^+, *m*/*z* (%)): 351 ($M+H^+$).

2.2.2.3. Synthesis of 2-bromo-6-hydroxy-3,4-methylenedioxybenzaldehyde 16. To a solution of 2-[6-(*t*-butyldimethylsilanyloxy)benzo-1,3-dioxol-5-yl]-1,3-dimethyl-imidazolidine **15** (3 mmol, 1.05 g) in diethyl ether (40 mL) at −90 °C was added dropwise 4 mL of a 1.5 N solution of *t*-butyllithium in hexane and the resulting reaction mixture was stirred at −90 °C under nitrogen atmosphere for 30 min. Bromine (6 mmol, 0.96 g) was then introduced under vigorous stirring and the reaction mixture was slowly warmed up to room temperature over a period of 4 h. After 4 h, a saturated sodium bisulfite solution (20 mL) was added to neutralize unreacted bromine, followed by 6 M aqueous HCl (50 mL). After stirring for 15 min, the organic layer was separated and the aqueous layer was diluted with water (50 mL) and extracted with dichloromethane (2 × 50 mL). The combined organic layers were washed with water (3 × 50 mL) and dried ($MgSO_4$). After filtration and evaporation of the solvent under reduced pressure, a black residue was obtained, which was dissolved in tetrahydrofuran (40 mL). A solution of 1 M tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (3 mL) was then added and the reaction mixture was stirred at room temperature for 4 h. Subsequently, tetrahydrofuran was evaporated in vacuo from the reaction mixture, water (50 mL) was added and the reaction mixture was extracted with dichloromethane (3 × 50 mL). The combined organic layers were washed with brine (50 mL) and water (2 × 50 mL) and dried ($MgSO_4$). After filtration and evaporation of the solvent under reduced pressure, a solid residue was obtained (1.2 g) which was chromatographed over silica gel (10% Et_2O/90% hexane) to yield 0.40 g (54% yield) pure 2-bromo-6-hydroxy-3,4-methylenedioxybenzaldehyde **16** as yellow crystals. Mp: 147 °C. IR (cm^{-1}): 3436 (br s, OH); 1682 (C═O); 1624; 1501; 1460; 1373; 1304; 1249; 1217; 1175; 1043; 937; 858. ^1H NMR (300 MHz, $CDCl_3$): δ 6.09 (s, 2H, OCH_2O); 6.41 (s, 1H, CH_{ar}); 10.01 (s, 1H, CHO); 12.78 (s, 1H, OH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 97.7 (CH_{ar}); 102.3 (C_qBr); 102.4 (OCH_2O); 110.8 (C_qCHO); 140.2 (OCH_2OC_qCBr); 154.6 (C_qOH); 163.9 (OCH_2OC_qCH); 194.4 (CHO). MS (70 eV, ES^-, *m*/*z* (%)): 243/245 ($M-H^+$).

2.2.2.4. Synthesis of 5-bromo-6,7-methylenedioxycoumarin BA-3. 2-Bromo-6-hydroxy-3,4-methylenedioxybenzaldehyde **16** (245 mg, 1 mmol) and methyl (triphenylphosphoranylidene)acetate (401 mg, 1.2 mmol) were dissolved in *N,N*-diethylaniline (15 mL) and the resulting mixture was stirred under reflux for 4 h. The reaction mixture was slowly cooled down to room temperature and left at room temperature for 24 h upon which crude 5-bromo-6,7-methylenedioxycoumarin **BA-3** crystallized from the reaction mixture. The crude coumarin **BA-3** was filtered off, dissolved in dichloromethane (40 mL) and the organic layer was washed with 1 M HCl (50 mL), saturated sodium bicarbonate solution (50 mL), and water (50 mL). After filtration and evaporation of the solvent, 5-bromo-6,7-methylenedioxycoumarin **BA-3** was obtained as yellow crystals. Mp: 230 °C. IR (cm^{-1}): 1727 (C═O); 1626; 1575; 1468; 1453; 1386; 1267; 1241; 1124; 1041; 934; 866. ^1H NMR (300 MHz, $CDCl_3$): δ 6.16 (s, 2H, OCH_2O); 6.36 (d, J = 9.9 Hz, 1H, 3-CH); 6.80 (s, 1H, CH_{ar}); 7.93 (d, J = 9.9 Hz, 1H, 4-CH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 97.7 (CH_{ar}); 98.6 (C_q); 102.7 (OCH_2O); 112.5 (C_q); 114.1 (3-CH); 141.7 (4-CH); 144.0 (C_q); 150.6 (C_q); 151.8 (C_q); 160.6 (CHO). MS (70 eV, ES^-, *m*/*z* (%)): 269/271 ($M-H^+$).

2.2.2.5. Synthesis of 2-fluoro-6-hydroxy-3,4-methylenedioxybenzaldehyde 17. To a solution of 2-[6-(*t*-butyldimethylsilanyloxy)benzo-1,3-dioxol-5-yl]-1,3-dimethyl-imidazolidine **15** (3 mmol, 1.05 g) in THF (40 mL) at −90 °C was added dropwise 4 mL of a 1.5 M solution of *t*-butyllithium in hexane and the resulting reaction mixture was stirred at −90 °C under a nitrogen atmosphere for 30 min. A solution of *N*-fluorobenzenesulfonimide (NFSI) (6 mmol, 1.57 g) in THF was then added and the reaction mixture was slowly warmed up to 0 °C. After keeping the reaction at 0 °C for 2 h, 1 M HCl (50 mL) was added, and the resulting reaction mixture was warmed up to room temperature. After 2 h at room temperature, the reaction mixture was extracted with diethyl ether (3 × 30 mL). The combined organic layers were washed with water (2 × 50 mL) and dried ($MgSO_4$). After filtration and evaporation of the solvent in vacuo, a solid residue was obtained, which was further purified by column chromatography (10% ethyl acetate/90% hexane) to give 2-fluoro-6-hydroxy-3,4-methylenedioxybenzaldehyde **17** (0.24 g, 43% yield) as a yellow solid. Mp: 107 °C. IR (cm^{-1}): 3429 (br s, OH); 1674 (C═O); 1645; 1608; 1501; 1467; 1397; 1279; 1229; 1162; 1078; 1040. ^1H NMR (300 MHz, $CDCl_3$): δ 6.06 (s, 2H, OCH_2O); 6.30 (t, J_{H-F} = 0.8 Hz, 1H, CH_{ar}); 10.00 (s, 1H, CHO); 12.15 (s, 1H, OH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 94.6 (d, J_{C-F} = 3.5 Hz, $OC_qCH_{ar}C_qOH$); 103.1 (s, OCH_2O); 105.5 (d, J_{C-F} = 9.2 Hz, C_qCHO); 126.6 (d, J_{C-F} = 11.5 Hz, OC_qCF); 145.7 (d, J_{C-F} = 256.1 Hz, C_qF); 157.6 (d, J_{C-F} = 9.2 Hz, $OC_qCH_{ar}C_qOH$); 161.6 (d, J_{C-F} = 3.5 Hz, $OC_qCH_{ar}C_qOH$); 189.4 (d, J_{C-F} = 8.1 Hz, CHO). ^{19}F NMR (282 MHz, $CDCl_3$): δ −148.7. MS (70 eV, ES^-, *m*/*z* (%)): 183 ($M-H^+$).

2.2.2.6. Synthesis of 5-fluoro-6,7-methylenedioxycoumarin BA-2. The synthesis of 5-fluoro-6,7-methylenedioxycoumarin **BA-2** (154 mg, 74%) from 2-fluoro-6-hydroxy-3,4-methylenedioxybenzaldehyde **17** (184 mg, 1 mmol) and methyl (triphenylphosphoranylidene)acetate (401 mg, 1.2 mmol) was analogous

to the synthesis of 5-bromo-6,7-methylenedioxycoumarin **BA-3** (vide supra). Mp: 169 °C. IR (cm^{-1}): 1720 (C=O); 1664; 1580; 1501; 1483; 1401; 1311; 1257; 1126; 1079; 1030; 926; 827. ^1H NMR (300 MHz, $CDCl_3$): δ 6.13 (s, 2H, OCH_2O); 6.33 (d, J = 9.6 Hz, 1H, 3-CH); 6.69 (t, J_{H-F} = 0.8 Hz, 1H, CH_{ar}); 7.85 (d, J = 9.6 Hz, 1H, 4-CH) ^{13}C NMR (75 MHz, $CDCl_3$): δ 94.7 (d, J_{C-F} = 9.2 Hz, 8-CH_{ar}); 103.5 (OCH_2O); 104.5 (d, J_{C-F} = 16.2 Hz, 4a-C_q); 113.5 (s, 3-CH); 130.9 (d, J_{C-F} = 12.7 Hz, 6-C_q); 136.3 (d, J_{C-F} = 3.5 Hz, 4-CH); 140.6 (d, J_{C-F} = 253.8 Hz, 5-C_q); 150.6 (d, J_{C-F} = 3.5 Hz, 8a-C_q); 153.0 (d, J_{C-F} = 6.9 Hz, 7-C_q); 160.4 (s, C=O). ^{19}F NMR (282 MHz, $CDCl_3$): δ −146.1. MS (70 eV, ES^+, m/z (%)): 209 ($M+H^+$).

2.2.2.7. Synthesis of 5-methoxy-6,7-methylenedioxycoumarin C-1. A mixture of 5-bromo-6,7-methylenedioxycoumarin **BA-3** (1 mmol, 269 mg) and $CuCl_2$ was dissolved in methanol/DMF (1/1, 6 mL). Under a nitrogen atmosphere, 6 mL of 2 M NaOMe was added and the resulting reaction mixture was boiled under reflux for 32 h. Aqueous HCl (2 M) (20 mL) was then added and the reaction mixture was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with water and brine (each 20 mL), dried ($MgSO_4$), filtered and the solvent was removed in vacuo to yield 160 mg of crude methyl (2*E*)-3-(6-hydroxy-4-methoxy-1,3-benzodioxol-5-yl)prop-2-enoate which was not further purified. The crude (2*E*)-3-(6-hydroxy-4-methoxy-1,3-benzodioxol-5-yl)prop-2-enoate was dissolved in *N,N*-diethylaniline and boiled under reflux for 4 h. Subsequently, the solvent was removed by vacuum distillation and the residue was purified by column chromatography (50% Et_2O/50% hexane) to give 55% (120 mg) of pure 5-methoxy-6,7-methylenedioxycoumarin **C-1** as a pale yellow powder. Mp: 192 °C (lit. 192–194[12]). ^1H NMR (300 MHz, CD_3OD): δ 4.14 (3H, s, OCH_3); 6.01 (2H, s, OCH_2O); 6.21 (1H, d, J = 9.9 Hz, 3-CH); 6.54 (1H, s, 8-CH); 7.95 (1H, d, J = 9.9 Hz, 4-CH). All spectral data corresponded with the reported data of the natural product.[12]

2.2.2.8. Synthesis of 2,6-dihydroxy-3,4-methylenedioxybenzaldehyde 18. To a solution of 2-[6-(*t*-butyldimethylsilanyloxy)benzo-1,3-dioxol-5-yl]-1,3-dimethylimidazolidine **15** (1 mmol, 350 mg) in diethyl ether (15 mL) at −90 °C was added 1 mL of a 1.5 M solution of *t*-butyllithium in hexane and the resulting reaction mixture was stirred at −90 °C under a nitrogen atmosphere for 30 min. Trimethyl borate (1.5 mmol, 156 mg) was added and the reaction mixture was slowly warmed to 0 °C and left at 0 °C for 1 h. Acetic acid (0.5 mL) was then added, followed by hydrogen peroxide (30%, 1.2 mL) and the reaction mixture was stirred at room temperature for 14 h. The reaction mixture was poured in an aqueous HCl solution (6 M) (40 mL) and stirred at room temperature for 2 h. The organic layer was separated and the aqueous layer was extracted with dichloromethane (3 × 50 mL). The combined organic layers were subsequently washed with saturated aqueous sodium bicarbonate and water and subjected to preparative TLC (80% EtOAc/20% hexane). After desorption from the silica gel by stirring the collected fraction in ethyl acetate (50 mL) for 2 h, filtration and evaporation of the solvent in vacuo, 80 mg (44% yield) of 2,6-dihydroxy-3,4-methylenedioxybenzaldehyde **18** was obtained as a pale yellow powder. Mp: 181 °C. IR (cm^{-1}): 3436 (br s, OH); 1650 (C=O); 1633; 1597; 1480; 1463; 1375; 1248; 1131; 1091; 931. ^1H NMR (300 MHz, CD_3OD): δ 5.94 (s, 2H, OCH_2O); 5.97 (s, 1H, CH_{ar}); 10.07 (s, 1H, CHO). ^{13}C NMR (75 MHz, CD_3OD): δ 90.3 (CH_{ar}); 103.5 (OCH_2O); 107.7 (C_qCHO); 127.9 (C_q); 144.9 (C_q); 158.1 (C_q); 162.6 (C_q); 193.4 (CHO). MS (70 eV, ES^-, m/z (%)): 181 ($M-H^+$).

2.2.2.9. Synthesis of 5-hydroxy-6,7-methylenedioxycoumarin BA-1. 2,6-Dihydroxy-3,4-methylenedioxybenzaldehyde **18** (72 mg, 0.4 mmol) and methyl (triphenylphosphoranylidene)acetate (160 mg, 0.48 mmol) were dissolved in *N,N*-diethylaniline (15 mL) and the resulting mixture was boiled under reflux for 4 h. The reaction mixture was cooled down and *N,N*-diethylaniline was distilled off in vacuo. The resulting brown oil was purified by preparative TLC (50% EtOAc/50% hexane) to give 5-hydroxy-6,7-methylenedioxycoumarin **BA-1** (28 mg, 34% yield). Mp: 256 °C (lit. not reported[13]). ^1H NMR (300 MHz, CD_3OD): δ 5.98 (2H, s, OCH_2O); 6.13 (1H, d, J = 9.9 Hz, 3-CH); 6.42 (1H, s, 8-CH); 8.08 (1H, d, J = 9.9 Hz, 4-CH). For full spectral data, see Ref. 13.

2.2.2.10. Synthesis of 5-(3-methyl-2-butenyloxy)-6,7-methylenedioxycoumarin C-2. 5-Hydroxy-6,7-methylenedioxycoumarin **BA-1** (103 mg, 0.5 mmol) was dissolved in THF (2.5 mL). Potassium carbonate (138 mg, 1 mmol) and prenyl bromide (372 mg, 2.5 mmol) were added and the reaction mixture was stirred at room temperature for 24 h. Diethyl ether was added (5 mL), the reaction mixture was filtered and the organic phase was washed with water (5 mL). After drying ($MgSO_4$), filtration and evaporation of the solvent in vacuo, 142 mg (91% yield) of 5-(3-methyl-2-butenyloxy)-6,7-methylenedioxycoumarin **C-2** was obtained, which appeared as white crystals after crystallization from diethyl ether/hexane. Mp: 128 °C (lit. 128–129 °C[14]). ^1H NMR (300 MHz, $CDCl_3$): δ = 1.78 (s, 3H, CH_3); 1.83 (s, 3H, CH_3); 4.85 (d, J = 7.4 Hz, 2H, =CHCH_2O); 5.48 (t, J = 7.4 Hz, 1H, =$CH$$CH_2$O); 6.02 (s, 2H, OCH_2O); 6.20 (d, J = 9.8 Hz, 1H, 3-CH); 6.53 (s, 1H, 8-CH_{ar}); 7.95 (d, J = 9.8 Hz, 1H, 4-CH). For full spectral data, see Ref. 12.

2.2.2.11. Hydrolysis of 5-(3-methyl-2-butenyloxy)-6,7-methylenedioxycoumarin C-2 to 5-hydroxy-6,7-methylenedioxycoumarin BA-1. 5-(3-Methyl-2-butenyloxy)-6,7-methylenedioxycoumarin **C-2** (27.5 mg, 0.1 mmol) was dissolved in methanol (1.5 mL) and a solution of 1 M aqueous HCl (0.5 mL) was added dropwise. The reaction mixture was heated for 25 min at 70 °C. After evaporation of the methanol in vacuo, the reaction mixture was poured in aqueous $NaHCO_3$ (4 mL) and extracted with ethyl acetate (3 × 4 mL). After drying ($MgSO_4$) of the combined organic phases, filtration and evaporation of the solvent in vacuo, 19.3 mg (94% yield) pure 5-hydroxy-6,7-methylenedioxycoumarin **BA-1** was obtained.

2.2.2.12. Methylation of 5-hydroxy-6,7-methylenedioxycoumarin BA-1 to 5-methoxy-6,7-methylenedioxycoumarin C-1. Dimethyl sulfate (10.4 mg, 0.075 mmol) was added to a solution of 5-hydroxy-6,7-methylenedioxycoumarin **BA-1** and potassium carbonate (9.5 mg, 0.075 mmol) in acetone (2 mL). The reaction mixture was boiled under reflux for 18 h. After evaporation of the solvent in vacuo, the residue was dissolved in dichloromethane (4 mL). The reaction mixture was poured in aqueous $NaHCO_3$ solution (5 mL) and extracted with dichloromethane (3 × 4 mL). After drying ($MgSO_4$) of the combined organic phases, filtration and evaporation of the solvent in vacuo, 10.1 mg (92% yield) of 5-methoxy-6,7-methylenedioxycoumarin **C-1** was obtained.

2.2.2.13. Synthesis of 5-(2,3-epoxy-3-methylbutoxy)-6,7-methylenedioxycoumarin D-4. At 0 °C, *m*CPBA (31.1 mg, 0.18 mmol) was added to a solution of 5-(3-methyl-2-butenyloxy)-6,7-methylenedioxycoumarin **C-2** in dichloromethane (2.5 mL). The reaction mixture was stirred for 4 h at room temperature, poured into saturated aqueous $NaHCO_3$ (8 mL) and extracted with dichloromethane (3 × 5 mL). The combined organic phases were washed with saturated aqueous $NaHCO_3$ (4 mL), water (4 mL) and dried ($MgSO_4$). After filtration and evaporation of the solvent in vacuo, 35.2 mg (81% yield) of pure 5-(2,3-epoxy-3-methylbutoxy)-6,7-methylenedioxycoumarin **D-4** was obtained. Mp: 96 °C (lit. 81

°C[15]). IR (cm^{-1}): 1720 (C=O); 1639 (C=C); 1580. ^1H NMR (270 MHz, $CDCl_3$): δ 1.35 (s, 3H, CH_3); 1.39 (s, 3H, CH_3); 3.15 (dd, J = 6.6, 4.3 Hz, 1H, CHO); 4.38 (dd, J = 11.6, 6.6 Hz, 1H, OC*H*(H)); 4.59 (dd, J = 11.6, 4.3 Hz, 1H, OCH(*H*)); 6.02 (s, 2H, OCH_2O); 6.23 (d, J = 9.6 Hz, 1H, 3-CH); 6.56 (s, 1H, 8-CH); 8.01 (d, J = 9.6 Hz, 1H, 4-CH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 19.1 and 24.6 (each CH_3); 58.1 ($C(CH_3)_2$); 61.2 (CHO); 71.3 (CH_2O); 93.0 (8-CH); 102.0 (OCH_2O); 107.0 (4a-C_q); 112.1 (3-CH); 132.1 (6-C_q); 136.8 (5-C_q); 138.7 (4-CH); 151.6 (C_q); 152.5 (C_q); 161.2 (C=O). MS (70 eV, ES^+, m/z (%)): 291 ($M+H^+$). The ^1H NMR spectral data corresponded with the reported data of the natural coumarin, erroneously reported as 5,6-methylenedioxy-7-(2,3-epoxy-3-methylbutoxy)coumarin in Ref. 15, but structurally revised as 5-(2,3-epoxy-3-methylbutoxy)-6,7-methylenedioxycoumarin **D-4** in Ref. 18.

2.2.2.14. Synthesis of 5-(2,3-dihydroxy-3-methylbutoxy)-6,7-methylenedioxycoumarin D-3. *Synthesis from 5-(2,3-epoxy-3-methylbutoxy)-6,7-methylenedioxycoumarin **D-4**:* 5-(2,3-Epoxy-3-methylbutoxy)-6,7-methylenedioxycoumarin **D-4** (29 mg, 0.1 mmol) was dissolved in a mixture of tetrahydrofuran (1 mL) and water (1 mL). A catalytic amount of trifluoroacetic acid (2.3 mg, 0.02 mmol) was added and the reaction mixture was stirred at room temperature for 8 h. After evaporation of the tetrahydrofuran under reduced pressure, the aqueous phase was extracted with dichloromethane (2 × 4 mL). The combined organic phases were dried ($MgSO_4$), filtered and evaporated in vacuo which afforded 30 mg (97% yield) of 5-(2,3-dihydroxy-3-methylbutoxy)-6,7-methylenedioxycoumarin **D-3**.

*Synthesis from 5-(3-methyl-2-butenyloxy)-6,7-methylenedioxycoumarin **C-2**:* *N*-Methylmorpholine-*N*-oxide (16.4 mg, 0.14 mmol) and osmium(VIII) oxide (0.3 mg, 0.01 mmol) were dissolved in a mixture of tetrahydrofuran (0.6 mL) and water (0.4 mL). At 0 °C, 5-(3-methyl-2-butenyloxy)-6,7-methylenedioxycoumarin **C-2** (27.4 mg, 0.1 mmol) was added. The reaction mixture was stirred at room temperature for 14 h. After evaporation of tetrahydrofuran under reduced pressure, a saturated aqueous sodium bisulfite solution (5 mL) was added and the mixture was stirred for 30 min at room temperature. The aqueous phase was extracted with ethyl acetate (3 × 5 mL). After drying ($MgSO_4$) of the combined organic phases, filtration and evaporation of the solvent in vacuo, the residue was purified by column chromatography to afford 12 mg (39% yield) of 5-(2,3-dihydroxy-3-methylbutoxy)-6,7-methylenedioxycoumarin **D-3**. Mp: 156–157 °C (lit. 150.4–151 °C,[16] 160–161 °C[17]). IR (cm^{-1}): 3422 (br, OH), 1706 (C=O); 1686 (C=C), 1626 ($C=C_{Ar}$). ^1H NMR (300 MHz, $CDCl_3$): δ 1.27 (s, 3H, CH_3); 1.32 (s, 3H, CH_3); 2.68 (br s, 2H, 2 × OH); 3.83 (dd, J = 8.0, 2.8 Hz, 1H, CHO); 4.37 (dd, J = 10.5, 8.0 Hz, 1H, OC*H*(H)); 4.52 (dd, J = 10.5, 2.8 Hz, 1H, OCH(*H*)); 6.025 (d, J = 1.2 Hz, 1H, OC*H*(H)O); 6.032 (d, J = 1.2 Hz, 1H, OCH(*H*)); 6.21 (d, J = 9.8 Hz, 1H, 3-CH); 6.55 (s, 1H, 8-CH); 7.96 (d, J = 9.8 Hz, 1H, 4-CH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 24.9 and 26.8 (each CH_3); 71.7 ($C(CH_3)_2$); 73.8 (CH_2O); 76.5 (CHO); 93.2 (8-CH); 102.2 (OCH_2O); 107.1 (4a-C_q); 112.1 (3-CH); 132.4 (6-C_q); 136.8 (5-C_q); 138.8 (4-CH); 151.6 (C_q); 152.6 (C_q); 161.4 (C=O). MS (70 eV, ES^+, m/z (%)): 309 ($M+H^+$). The ^1H and ^{13}C NMR data were in accordance with reported data for the natural product, see Ref. 18.

2.2.2.15. Synthesis of 5-(2-hydroxy-3-methoxy-3-methylbutoxy)-6,7-methylenedioxy-coumarin D-2. To a solution of 5-(2,3-epoxy-3-methylbutoxy)-6,7-methylenedioxycoumarin **D-4** (14.5 mg, 0.05 mmol) in dry methanol (2 mL) was added a catalytic amount of *p*-toluenesulfonic acid (1.7 mg, 0.01 mmol) at 0 °C. The reaction mixture was stirred for 4 h at room temperature. Subsequently, the solvent was evaporated under reduced pressure and the residue was dissolved in dichloromethane (5 mL). Water was added (5 mL) and after separation of the organic layer, the aqueous layer was again extracted with dichloromethane (2 × 5 mL). The combined organic layers were dried ($MgSO_4$), filtered, and evaporated in vacuo to afford 15 mg (93% yield) of 5-(2-hydroxy-3-methoxy-3-methylbutoxy)-6,7-methylenedioxycoumarin **D-2**. IR (cm^{-1}): 3431 (br, OH), 1722 (C=O); 1629 ($C=C_{Ar}$). ^1H NMR (300 MHz, $CDCl_3$): δ 1.21 (s, 3H, CH_3); 1.25 (s, 3H, CH_3); 2.50 (br s, 1H, OH); 3.26 (s, 3H, OCH_3); 3.86 (dd, J = 8.3, 3.0 Hz, 1H, CHO); 4.32 (dd, J = 10.5, 8.3 Hz, 1H, OCH(*H*)); 4.52 (dd, J = 10.5, 3.0 Hz, 1H, OCH(*H*)); 6.019 (d, J = 1.4 Hz, 1H, OC*H*(H)O); 6.032 (d, J = 1.4 Hz, 1H, OCH(*H*)); 6.22 (d, J = 9.8 Hz, 1H, 3-CH); 6.55 (s, 1H, 8-CH); 8.03 (d, J = 9.8 Hz, 1H, 4-CH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 20.6 and 20.9 (each CH_3); 49.3 (OCH_3); 73.6 (CH_2O); 76.0 ($C(CH_3)_2$); 78.0 (CHO); 92.9 (8-CH); 102.0 (OCH_2O); 107.2 (4a-C_q); 112.0 (3-CH); 132.3 (6-C_q); 137.2 (5-C_q); 139.1 (4-CH); 151.6 (C_q); 152.6 (C_q); 161.5 (C=O). MS (70 eV, ES^+, m/z (%)): 323 ($M+H^+$). All spectroscopic data were in accordance with reported data for the natural product, see Ref. 13.

2.2.2.16. Synthesis of 5-(3-chloro-2-hydroxy-3-methylbutoxy)-6,7-methylenedioxycoumarin D-5. 5-(2,3-Epoxy-3-methylbutoxy)-6,7-methylenedioxycoumarin **D-4** (29 mg, 0.1 mmol) was dissolved in dry diethyl ether (5 mL). At 0 °C, dry HCl gas was bubbled through the reaction mixture for 1 h. The reaction mixture was warmed to room temperature and evaporated in vacuo to yield 32 mg of the crude coumarin **D-5** which was recrystallized to afford 28 mg (88% yield) of pure 5-(3-chloro-2-hydroxy-3-methylbutoxy)-6,7-methylenedioxycoumarin **D-5**. IR (cm^{-1}): 3439 (br s, OH); 1717 (C=O); 1623 (C=C). ^1H NMR (270 MHz, $CDCl_3$): δ 1.67 (s, 3H, CH_3); 1.68 (s, 3H, CH_3); 2.55 (br s, 1H, OH); 3.97 (dd, J = 7.6, 3.0 Hz, 1H, C*H*OH); 4.39 (dd, J = 10.6, 7.6 Hz, 1H, OC*H*(H)); 4.68 (dd, J = 10.6, 3.0 Hz, 1H, OCH(*H*)); 6.03 (s, 2H, OCH_2O); 6.23 (d, J = 9.8 Hz, 1H, 3-CH); 6.58 (s, 1H, 8-CH); 8.03 (d, J = 9.8 Hz, 1H, 4-CH). ^{13}C NMR (68 MHz, $CDCl_3$): δ 28.8 and 28.9 (each CH_3); 71.7 (CCl); 73.2 (CH_2O); 77.3 (CHOH); 93.1 (8-CH); 102.1 (OCH_2O); 107.0 (4a-C_q); 112.1 (3-CH); 132.2 (6-C_q); 136.7 (5-C_q); 138.7 (4-CH); 151.5 (C_q); 152.5 (C_q); 161.2 (C=O). MS (70 eV, ES^+, m/z (%)): 327/29 ($M+H^+$).

2.2.2.17. Synthesis of 7,8-methylenedioxycoumarin D-7. The synthesis of 7,8-methylenedioxycoumarin **D-7** from 2-hydroxy-3,4-methylenedioxybenzaldehyde **21** (332 mg, 2 mmol) and methyl (triphenylphosphoranylidene)acetate (802 mg, 2.4 mmol) was analogous to the synthesis of 5-bromo-6,7-methylenedioxycoumarin **BA-3** (vide supra). In this case, after the reaction, *N,N*-diethylaniline was distilled from the reaction mixture in vacuo (1 mmHg, 52 °C) and the residue was chromatographed over silica gel (40% diethyl ether/60% hexane) to give 280 mg (74%) 7,8-methylenedioxycoumarin **D-7**. Mp: 178 °C (lit. 187–189 °C[19]). IR (cm^{-1}): 1726 (C=O); 1714; 1639; 1582; 1497; 1460; 1280; 1267; 1120; 1074; 1044; 834. ^1H NMR (300 MHz, $CDCl_3$): δ 6.15 (s, 2H, OCH_2O); 6.25 (d, J = 9.6 Hz, 1H, 3-CH); 6.81 (d, J = 8.3 Hz, 1H, 6-CH); 7.00 (d, J = 8.3 Hz, 1H, 5-CH); 7.62 (d, J = 9.6 Hz, 1H, 4-CH). ^{13}C NMR (75 MHz, $CDCl_3$): δ 103.1 (OCH_2O); 105.7 (6-CH); 113.7 (3-CH); 115.2 (4a-C_q); 122.0 (5-CH); 133.9 (C_q); 138.4 (C_q); 143.9 (4-CH); 151.6 (C_q); 159.7 (C=O). MS (70 eV, ES^+, m/z (%)): 191 ($M+H^+$). HRMS Calcd for $C_{10}H_7O_4$ ($M+H^+$) 191.03389, Found 191.03396.

2.3. Coumarins

Coumarin (purity >99.9%) was obtained from Sigma Chemical Co. (St. Louis, USA). All synthesized, natural, and commercial coumarins (Table 1) were dissolved in 0.01% (v/v) DMSO and stored at −20 °C.

2.4. Cell culture

The U-937 cell line (American Type Culture Collection, Rockville, MD) was cultured at 37 °C in a humidified atmosphere with 5% CO_2 in RPMI 1640 medium, supplemented with 10% fetal calf serum and 50 μg/mL gentamicin. The cell suspension was split at the third day and diluted one day before experimental procedures.

2.5. Measurement of the cytotoxicity concentration 50 (CC_{50})

Cells growing in exponential phase were seeded at 10^5 cells in 1 mL of RPMI 1640 in a 24-well culture plate and incubated in a 5% CO_2 atmosphere. Cells were exposed at different coumarin concentrations (0.15 μM to 2.0 mM) or 0.0001% (v/v) DMSO (control group). After incubation for 48 h, an aliquot of the medium was mixed with an equal volume of 0.4% trypan blue and incubated for 5 min after which the number of viable cells was estimated by a hemocytometer chamber. CC_{50} values were calculated with the equation for sigmoidal dose response using Prism 4.00 for Windows (GraphPad Software, San Diego, CA). Assays were carried out in quadruplicate in at least four independent experiments.

2.6. Cell growth inhibition assays (IC_{50})

Cells growing in exponential phase were seeded at 10^4 cells in 150 μl of RPMI 1640 in a 96-well culture plate and incubated under a 5% CO_2 atmosphere. U-937 cells were exposed to different concentrations of coumarins ranging from 0.15 μM to 2.0 mM or 0.0001% (v/v) DMSO (control group), followed by incubation with 0.5 μCi of [^{3}H] 5-methyl thymidine (Perkin–Elmer, USA) (added 12 h before the end of the experiment) and then harvested in an automatic cell harvester (Nunc, Maryland, USA). The incorporation of the radioactive nucleotide was measured in a Pharmacia Wallac 1410 liquid scintillation counter and expressed as incorporation percentage respect to the control group (cells treated with DMSO). IC_{50} values were calculated using the equation for sigmoidal dose response using Prism 4.00 for Windows (GraphPad Software, San Diego, CA). Assays were performed in quadruplicate in at least four independent experiments. On the other hand, to evaluate U-937 proliferation in time, the number of cells was determined using a cellular meter Coulter Z-1. Briefly, 10^5 cells/mL were seeded in 24 wells plates and treated with different pure compounds concentrations, 400 μM dbcAMP (positive control), or 0.0001% (v/v) DMSO (control group) for 3 days. Cells were then collected at different times according to each experiment and the number of cells was determined by a Coulter Z-1. Cell density in culture did not exceed 1×10^6 cells/mL.

2.7. Determination of U-937 cell differentiation markers

2.7.1. Nitrobluetetrazolium differentiation assay

U-937 cells (2×10^5/mL) were exposed for 48 h to different coumarin concentrations, 1 μM ATRA (positive control) or 0.0001% (v/v) DMSO (control group). Cells were then washed and resuspended in 200 μl RPMI 1640 media containing 1 mg/mL NBT and 1 μg/mL PMA. After incubation at 37 °C for 30 min, cells were pelleted and dissolved in 200 μl DMSO and absorbance determined at 570 nm.

2.7.2. Surface myeloid CD11b antigen assay

The expression of CD11b was detected by direct immunofluorescence staining. U-937 cells (2×10^5 cells/mL) were treated with different concentrations of coumarins, 400 μM dbcAMP (positive control) or 0.0001% (v/v) DMSO (control group) for 48 h. Treated and control cells were washed twice in PBS and incubated with a saturated concentration of phycoerythrin anti-CD11b antibody (Coulter-Immunotech, France) or an equivalent concentration of isotype-matched control at 4 °C for 30 min. Cells were washed twice with PBS supplemented with 1% FCS and immediately analyzed in a FACScan flow cytometer (Becton-Dickinson, CA, USA). For each sample, a minimum of 5000 events were acquired. Percentages of positive cells between specific CD-immunolabelled cells and their negative controls were established for both untreated and treated cells.

2.7.3. Expression of the C5a receptor (CD88) assay

The C5a receptor (CD88) is a G-protein coupled receptor associated with Ca^{2+} release from intracellular stores.[20] Thus, we evaluated its expression by measuring Ca^{2+} intracellular release after rhC5a stimulus, using Fura 2-AM as a fluorescent indicator. 1×10^6 U-937 cells/mL were treated with different concentrations of coumarins, 400 μM dbcAMP (positive control), or 0.0001% (v/v) DMSO (control group) for 48 h. Cells of each experimental group were washed, resuspended and incubated in a buffered saline solution (BSS; 140 mM NaCl, 3.9 mM KCl, 0.7 mM KH_2PO_4, 0.5 mM $Na_2HPO_4{\cdot}12H_2O$, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 20 mM HEPES, 10 mM glucose, and 0.1% BSA, pH 7.5) in the presence of 2 μM Fura 2-AM. Stock solution of 2 mM of Fura 2-AM was prepared in DMSO. Cells were incubated for 30 min at 37 °C in an atmosphere of 5% CO_2, time by which Fura 2-AM was trapped intracellularly by esterase cleavage. Cells were then washed twice in BSS, and brought to a density of 2×10^6 cells/mL in BSS. Fluorescence was measured in a spectrofluorometer (Jasco, Tokyo, Japan) provided with the CA-61 accessory to measure Ca^{2+} with continuous stirring, with the thermostat adjusted to 37 °C and an injection chamber. During 8 min intracellular Ca^{2+} ($[Ca^{2+}]i$) levels were registered every second by exposure to alternating 340 and 380 nm light beams, and the intensity of light emission at 505 nm was measured. In this way, light intensities and their ratio (F340/F380) were tracked. Different agents (rhC5a or ATP) were injected (5 μl) into the chamber as a 100-fold concentrated solution without interrupting recording. The preparation was calibrated by determining maximal fluorescence induced by 0.1% Triton X-100, and minimal fluorescence in the presence of 6 mM EGTA (pH 8.3). $[Ca^{2+}]i$ was calculated according to Grynkiewicz et al.[21]

3. Results

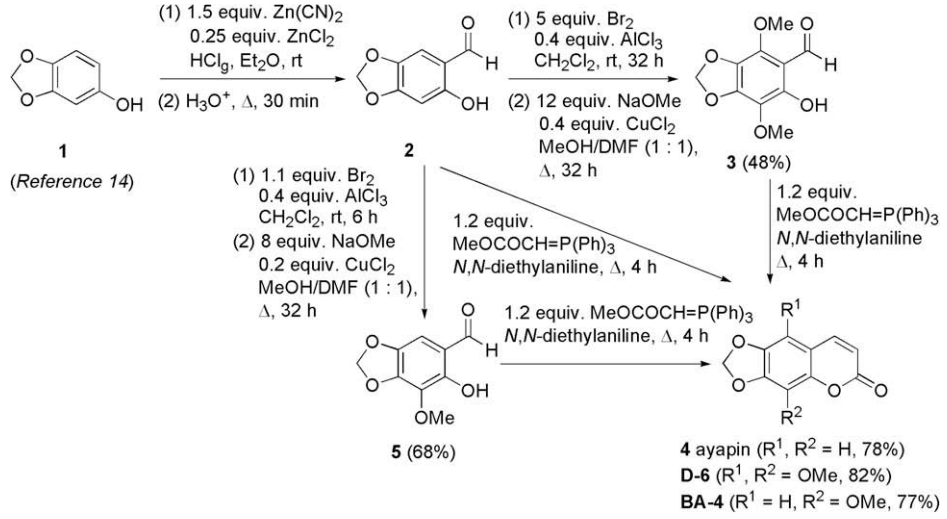
3.1. Synthesis of oxygenated coumarins

The coumarins used in the present biological study were isolated from their natural source, that is, from *P. polystachyum* DC (Compositae) for 6-hydroxy-7-(3-methyl-2-butenyloxy)coumarin **D-11**,[22] or were prepared synthetically. The total syntheses of some of the oxygenated coumarins were reported earlier,[14,23,24] and are briefly summarized below (Schemes 1–3), while the unreported synthesis of the 5-substituted ayapin derivatives **BA-1**-**BA-3**, **C-1**, **C-2**, **D-2**-**D-5**, and **D-7** is shown in Schemes 4–6.

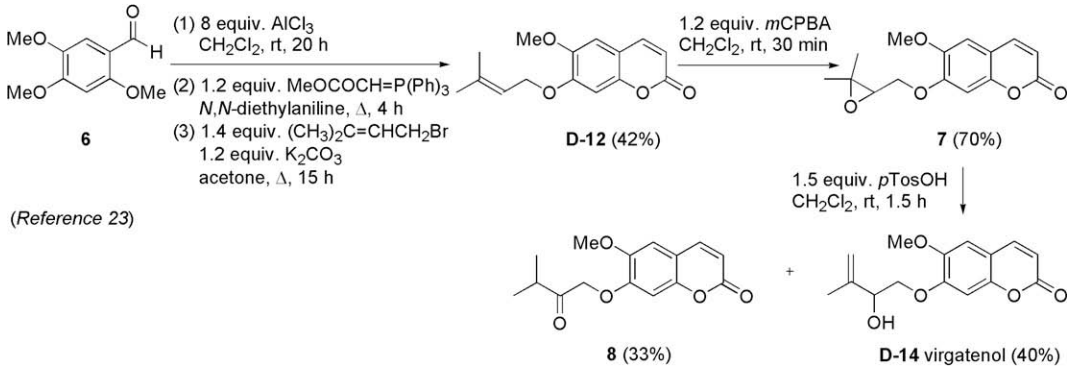
The total synthesis of the natural product ayapin **4** and its naturally occurring derivatives **D-6** and **BA-4** started with a Gattermann formylation of sesamol **1** (Scheme 1). Then, the resulting benzaldehyde **2** was brominated selectively at the 3-position or it was dibrominated, depending on the reaction conditions, and subsequent aromatic substitution of the aryl bromides with sodium methoxide furnished the methoxy-substituted benzaldehydes **3** and **5**. Finally, a Wittig reaction of aldehydes **2**, **3**, and **5** with methoxycarbonylmethylenephosphorane and subsequent

intramolecular cyclization delivered the target coumarins ayapin **4**, **D-6** and **BA-4**.[14]

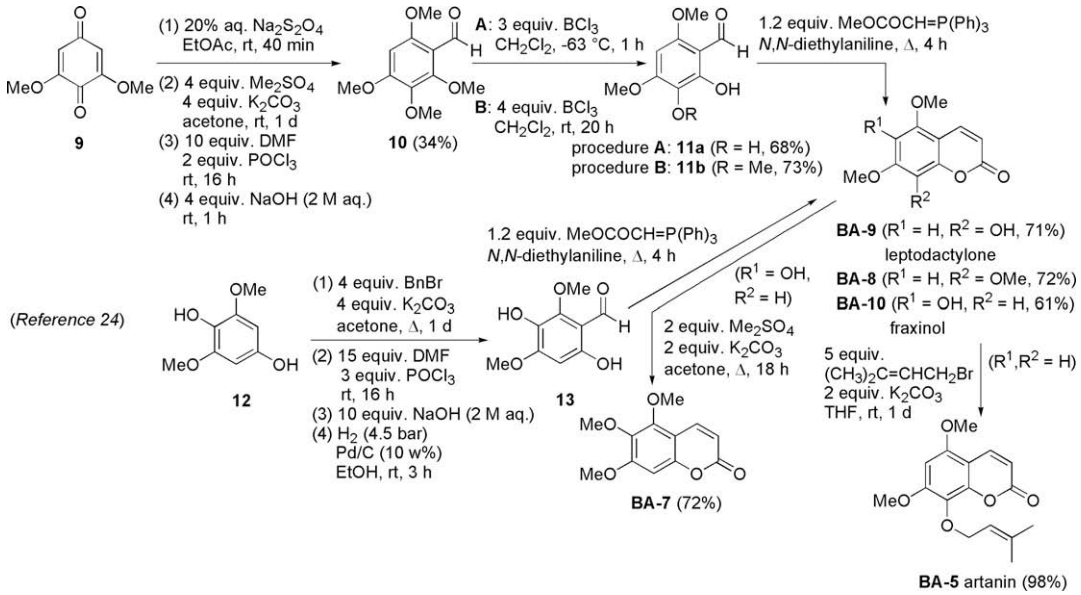
The synthesis of coumarins **D-12** and **D-14** was accomplished by aluminum(III) chloride-mediated deprotection of the methyl ether-protected alcohol at the 2-position of 2,4,5-trimethoxybenzaldehyde **6** (Scheme 2). Then, a Wittig reaction with methoxycarbonylmethylenephosphorane, followed by intramolecular cyclization and O-alkylation of the intermediate



Scheme 1.



Scheme 2.

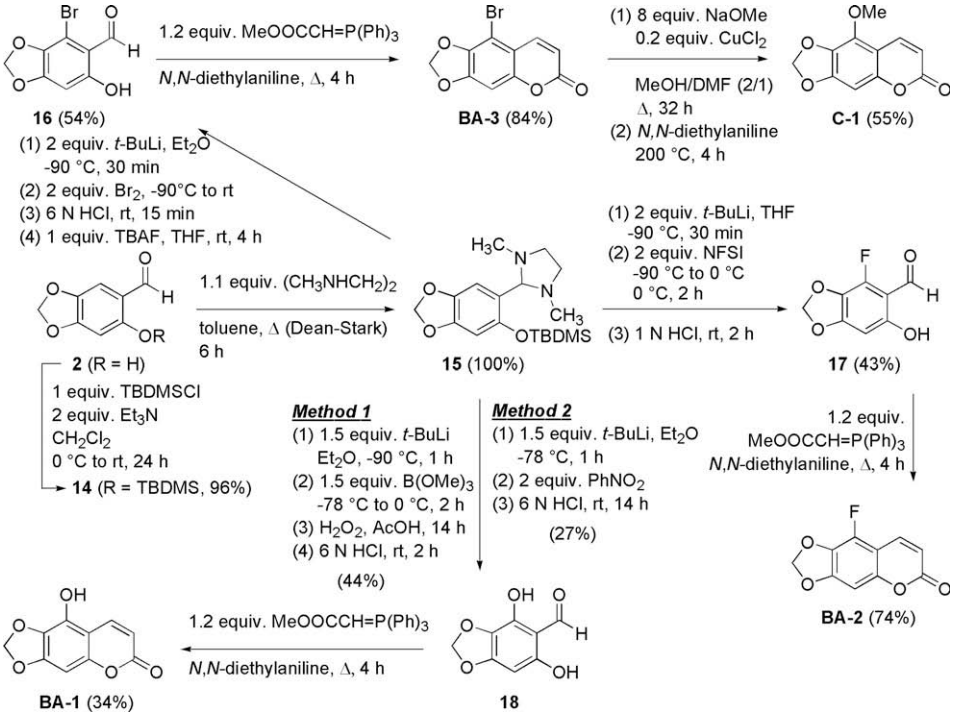


Scheme 3.

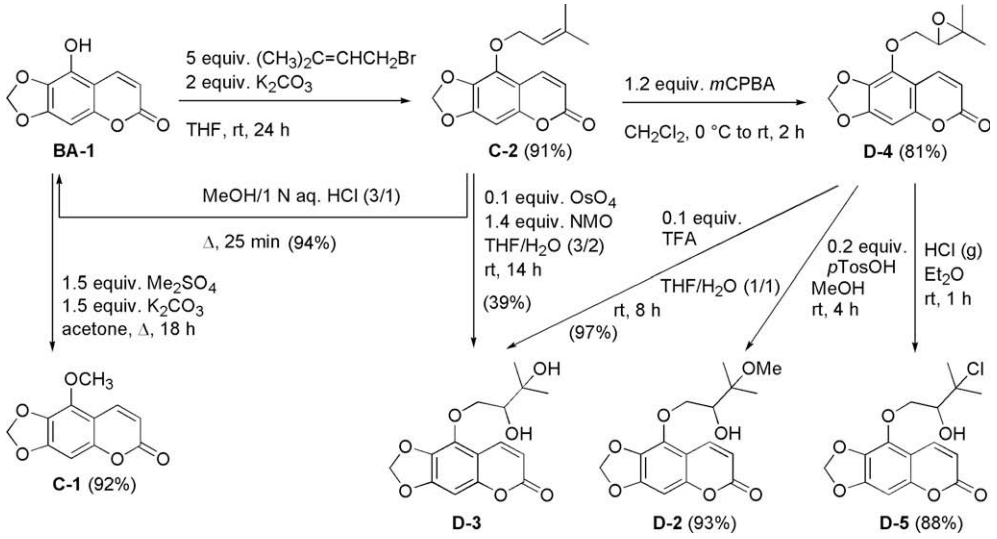
alcohol with prenyl bromide, furnished 6-methoxy-7-(3-methyl-2-butenyloxy)coumarin **D-12**. Reaction of this prenylated compound **D-12** with *m*CPBA to the corresponding epoxide **7** and acid-catalyzed ring opening of the epoxide with *p*TosOH gave a mixture of coumarin **8** and the target compound virgatenol **D-14**, which was purified by column chromatography.[23]

In a following reaction pathway, the total synthesis of 5,7,8-trioxygenated coumarins artanin **BA-5**, **BA-8**, leptodactylone **BA-9** and of 5,6,7-trioxygenated coumarins **BA-7**, fraxinol **BA-10** is described (Scheme 3). For each of these compounds, the coumarin skeleton was constructed by a Wittig reaction of the tetraoxygenated benzaldehydes **11a**, **11b**, and **13** with methoxycarbonylmethylenephosphorane. The intermediate benzaldehydes **11a** and **11b** were obtained from 2,6-dimethoxy-1,4-benzoquinone **9** after reduction of the quinone, protection of the intermediate hydroquinone as the corresponding methyl ether, a Villsmeier formylation and boron(III) chloride-mediated demethylation. In addition, protection of hydroquinone **12** as its benzyl ether, followed by a Villsmeier formylation and deprotection of the benzyl ether delivered the third tetraoxygenated benzaldehyde **13**.[24]

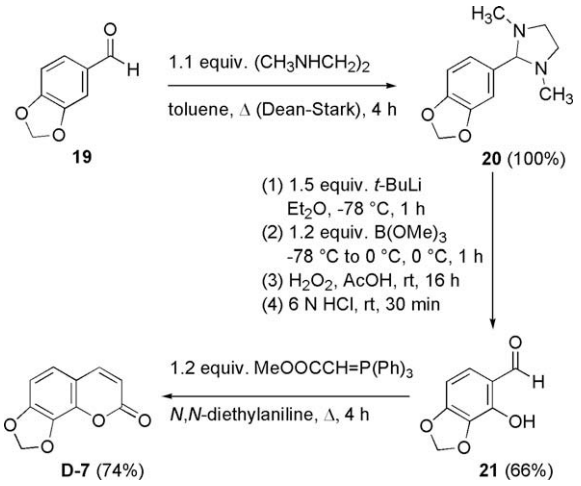
In the next part, the unreported synthesis of the 5-substituted ayapin derivatives **BA-1**-**BA-3**, **C-1**, **C-2**, **D-2**-**D-5**, and **D-7** is disclosed. The first idea was to synthesize 5-oxygenated 6,7-methylenedioxycoumarins through a substitution reaction on 5-bromo-6,7-methylenedioxycoumarin **BA-3**. The required precursor for this strategy, 2-bromo-6-hydroxy-3,4-methylenedioxybenzaldehyde



Scheme 4.



Scheme 5.



Scheme 6.

16, cannot be prepared via direct bromination of 2-hydroxy-4,5-methylenedioxybenzaldehyde **2** with bromine and aluminum(III) chloride, as this leads to the non-desired regioisomer (vide supra, Scheme 1).[14] In order to brominate the *ortho* position with respect to the formyl group, the free phenolic hydroxyl group of aldehyde **2** was protected as the *t*-butyldimethylsilyl ether **14** and the aldehyde was turned into an *ortho*-directing lithiation group by protection as the imidazolidine **15** (Scheme 4). Treatment of imidazolidine **15** with *t*-butyllithium at −90 °C, followed by quenching with bromine, hydrolysis of the imidazolidine and deprotection of the hydroxyl group afforded 2-bromo-6-hydroxy-3,4-methylenedioxybenzaldehyde **16** in 54% yield. The development of this one-pot reaction procedure avoided tedious purification procedures of reaction products. Benzaldehyde **16** was converted to 5-bromo-6,7-methylenedioxycoumarin **BA-3** by a Wittig reaction in *N,N*-diethylaniline in 84% yield. The purification of coumarin **BA-3** was easily achieved by crystallization from the crude reaction mixture.

In recent years, there is a continuously growing interest in fluorinated compounds and fluorinated building blocks as the basis of valuable bioactive molecules.[25] Therefore, it was decided to investigate also the synthesis of 5-fluoro-6,7-methylenedioxycoumarin **BA-2**. Lithiation of imidazolidine **15** with *t*-butyllithium at −90 °C, followed by quenching of the lithium salt with *N*-fluorobenzenesulfonimide (NFSI) and deprotection of the aldehyde as well as the hydroxyl group via acid hydrolysis gave 2-fluoro-6-hydroxy-3,4-methylenedioxybenzaldehyde **17** in 43% yield. The *o*-hydroxybenzaldehyde **17** was converted into the corresponding coumarin **BA-2** in 74% yield by a Wittig reaction. Also in this case the product could be recovered by crystallization from the reaction mixture.

All attempts to directly substitute the 5-bromo substituent of coumarin **BA-3** to a hydroxyl function via bromo-lithium exchange followed by treatment with a suitable oxidant such as trimethylborate–hydrogen peroxide or nitrobenzene,[26] or by palladium-catalyzed substitution reaction with potassium hydroxide failed,[27] with the former conditions giving mainly reduced 6,7-methylenedioxycoumarin (ayapin) **4**, while the palladium-catalyzed reaction led to opening of the lactone ring. An alternative approach via Cu-catalyzed methoxylation was more successful in functionalizing 5-bromo-6,7-methylenedioxycoumarin **BA-3**. Treatment of coumarin **BA-3** with sodium methoxide and $CuCl_2$ as catalyst in a mixture of MeOH/DMF and heating of the reaction mixture under reflux for 32 h,[14] afforded methyl (2*E*)-3-(6-hydroxy-4-methoxy-1,3-benzodioxol-5-yl)prop-2-enoate as a crude intermediate which was dissolved in *N,N*-diethylaniline and heated at 200 °C for 4 h to get isomerization and ring closure toward 5-methoxy-6,7-methylenedioxycoumarin **C-1**. In this way, coumarin **C-1** was obtained in 55% overall yield. All attempts to selectively deprotect the methyl ether of coumarin **C-1**, while preserving the methylenedioxy moiety, toward the synthesis of 5-hydroxy-6,7-methylenedioxycoumarin **BA-1** were unsuccessful. No reaction occurred upon treatment of 5-methoxy-6,7-methylenedioxycoumarin **C-1** with boron(III) chloride at 0 °C,[28] or aluminum(III) chloride at room temperature. On the other hand, complex reaction mixtures were obtained with boron(III) chloride at room temperature and boron(III) bromide at 0 °C. Attempted demethylation of coumarin **C-1** using lithium chloride in boiling *N,N*-dimethylformamide also gave rise to a complex reaction mixture.[29] Since 5-hydroxy-6,7-methylenedioxycoumarin **BA-1** could not be synthesized from 5-bromo-6,7-methylenedioxycoumarin **BA-3**, another synthetic strategy had to be developed. An alternative method consisted of the introduction of the hydroxyl group already in aldehyde **18**, starting the synthesis from imidazolidine **15**. Lithiation of imidazolidine **15** with *t*-butyllithium in diethyl ether at −90 °C for 1 h was followed by treatment of the lithium salt with trimethylborate and oxidation with hydrogen peroxide. When a 2 M aqueous HCl solution was subsequently used for the hydrolysis of the imidazolidine, it was observed that also the silyl ether was partially cleaved. Whereas performing the reaction using 6 M aqueous HCl for longer times (16 h), the imidazolidine as well as the *t*-butyldimethylsilyl group were cleaved. Attempts to purify aldehyde **18** by column chromatography resulted in very low yields of **18** (23% yield). Slightly better results were obtained when preparative thin layer chromatography was used (30% yield). A better procedure consisted in extracting the reaction mixture extensively with dichloromethane, followed by basic extraction of the organic phase. Acidification of the aqueous phase and extractive workup with ethyl acetate afforded 2,6-dihydroxy-3,4-methylenedioxybenzaldehyde **18** as a white crystalline solid in 44% yield. Considerably lower yields (27% yield) were obtained when nitrobenzene was used as an oxidans. It could be expected that, when 2,6-dihydroxy-3,4-methylenedioxybenzaldehyde **18** is treated with methyl (triphenylphosphoranylidene)acetate in *N,N*-diethylaniline, both 5-hydroxy-6,7-methylenedioxycoumarin **BA-1** and 5-hydroxy-7,8-methylenedioxycoumarin would be formed. However, only 5-hydroxy-6,7-methylenedioxycoumarin **BA-1** was isolated. In this case, the coumarin **BA-1** did not crystallize from the reaction mixture. Careful chromatographic purification, using the natural coumarin **BA-1** as a reference,[13] allowed to isolate 5-hydroxy-6,7-methylenedioxycoumarin **BA-1** in very low yields (11–16%) when purification was done by column chromatography and in much better yield (34%) when the reaction mixture was purified by preparative TLC.

5-Hydroxy-6,7-methylenedioxycoumarin **BA-1** is the ideal precursor for the synthesis of other 5-oxygenated ayapin derivatives. Treatment of 5-hydroxy-6,7-methylenedioxycoumarin **BA-1** with 5 equiv of prenyl bromide and 2 equiv of sodium carbonate in tetrahydrofuran yielded 91% of pure 5-(3-methyl-2-butenyloxy)-6,7-methylenedioxycoumarin **C-2** (Scheme 5). The reverse reaction, the acid-mediated hydrolysis of the natural product **C-2** toward coumarin **BA-1**, was also possible upon heating in methanol in the presence of 1 N aqueous hydrochloric acid. The obtained 5-hydroxy-6,7-methylenedioxycoumarin **BA-1** was further converted to 5-methoxy-6,7-methylenedioxycoumarin **C-1** in 92% yield with dimethyl sulfate and potassium carbonate in boiling acetone. The double bond in the prenyloxy side chain of coumarin **C-2** was epoxidized in 81% yield using 3-chloroper-

benzoic acid in dichloromethane. Acid catalyzed ring opening of 5-(2,3-epoxy-3-methylbutoxy)-6,7-methylenedioxycoumarin **D-4** in a mixture of tetrahydrofuran and water (ratio 1:1) resulted in 97% of 5-(2,3-dihydroxy-3-methylbutoxy)-6,7-methylenedioxycoumarin **D-3**. When the epoxide was opened in the presence of methanol, 5-(2-hydroxy-3-methoxy-3-methylbutoxy)-6,7-methylenedioxycoumarin **D-2** was obtained in 93% yield. Opening of the epoxide **D-4** with dry hydrogen chloride gas in diethyl ether gave 88% of 5-(3-chloro-2-hydroxy-3-methylbutoxy)-6,7-methylenedioxycoumarin **D-5**. The latter coumarin has not yet been isolated from a natural source, but is related to other coumarinic chlorohydrins found in plants or prepared synthetically.[23] Direct dihydroxylation of coumarin **C-2** with osmium tetroxide in the presence of *N*-methylmorpholine-*N*-oxide (NMO) resulted in 39% of 5-(2,3-dihydroxy-3-methylbutoxy)-6,7-methylenedioxycoumarin **D-3**.

In the final part, a new synthesis of the natural coumarin 7,8-methylenedioxycoumarin **D-7** is reported. The only synthesis known for 7,8-methylenedioxycoumarin **D-7** is the methylenation of natural 7,8-dihydroxycoumarin.[30] In contrast to this synthesis, the present synthesis starts from readily available piperonal **19**. Treatment of piperonal **19** with *N,N′*-dimethylethylene-1,2-diamine under reflux in toluene under Dean–Stark conditions resulted in 2-benzo-1,3-dioxol-5-yl-1,3-dimethylimidazolidine **20** quantitatively (Scheme 6). Further reaction of this imidazolidine **20** with *t*-butyllithium at −78 °C, followed by treatment with trimethyl borate and hydrogen peroxide gave 2-hydroxy-3,4-methylenedioxybenzaldehyde **21** in 66% yield. This aldehyde **21** was treated with 1.2 equiv of methyl (triphenylphosphoranylidene)acetate in *N,N*-diethylaniline and boiled under reflux for 4 h. After purification, 7,8-methylenedioxycoumarin **D-7** was obtained in 74% yield.

3.2. Evaluation of the cytotoxicity and U-937 growth inhibition by polyoxygenated coumarins

The first parameter determined was the cytotoxicity of the tested coumarins in the U-937 cell line. In order to be useful as a differentiation inducing agent in human leukemia cells, compounds should not possess high cytotoxicity in U-937 cells at the effective differentiation concentration. Different concentrations of the coumarins (0.15 μM to 2 mM) were added to U-937 cells for 5 days, and cell viability was assayed by the trypan blue exclusion test. The most potent cytotoxic activity was observed for coumarins **D-2**, followed by compound **D-3** (Table 2). The CC_{50} of coumarins **BA-5**, **D-5**, **D-4**, **D-11**, **D-12**, **D-14**, and **BA-7** show a 500–900 μM range, whereas the remaining compounds did not affect cell viability at a concentration lower than 2 mM (Table 2). The U-937 treatment with the vehicle alone (0.0001% (v/v) DMSO) exhibited more than 90% of cell viability.

The rate in which the test compounds inhibit cell proliferation was measured by U-937 cell growth experiment, assessed by [^{3}H]-thymidine incorporation after 48 h of coumarin treatment. The results of this assay are depicted in Table 2, showing the highest anti-proliferative activity for coumarin **D-2** with an IC_{50} value of 25.3 ± 1.1 μM. Other coumarins which showed anti-proliferative activity were **BA-1**, **D-3**, **D-5**, **BA-5**, and **BA-7**. The other polyoxygenated coumarins evaluated did not inhibit U-937 cells proliferation in the concentrations tested.

3.3. Evaluation of the U-937 differentiation by polyoxygenated coumarins

The U-937 cell line derived from a histiocytic lymphoma is an appropriate model to evaluate monocyte cell differentiation.[31] In this cell line, dibutyryl cAMP (dbcAMP) as all-*trans*-retinoic acid (ATRA) induce monocyte maturation[31,32] which can be monitored by changes of morphological, biochemical, and immunological properties.

As part of the present screening, to determine whether tested coumarins evoked cell growth inhibition in U-937 cells resulting from the induction of maturation of the leukemic cells, the expression of the CD88 receptor as marker of monocyte maturation was determined. U-937 cells were treated with different concentrations (5, 25, and 50 μM) of the coumarins **D-2**, **BA-1**, **D-3**, **D-5**, **BA-5**, and **BA-7** for 48 h. U-937 cells treated with dibutyryl cyclic adenosine monophosphate (dbcAMP) were used as a positive control of U-937 cell differentiation. As shown in Figure 1, cells pretreated with **D-2** (25 and 50 μM) and **D-3** (50 μM) for 2 days, displayed an increased intracellular [Ca^{2+}] level induced by rhC5a stimulus, indicating the expression of the marker CD88 in the cellular surface of the treated cells, similar to that evoked by dbcAMP. The pretreatment with 5 μM **D-2** and **D-3** did not induce CD88 expression (data not shown). Moreover, for all the tested concentrations of the other four coumarins, no release of Ca^{2+} from intracellular stores was observed upon addition of C5a. No response was observed in vehicle pretreated cells, but when these cells were stimulated with ATP, known to elevate [Ca^{2+}]i levels,[33] they showed the typical spike, indicating that these cells were able to evoke a Ca^{2+} response.

3.4. Effects of coumarins D-2 and D-3 on U-937 cell proliferation

In order to gain insight into the anti-proliferative and differentiation activity described for compounds **D-2** and **D-3**, the activity of these compounds on U-937 cells was further depicted. Figure 2 shows that both coumarins, **D-2** and **D-3**, inhibited the growth of human leukemia U-937 cells in a time dependent manner. These results showed that 50 μM **D-2** and 50 μM **D-3** significantly inhibited the growth of U-937 cells at 72 h, whereas the percentage of cell inhibition with respect to control cells were 62.5% for **D-2** and 38.0% for **D-3** (p <0.01 and p <0.05, respectively).

Table 2
Effect of coumarins on U-937 cell proliferation and cytotoxicity

	CC_{50} (mM)	IC_{50} (μM)
Ayapin	>2	>400
BA-1	>2	255.4 ± 1.2
BA-2	>2	>400
BA-3	>2	>400
D-2	0.365 ± 0.002	25.3 ± 1.1
D-3	0.420 ± 0.002	191.9 ± 1.2
D-4	0.899 ± 0.008	>400
D-5	0.567 ± 0.005	226.9 ± 1.2
D-6	>2	>400
BA-4	>2	>400
D-7	>2	>400
Coumarin	>2	>400
D-11	0.820 ± 0.008	>400
D-12	0.799 ± 0.002	>400
D-14	0.704 ± 0.010	>400
BA-7	0.790 ± 0.001	182.1 ± 1.9
BA-10	>2	>400
BA-9	>2	>400
BA-8	>2	>400
BA-5	0.535 ± 0.006	177.3 ± 1.9
C-1[1]	0.252 ± 0.001	2.2 ± 0.3
C-2[1]	0.266 ± 0.001	3.5 ± 0.4

Cell growth of control and treated U-937 cells was assessed by [^{3}H]-thymidine incorporation. Cellular toxicity was evaluated by the Trypan blue assay. IC_{50} and CC_{50} values were calculated with the equation for sigmoidal dose response using Prism 4.00 for Windows (GraphPad Software, San Diego, CA, USA). Data are means ± SEM (n = 4).

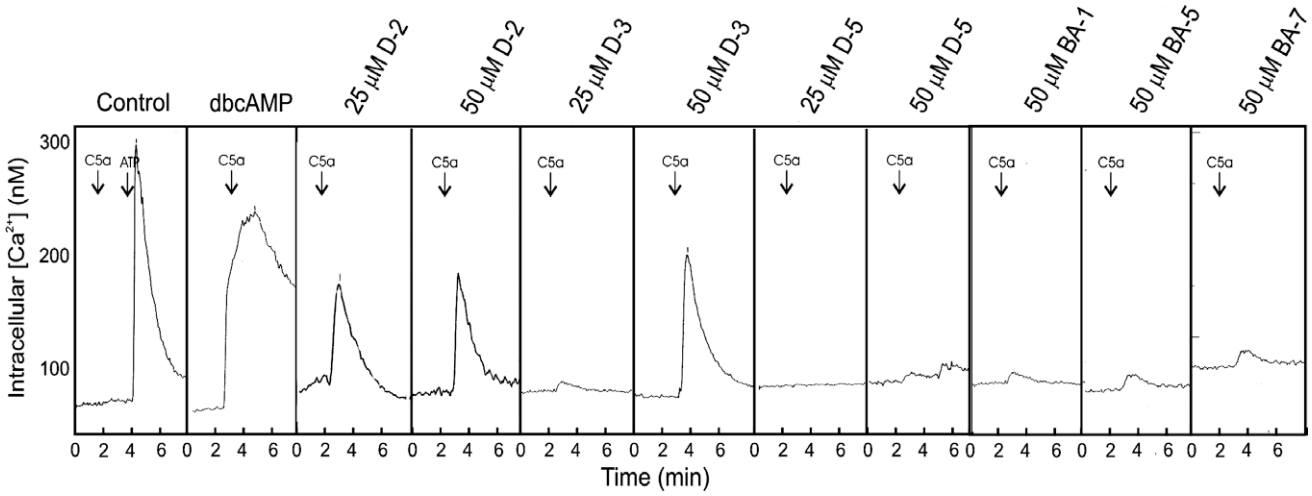


Figure 1. Effect of anti-proliferative coumarins on $[Ca^{2+}]i$ response induced by rhC5a. U-937 cells were treated with 25 and 50 μM of the different tested compounds, 400 μM dbcAMP, or 0.0001% (v/v) DMSO, for 48 h. $[Ca^{2+}]i$ was determined as described in Section 2. Arrows indicate the addition of rhC5a (C5a) or ATP. Similar results were obtained in at least three independent experiments.

3.5. Effects of coumarins D-2 and D-3 on U-937 cell differentiation

In addition of CD88 expression evaluated as part of our initial screening, we determined others U-937 cell differentiation markers after treatment with these 5-oxygenated-6,7-methylenedioxycoumarins. The burst oxidative capacity of differentiated U-937 cells was determined by NBT reduction assays. The rate of NBT reduction by differentiated U-937 cells was determined by the measure of formazan at 570 nm. A significant increase in the production of formazan was observed following 72 h exposure to **D-2** (25 and 50 μM) and **D-3** (50 μM) with respect to control cells (DMSO treated cells) (Fig. 3). For this assay, ATRA was employed as positive control of cell differentiation. As shown in Figure 4, similar treatments of U-937 induced an increase in the expression of other monocyte differentiation marker CD11b, the α-subunit of the integrin αβ-heterodimer CD11b/CD18.[34] In U-937 cells treated with 5 μM of both compounds, the differentiation markers evaluated were not significantly different with respect to control cells (data not shown). These findings show that exposure of leukemic cells to the polyoxygenated coumarins **D-2** and **D-3** induced U-937 cell maturation, coumarin **D-2** being a more effective differentiation agent than **D-3**.

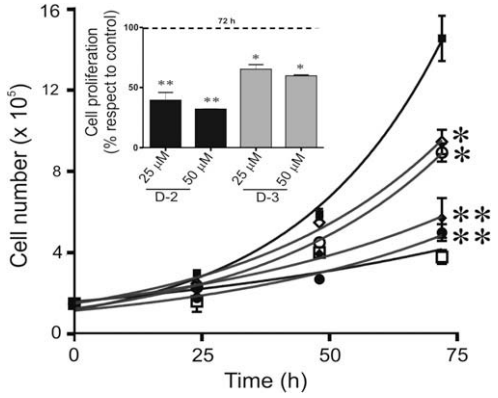


Figure 2. Effect of coumarins **D-2** and **D-3** on U-937 cell proliferation. Cells were treated with (♦) 25 μM **D-2**; (●) 50 μM **D-2**; (○) 25 μM **D-3**; (◇) 50 μM **D-3**; (□) 400 μM dbcAMP (positive control), or (■) 0.0001% (v/v) DMSO for 24, 48, and 72 h as described in Section 2. Inset U-937 cell proliferation after 72 h of treatment with compounds **D-2** and **D-3**. Data are expressed as proliferation percentage with respect to the control group. Control group proliferation considered 100% is shown as a dotted line. The results represent the mean ± SEM ($n = 3$). $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control group (DMSO treated cells).

4. Discussion

Nowadays, the most commonly used therapeutic modalities employed in leukemias include cytotoxic drugs treatments, bone marrow transplantation as well as combination therapies with deleterious side effects.[35] Thus, therapy with minimal side effects is highly demanded in the clinical field. Leukemic cells are blocked

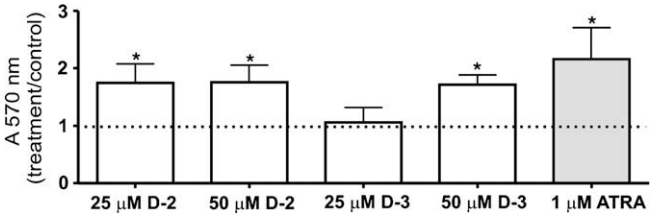


Figure 3. Nitrobluetetrazolium reduction by U-937 cells treated with **D-2** and **D-3**. Cells were treated with 1 μM ATRA (positive control), 25 μM and 50 μM **D-2** or **D-3**, or 0.0001% (v/v) DMSO (vehicle) for 72 h. Formazan production was determined as described in Section 2. Control group (DMSO-treated cells) absorbance considered 1 is shown as a dotted line. Results are expressed as means ± SEM ($n = 3$). $^{*}p < 0.05$ versus control group.

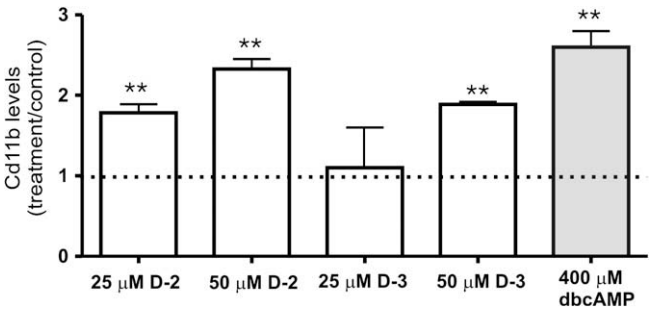


Figure 4. CD11b levels in U-937 cells treated with **D-2** and **D-3**. Cells were treated with 400 μM dbcAMP (positive control), 25 μM and 50 μM **D-2** or **D-3**, or 0.0001% (v/v) DMSO (vehicle) for 72 h. CD11b levels were determined as described in Section 2. CD11b expression in control group (DMSO-treated cells) was considered as 1, shown as a dotted line. Results are expressed as means ± SEM ($n = 3$). $^{**}p < 0.01$ versus control group.

in an early stage of their normal maturation, so differentiation induction therapy has attracted world-wide attention due to its higher specificity compared to the traditional approaches.[36]

In the last years we started working in the development of new potential and selective anti-leukemic compounds employing natural and synthetic coumarins as lead molecules. In the present work, 20 related coumarin compounds were made easily accessible via chemical synthesis. The natural and synthetic 5-substituted ayapin derivatives **BA-1**, **BA-3**, **C-1**, **C-2**, **D-2**, **D-5** and the natural 7,8-methylenedioxycoumarin **D-7** were newly synthesized. The lithiation and electrophilic quenching of functionalized imidazolidine **15** and **20** are key-steps in the syntheses of 5-hydroxy-, 5-fluoro- and 5-bromoayapins **BA-1**, **BA-3** and coumarin **D-7**, respectively. 5-Hydroxyayapin **BA-1** proved to be a suitable precursor for functional group transformations to other 5-oxygenated ayapin derivatives **C-1**, **C-2** and **D-2**, **D-5**.

The biological evaluation of the compounds revealed that **D-2**, **D-3**, **BA-1**, **D-5**, **BA-5**, and **BA-7**,[24] showed anti-proliferative activity in U-937 cells (Table 2). Nevertheless, as seen in Figures 1, 3, and 4, only treatments with 5-(2,3-dihydroxy-3-methylbutoxy)-6,7-methylenedioxycoumarin (**D-3**) and 5-(2-hydroxy-3-methoxy-3-methylbutoxy)-6,7-methylenedioxycoumarin (**D-2**) were able to induce the differentiation of U-937 cells along the monocytic lineage analyzed by the expression of cell surface markers as CD88, CD11b, increased oxidative capacity of mature cells following 48 h treatment. On the other hand, other related oxygenated coumarins did not display differentiation activity in the human leukemia U-937 cell line (Fig. 1). **D-2** and **D-3** coumarins show similar activities as previously described for the natural coumarins 5-(3-methyl-2-butenyloxy)-6,7-methylenedioxycoumarin (**C-2**) and 5-methoxy-6,7-methylenedioxy coumarin (**C-1**) isolated from *P. polystachyum*.[1] However, **D-2** and **D-3** conform a racemic mixture, hence it is not possible to predict if both enantiomers are active or just one of them.

Our results provide insight into the correlation between some structural properties of polyoxygenated coumarins and their in vitro leukemic differentiation activity. The most important features in terms of the structure–activity relationship (SAR) are the presence of the 6,7-methylendioxy arrangement and the presence of an alkoxy substituent only at position 5 of the coumarin nucleus. In this work, only the 5-oxygenated-6,7-methylenedioxycoumarins **D-2** and **D-3** showed anti-proliferative and differentiation activity in U-937 cells. 6,7-Methylenedioxycoumarins such as ayapin, **BA-2**, **BA-3**, and **BA-4**, which do not possess an alkoxy substituent on carbon 5, did not inhibit U-937 proliferation. Remarkably, compound **D-6,** which bears methoxy groups at positions 5 and 8, did not display an anti-proliferative effect on leukemic cells. Furthermore, 5-hydroxy-6,7-methylenedioxycoumarin (**BA-1**) was able to inhibit cell growth in the tested concentration range, but failed to induce the expression of CD88 receptor after 48 h of treatment. The set of results obtained with compounds **D-7**, **D-11**, **D-12**, **D-14**, **BA-6**, **BA-10**, **BA-9**, **BA-8**, and **BA-5** supports that the presence of the methylendioxy group at positions 6 and 7 of the nucleus would be a requirement for the differentiation activity. These findings support the results obtained with the natural coumarins **C-1** and **C-2** isolated from *P. polystachyum*.[1]

In summary, the present study with natural and synthetic coumarins indicates that these compounds can be considered as novel candidates for leukemia therapy, since they are able to inhibit cancer growth and induce differentiation of leukemic cells without inducing a relevant cytotoxic effect.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.08.002.

References and notes

1. Riveiro, M. E.; Shayo, C.; Monczor, F.; Fernandez, N.; Baldi, A.; De Kimpe, N.; Rossi, J.; Debenedetti, S.; Davio, C. *Cancer Lett.* **2004**, *210*, 179.
2. Leszczyniecka, M.; Roberts, T.; Dent, P.; Grant, S.; Fisher, P. B. *Pharmacol. Ther.* **2001**, *90*, 105.
3. Tsiftsoglou, A. S.; Pappas, I. S.; Vizirianakis, I. S. *Pharmacol. Ther.* **2003**, *100*, 257.
4. O'Kennedy, R.; Thornes, R. D. *Coumarins: Biology, Applications and Mode of Action*; Chichester, 1997.
5. Riveiro, M. E.; Moglioni, A.; Vazquez, R.; Gomez, N.; Facorro, G.; Piehl, L.; de Celis, E. R.; Shayo, C.; Davio, C. *Bioorg. Med. Chem.* **2008**, *16*, 2665.
6. Yim, D.; Singh, R. P.; Agarwal, C.; Lee, S.; Chi, H.; Agarwal, R. *Cancer Res.* **2005**, *65*, 1035.
7. Riveiro, M. E.; Vazquez, R.; Moglioni, A.; Gomez, N.; Baldi, A.; Davio, C.; Shayo, C. *Biochem. Pharmacol.* **2008**, *75*, 725.
8. Kostova, I.; Raleva, S.; Genova, P.; Argirova, R. *Bioinorg. Chem. Appl.* **2006**, 1.
9. Gage, B. F. *Hematology Am. Soc. Hematol. Educ. Program* **2006**, 467.
10. Ostrov, D. A.; Hernandez Prada, J. A.; Corsino, P. E.; Finton, K. A.; Le, N.; Rowe, T. C. *Antimicrob. Agents Chemother.* **2007**, *51*, 3688.
11. Kontogiorgis, C. A.; Savvoglou, K.; Hadjipavlou-Litina, D. J. J. *Enzyme Inhib. Med. Chem.* **2006**, *21*, 21.
12. Debenedetti, S. L.; Palacios, P. S.; Nadinic, E. L.; Coussio, J. D.; De Kimpe, N.; Boeykens, M.; Feneau-Dupont, J.; Declercq, J.-P. *J. Nat. Prod.* **1994**, *57*, 1539.
13. Maes, D.; Debenedetti, S.; De Kimpe, N. *Biochem. System. Ecol.* **2006**, *34*, 165.
14. Maes, D.; Vervisch, S.; Debenedetti, S.; Davio, C.; Mangelinckx, S.; Giubellina, N.; De Kimpe, N. *Tetrahedron* **2005**, *61*, 2505.
15. Bohlmann, F.; Grenz, M.; Zdero, C. *Chem. Ber.* **1975**, *108*, 2955.
16. Debenedetti, S. L.; Ferraro, G. E.; Coussio, J. D. *Planta Medica* **1981**, *42*, 97.
17. Magalhães, A. F.; Magalhães, E. G.; Leitão Filho, H. F.; Frighetto, R. T. S.; Barros, S. M. G. *Phytochemistry* **1981**, *20*, 1369.
18. Debenedetti, S. L.; De Kimpe, N.; Boeykens, M.; Coussio, J. D.; Kesteleyn, B. *Phytochemistry* **1997**, *45*, 1515.
19. Herz, W.; Bhat, S. V.; Santhanam, P. S. *Phytochemistry* **1970**, *9*, 891.
20. Burg, M.; Martin, U.; Rheinheimer, C.; Kohl, J.; Bautsch, W.; Bottger, E. C.; Klos, A. *J. Immunol.* **1995**, *155*, 4419.
21. Grynkiewicz, G.; Poenie, M.; Tsien, R. Y. *J. Biol. Chem.* **1985**, *260*, 3440.
22. Palacios, R. S.; Rojo, A. A.; Coussio, J. D.; De Kimpe, N.; Debenedetti, S. L. *Planta Medica* **1999**, *65*, 294.
23. Demyttenaere, J.; Vervisch, S.; Debenedetti, S.; Coussio, J.; Maes, D.; De Kimpe, N. *Synthesis* **2004**, *11*, 1844.
24. Maes, D.; Riveiro, M. E.; Shayo, C.; Davio, C.; Debenedetti, S.; De Kimpe, N. *Tetrahedron* **2008**, *64*, 4438.
25. Dolbier, W. R., Jr. *J. Fluorine Chem.* **2005**, *126*, 157.
26. (a) Buck, P.; Köbrich, G. *Tetrahedron Lett.* **1967**, 1563; (b) Köbrich, G.; Buck, P. *Chem. Ber.* **1970**, *103*, 1412; (c) Sinhababu, A. K.; Borchardt, R. T. *J. Org. Chem.* **1983**, *48*, 1941; (d) Comins, D. L.; Brown, J. D. *Tetrahedron Lett.* **1981**, *22*, 4213; (e) Wiriyachitra, P.; Cava, M. P. *J. Org. Chem.* **1977**, *42*, 2274; (f) Katritzky, A. R.; CavaLong, Q.; He, H.-Y.; Qiua, G.; Wilcox, A. L. P. *Arkivoc* **2000**, *VI*, 868.
27. Anderson, K. W.; Ikawa, T.; Tundel, R. E.; Buchwald, S. L. *J. Am. Chem. Soc.* **2006**, *128*, 10694.
28. Teitel, S.; O'Brien, J.; Brossi, A. *J. Org. Chem.* **1972**, *37*, 3368.
29. Bernard, A. M.; Ghiani, M. R.; Piras, P. P.; Rivoldini, A. *Synthesis* **1989**, 287.
30. Castillo, P.; Rodriguez-Ubis, J. C.; Rodriguez, F. *Synthesis* **1986**, 839.
31. Harris, P.; Ralph, P. J. *Leukoc. Biol.* **1985**, *37*, 407.
32. Sundstrom, C.; Nilsson, K. *Int. J. Cancer* **1976**, *17*, 565.
33. Ansselin, A.; Davey, D.; Allen, D. *Neuroscience* **1997**, *76*, 947.
34. Arnaout, M. *Blood* **1990**, *75*, 1037.
35. Estey, E. H. *Best Pract. Res. Clin. Haematol.* **2003**, *16*, 521.
36. Sell, S. *Stem Cell Rev.* **2005**, *1*, 197.