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RESEARCH ARTICLE

Antiproliferative effect on HepaRG cell cultures of new calix[4]arenes

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Abstract

Cell cycle progression is dependent on the intracellular iron level, and chelators lead to iron depletion and decrease cell proliferation. This antiproliferative effect can be inhibited by exogenous iron. In this work, we present the synthesis of new synthetic calix[4]arene podands bearing alkyl acid and alkyl ester groups at the lower rim, designed as potential iron chelators. We report their effect on cell proliferation, in comparison with the new oral chelator ICL670 (4-[3,5-bis-(2-hydroxyphenyl)-1,2,4-triazol-1-yl]-benzoic acid). The antiproliferative effect of these new compounds was studied in human hepatocarcinoma HepaRG cell cultures using the MTT assay. Their cytotoxicity was evaluated by extracellular LDH activity. Preliminary results indicate that their antiproliferative effect is due to their cytotoxicity. The efficiency of these compounds, comparable to that of ICL670, was independent of iron depletion. This effect remains to be further explored. Moreover, it also shows that novel substituted calix[4]arenes could open the way to new valuable medicinal chemistry scaffolding.

Keywords: *Calix[4]arene; hepatocyte; HepaRG cell line; iron chelation; antiproliferative effect*

Abbreviations: MEM, minimum essential medium; LDH, lactate dehydrogenase; SDH, succinate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline

Introduction

Higher life forms such as humans have developed a highly efficient iron management system in which we absorb and excrete only about 1 mg of the metal daily; there is no mechanism for the excretion of excess iron[1,2]. As a result, a progressive accumulation of iron in the body, particularly in the liver, leads to iron overload, which is toxic and can induce hepatocellular carcinoma development as observed in genetic and secondary hemochromatosis[3,4]. Iron plays a critical role in a variety of metabolic processes, as Fe-containing proteins catalyze key reactions involved in energy production and DNA synthesis. In particular, Fe is critical for ribonucleotide reductase (RR) activity, which is the rate-limiting step in DNA synthesis[5]. RR comprises two subunits, R1 and R2. Iron is essential for the catalytic activity of RR and stabilizes the tyrosyl radical located within the R2 subunit, making iron an obvious target for chemotherapeutic agents[6]. Iron depletion by different iron chelators has been shown to inhibit proliferation of various cell lines and normal activated lymphocytes *in vitro*[7–9]. The iron depletion induced by iron chelators such as desferrioxamine (DFO) or *O*-Trensox decreases DNA synthesis in both normal and transformed hepatocytes[10,11]. Numerous cancer cell types are more susceptible to the effects of chelators than normal cells due to a higher Fe requirement for DNA synthesis and metabolism than the more slowly growing normal cells[12]. Several studies have shown that iron is implicated in tumor cell growth and some tumor cells are sensitive to iron chelation therapy[13–15]. The role of Fe chelators has previously been reviewed[16]. The effect of Fe chelators is complex due to various molecular targeting in addition to RR, and the molecular mechanisms involved in G1/S arrest after Fe deprivation remain poorly

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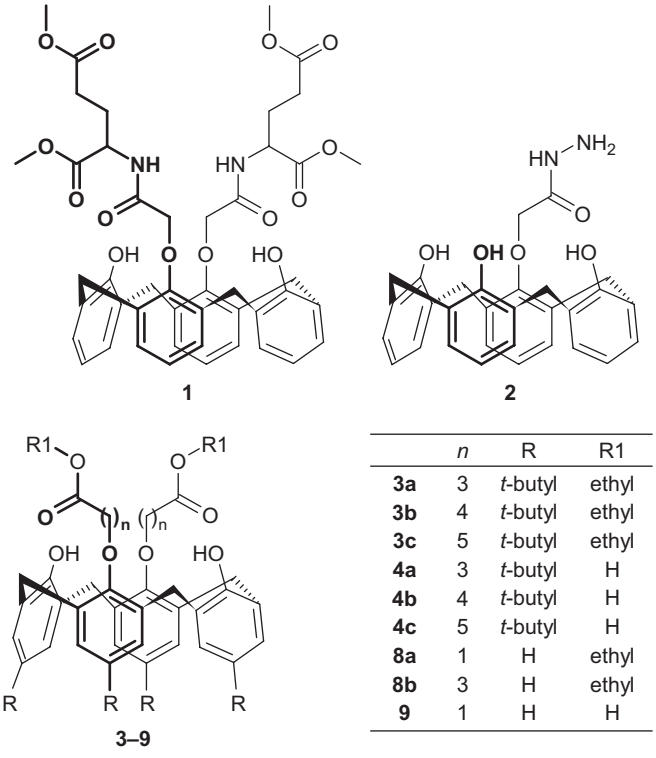
understood. Recent chelators such as 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone and analogs[17,18] and Triapine[19] have shown greater antiproliferative activity than DFO *in vitro*. However, DFO used for the treatment of iron overload, neuroblastoma, and other diseases such as malaria[20,21] is poorly absorbed by the gastrointestinal tract; furthermore, continuous exposure to DFO causes dose- and time-dependent cytotoxicity[22]. Other disadvantages of DFO are its short plasma half-life and its poor permeability, which lead to poor antitumor activity[23]. Therefore, various new iron chelators have been designed for clinical use. Among them, the bidentate hydroxypyridinone deferiprone (CP20) is the major molecule used for the treatment of secondary iron overload[24–26], but this chelator has been shown to induce severe neutropenia[27]. The orally active tridentate ICL670 is of special interest[21,22], because it induces cell cycle arrest in the S phase associated with a decrease of polyamine levels which could result from inhibition of polyamine biosynthesis, probably by ornithine decarboxylase (ODC) inactivation[28].

Clinically useful antitumor agents must show a significant therapeutic index (i.e. they exert little effect on normal cells while inhibiting tumor cell growth). Richardson and Lovejoy identified, in the case of pyridoxal isonicotinoyl hydrazone analogs, a significant chelator structure–activity relationship dependent on lipophilicity[29]. Within a series, or family, of ligands, the more lipophilic compounds generally have better iron-clearing efficiency, and lipophilicity has a profound effect on organ distribution of the chelator[30]. Hider and Liu synthethized a family of hydroxypyridinone ester analogs, which were designed especially to target the hepatocellular low molecular weight iron pool[31]. Chelators with high lipophilicity can easily enter cells and subsequently deplete iron from intracellular pools necessary for iron incorporation into the R2 subunit. Another design consideration to identify an ideal iron chelator for antiproliferative activity is metal selectivity[32]. The chelators should be selective for iron to minimize chelation of other biological metal cations such as Zn(II) and Cu(II).

Calix[4]arenes or derivatives have been tested as bioactive compounds such as antitumoral, antiviral, antimicrobial, anti-thrombotic, and antifungal agents[33,34]. Calix[4]arenes present interesting lipophilicity properties, and are selectively capable of binding cationic, anionic, or neutral species. Consequently, some calixarene derivatives possess affinity and selectivity for Fe(III), and much lower affinity for Mg(II), Ca(II), and Zn(II)[35–37]. Rao and colleagues reported a series of dinuclear transition metal complexes of calix[4]arenes. In the case of the Fe(III) complex the ligand acts as a trinegative, implying that the ligand uses a lower rim phenolic O^- in the binding[38,39]. Other authors also described calix[*n*]arene–iron(III) complexes in which the Fe(III) was six-coordinated by O donor atoms from calix[*n*]arenes[40]. Fe(III)complexes of some amide-substituted calix[4]arenes have also been reported. In these complexes, the Fe is bound to all seven O atoms of the calixarene with the shortest Fe–O distance to the phenolate O atom[41]. Thus, taking into account our experience in the field of the synthesis of new compounds of the calix[4]arene type[42–44], we previously prepared new calix[4]arene podands bearing two aspartic/glutamic acid or ornithine groups at the lower rim, and a mono hydrazidocalixarene[45]. In this series, two compounds **1–2** exhibited interesting antiproliferative activity in the rat hepatoma cell line Fao. Our preliminary published results need to be reinforced with complementary structure–activity (SAR) studies of other substituted calix[4]arenes in an aim to better understand, in particular, the level of substitution, or/and conformation of the corresponding substituted calix[4]arenes in regard to their potential antiproliferative activities. In continuation of our work, we present here the synthesis and proliferative activity in HepaRG cell line cultures of new alkyl ester or alkyl acid calix[4]arene derivatives **3–9** (Figure 1).

These functions are selectively introduced into calix[4]arenes locked in the cone or 1,3-alternate conformations to improve their antiproliferative activity, as well as their chelator behavior toward iron. The O donor atoms of the alkyl ester or alkyl acid calix[4]arene substituents could be implied in iron complexation in mono or dinuclear calyx[4]arene complexes as previously mentioned by Rao *et al.*[39]. The HepaRG cell line was isolated from a liver tumor of a patient suffering from a hepatocarcinoma[46]. This bipotent cell line differentiates progressively in both hepatocyte-like cells expressing highly differentiated functions and biliary-like cells. This cell line, which was characterized through a transcriptomic approach, exhibited an ability to



	n	R	R1
3a	3	*t*-butyl	ethyl
3b	4	*t*-butyl	ethyl
3c	5	*t*-butyl	ethyl
4a	3	*t*-butyl	H
4b	4	*t*-butyl	H
4c	5	*t*-butyl	H
8a	1	H	ethyl
8b	3	H	ethyl
9	1	H	H

Figure 1. Structures of peptidocalix[4]arene **1**, mono hydrazidocalix[4]arene **2**, and new ester or acid calix[4]arene derivatives **3–9**.

store iron within hepatocytes, when differentiated[47]. Such a model gives us the opportunity to evaluate the impact of iron chelators on hepatocyte metabolism at various phases of their proliferation/differentiation process.

Materials and methods

Chemistry

Instrumentation

Column chromatography was performed on Kieselgel 60 (40–63 µm, ASTM) (Merck). Reactions were analyzed on precoated silica gel 60 F_{254} plates (Merck) and the compounds were visualized with an ultraviolet (UV) lamp (254 nm) and phosphomolybdic acid reagent and heating. Calix[4]arene derivatives **5–7** were purified by reverse-phase high performance luiquid chromatrography (HPLC) using a Shimadzu semi-preparative HPLC system on a ProSphere 100 C18 5 µm column (10 × 250 mm) by elution with a linear gradient of (A): aqueous 0.1% trifluoroacetic acid (TFA) and (B): 0.1% (v/v) TFA in an acetonitrile/water mixture (80/20), at a flow rate of 3 mL/min with UV detection at 220 nm. Melting points were determined with an SM-LUX-POL Leitz hot-stage microscope and are reported uncorrected. Infrared (IR) spectra were recorded on a Jasco Fourier transform (FT)/IR-4200 spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 500 spectrometer (500 MHz). Chemical shifts refer to tetramethylsilane, which was used as an internal reference. *J* values are given in Hertz. Mass spectra were recorded on a Micromass (Waters) Q-TOF (quadrupole time-of-flight) Ultima spectrometer.

Synthesis of 25,27-dihydroxy-26,28-bis(ethoxycarbonylpropoxy) or (ethoxycarbonylbutoxy) or (ethoxycarbonylpentoxy)tert-butyl-calix[4]arenes, cone 3a–c

A suspension of *tert*-butyl-calix[4]arene (1.54 mmol) and K_2CO_3 (3.69 mmol) was reacted with alkyl bromide (3.39 mmol) in refluxing CH_3CN (50 mL) for 5 days. The solvent was then evaporated at reduced pressure, and the residue was taken up with 10% HCl (150 mL) and extracted with CH_2Cl_2 (200 mL). The organic layer was separated, washed twice with water, dried ($MgSO_4$), and evaporated to dryness. The residue was chromatographed on silica gel with cyclohexane:ethyl acetate (8:2, v/v) to give **3a–c**.

*25,27-Dihydroxy-26,28-bis(ethoxycarbonylpropoxy)tert-butyl-calix[4]arene, cone (**3a**)* Yield: 85%, white crystals, mp = 163°C, (Found MNa^+: 899.5449, $C_{56}H_{76}O_8{}^{23}Na$ requires 899.5438); IR ν_{max}/cm^{-1} 3337 (OH), 1708 (CO); ^1H NMR δ (500 MHz, $CDCl_3$) 7.78 (s, 2H, OH), 7.16 (s, 4H, Ar-H meta), 6.97 (s, 4H, Ar-H meta), 4.36 (d, *J* 12.88 Hz, 4H, $ArCH_2Ar$), 4.21 (m, 4H, OCH_2, ethyl), 4.14 (t, *J* 6.63 Hz, 4H, OCH_2), 3.43 (d, *J* 12.88 Hz, 4H, $ArCH_2Ar$), 2.95 (t, *J* 6.68 Hz, 4H, CH_2), 2.42 (m, 4H, CH_2), 1.39 (s, 18H, CH_3 *t*-butyl), 1.27 (m, 6H, CH_3 ethyl), 1.11 (s, 18H, CH_3 *t*-butyl). ^{13}C NMR δ (125 MHz, $CDCl_3$) 173.8 (COO), 151.2 (Cq, Ar ipso), 150.1 (Cq, Ar ipso), 147.5 (Cq, Ar para), 142.0 (Cq, Ar para), 133.2 (Cq, Ar ortho), 128.2 (Cq, Ar ortho), 126.1 (CH, Ar meta), 125.6 (CH, Ar meta), 75.6 (OCH_2), 60.8 (OCH_2, ethyl), 34.4 (Cq, *t*-butyl), 34.3 (Cq, *t*-butyl), 32.3 ($ArCH_2Ar$), 32.2 (CH_3, *t*-butyl), 31.5 (CH_3, *t*-butyl), 31.2 (CH_2), 26.0 (CH_2), 14.5 (CH_3, ethyl).

*25,27-Dihydroxy-26,28-bis(ethoxycarbonylbutoxy)tert-butyl-calix[4]arene, cone (**3b**)* Yield: 84%, white crystals, mp = 157°C, (Found MNa^+: 927.5707, $C_{58}H_{80}O_8{}^{23}Na$ requires 927.5751); IR ν_{max}/cm^{-1} 3340 (OH), 1720 (CO); ^1H NMR δ (500 MHz, $CDCl_3$) 7.56 (s, 2H, OH), 7.10 (s, 4H, Ar-H meta), 6.87 (s, 4H, Ar-H meta), 4.32 (d, *J* 12.91 Hz, 4H, $ArCH_2Ar$), 4.17 (m, 4H, OCH_2, ethyl), 4.04 (m, 4H, OCH_2), 3.36 (d, *J* 12.91 Hz, 4H, $ArCH_2Ar$), 2.53 (m, 4H, CH_2), 2.09 (m, 8H, CH_2), 1.34 (s, 18H, CH_3 *t*-butyl), 1.26 (m, 6H, CH_3 ethyl), 1.02 (s, 18H, CH_3 *t*-butyl). ^{13}C NMR δ (125 MHz, $CDCl_3$) 173.8 (COO), 151.1 (Cq, Ar ipso), 150.3 (Cq, Ar ipso), 147.2 (Cq, Ar para), 141.8 (Cq, Ar para), 133.0 (Cq, Ar ortho), 128.1 (Cq, Ar ortho), 125.9 (CH, Ar meta), 125.5 (CH, Ar meta), 76.3 (OCH_2), 60.6 (OCH_2, ethyl), 34.2 (CH_2), 33.0 (Cq, *t*-butyl), 32.9 (Cq, *t*-butyl), 32.2 ($ArCH_2Ar$), 32.1 (CH_3, *t*-butyl), 31.4 (CH_3, *t*-butyl), 29.8 (CH_2), 22.0 (CH_2), 14.6 (CH_3, ethyl).

*25,27-Dihydroxy-26,28-bis(ethoxycarbonylpentoxy)tert-butyl-calix[4]arene, cone (**3c**)* Yield: 93%, white crystal, mp = 147°C, (Found MNa^+: 955,6039, $C_{60}H_{84}O_8{}^{23}Na$ requires 955,6064); IR ν_{max}/cm^{-1} 3323 (OH), 1727 (CO); ^1H NMR δ (500 MHz, $CDCl_3$) 7.60 (s, 2H, OH), 7.10 (s, 4H, Ar-H meta), 6.88 (s, 4H, Ar-H meta), 4.33 (d, *J* 12.98 Hz, 4H, $ArCH_2Ar$), 4.14 (m, 4H, OCH_2, ethyl), 4.04 (t, *J* 6.66 Hz, 4H, OCH_2), 3.35 (d, *J* 12.98 Hz, 4H, $ArCH_2Ar$), 2.42 (t, *J* 7.2 Hz, 4H, CH_2), 2.08 (m, 4H, CH_2), 1.84 (m, 4H, CH_2), 1.76 (m, 4H, CH_2), 1.33 (s, 18H, CH_3 *t*-butyl), 1.26 (t, *J* 7.24 Hz, 6H, CH_3 ethyl), 1.05 (s, 18H, CH_3 *t*-butyl).^{13}C NMR δ (125 MHz, $CDCl_3$) 174.0 (COO), 151.2 (Cq, Ar ipso), 150.4 (Cq, Ar ipso), 147.2 (Cq, Ar para), 141.8 (Cq, Ar para), 133.1 (Cq, Ar ortho), 128.3 (Cq, Ar ortho), 125.9 (CH, Ar meta), 125.5 (CH, Ar meta), 76.5 (OCH_2), 60.7 (OCH_2, ethyl), 34.7 (CH_2), 34.3 (Cq, *t*-butyl), 34.2 (Cq, *t*-butyl), 32.2 ($ArCH_2Ar$), 32.1 (CH_3, *t*-butyl), 31.5 (CH_3, *t*-butyl), 30.4 (CH_2), 26.0 (CH_2), 25.2 (CH_2), 14.6 (CH_3, ethyl).

Synthesis of 25,27-dihydroxy-26,28-bis(acidbutoxy) or (acidpentoxy) or (acidhexoxy)tert-butyl-calix[4]arenes, cone 4a–c

The ethyl ester **3a–c** (0.7 mmol) was dissolved in a solution of NaOH (15%) in ethanol (50 mL) and stirred for 1 h at reflux. The solution was neutralized using excess HCl 6 M and the product was extracted in EtOAc (3 × 30 mL). The organic phase was washed with water, dried ($MgSO_4$), and evaporated to furnish a solid which was purified by column chromatography (cyclohexane:ethyl acetate 8:2, v/v) to give **4a–c**.

*25,27-Dihydroxy-26,28-bis(acidbutoxy)tert-butyl-calix[4]arene, cone (**4a**)* Yield: 85%, white crystals, mp = 196°C, (Found MNa^+: 843.4806, $C_{52}H_{68}O_8{}^{23}Na$ requires 843.4812); IR ν_{max}/cm^{-1} 3340 (OH), 1730 (CO); ^1H NMR δ (500 MHz, $CDCl_3$) 7.07 (s, 4H, Ar-H meta), 6.80 (s, 4H,

Ar-H meta), 4.26 (d, *J* 13.50 Hz, 4H, $ArCH_2Ar$), 3.94 (m, 4H, OCH_2), 3.31 (d, *J* 13.50 Hz, 4H, $ArCH_2Ar$), 2.86 (m, 4H, CH_2), 2.34 (m, 4H, CH_2), 1.31 (s, 18H, CH_3 *t*-butyl), 1.00 (s, 18H, CH_3 *t*-butyl). ^{13}C NMR δ (125 MHz, $CDCl_3$) 180.0 (COOH), 150.9 (Cq, Ar ipso), 150.3 (Cq, Ar ipso), 147.3 (Cq, Ar para), 141.9 (Cq, Ar para), 132.8 (Cq, Ar ortho), 128.2 (Cq, Ar ortho), 125.8 (CH, Ar meta), 125.5 (CH, Ar meta), 76.5 (OCH_2), 33.0 (Cq, *t*-butyl), 32.7 (Cq, *t*-butyl), 32.1(CH_3, *t*-butyl), 31.9 ($ArCH_2Ar$), 31.4 (CH_3, *t*-butyl), 31.6 (CH_2), 26.0 (CH_2).

*25,27-Dihydroxy-26,28-bis(acidpentoxy)tert-butyl-calix[4]arene, cone (**4b**)* Yield: 86%, yellow crystals, mp = 180°C, (Found MNa^+: 871.5103, $C_{54}H_{72}O_8{}^{23}Na$ requires 871.5125); IR ν_{max}/cm^{-1} 3310 (OH), 1731 (CO); ^{1}H NMR δ (500 MHz, $CDCl_3$) 8.72 (br s, 2H, OH), 7.56 (s, 2H, OH), 7.08 (s, 4H, Ar-H meta), 6.85 (s, 4H, Ar-H meta), 4.30 (d, *J* 13.00 Hz, 4H, $ArCH_2Ar$), 4.02 (m, 4H, OCH_2), 3.35 (d, *J* 13.00 Hz, 4H, $ArCH_2Ar$), 2.60 (m, 4H, CH_2), 2.08 (m, 8H, CH_2), 1.33 (s, 18H, CH_3, *t*-butyl), 1.00 (s, 18H, CH_3, *t*-butyl). ^{13}C NMR δ (125 MHz, $CDCl_3$) 179.8 (COOH), 151.0 (Cq, Ar ipso), 150.3 (Cq, Ar ipso), 147.3 (Cq, Ar para), 141.9 (Cq, Ar para), 133.0 (Cq, Ar ortho), 128.2 (Cq, Ar ortho), 125.9 (CH, Ar meta), 125.5 (CH, Ar meta), 76.3 (OCH_2), 34.2 (CH_2), 34.2 (Cq, *t*-butyl), 32.1 ($ArCH_2Ar$), 32.0 (CH_3, *t*-butyl), 31.4 (CH_3, *t*-butyl), 29.7 (CH_2), 21.9 (CH_2).

*25,27-Dihydroxy-26,28-bis(acidhexoxy)tert-butyl-calix[4]arene, cone (**4c**)* Yield: 89%, yellow crystals, mp = 105°C, (Found MNa^+: 899.5468, $C_{56}H_{76}O_8{}^{23}Na$ requires 899.5438); IR ν_{max}/cm^{-1} 3304 (OH), 1734 (CO), 1707 (CO); ^{1}H NMR δ (500 MHz, $CDCl_3$) 7.54 (s, 2H, OH), 7.50 (s, 2H, OH), 7.08 (s, 4H, Ar-H meta), 6.83 (s, 4H, Ar-H meta), 4.30 (d, *J* 13.20 Hz, 4H, $ArCH_2Ar$), 4.01 (m, 4H, OCH_2), 3.33 (d, *J* 13.20 Hz, 4H, $ArCH_2Ar$), 2.48 (m, 4H, CH_2), 2.06 (m, 4H, CH_2), 1.82 (m, 8H, CH_2), 1.31 (s, 18H, CH_3, *t*-butyl), 0.99 (s, 18H, CH_3, *t*-butyl).^{13}C NMR δ (125 MHz, $CDCl_3$) 179.8 (COOH), 151.1 (Cq, Ar ipso), 150.3 (Cq, Ar ipso), 147.2 (Cq, Ar para), 141.8 (Cq, Ar para), 133.0 (Cq, Ar ortho), 128.3 (Cq, Ar ortho), 125.9 (CH, Ar meta), 125.4 (CH, Ar meta), 76.6 (OCH_2), 76.5 (OCH_2), 34.4 (CH_2), 34.3 (Cq, *t*-butyl), 34.2 (Cq, *t*-butyl), 34.0 (Cq, *t*-butyl), 32.2 ($ArCH_2Ar$), 32.1 (CH_3, *t*-butyl), 31.3 (CH_3, *t*-butyl), 30.0 (CH_2), 26.0 (CH_2), 25.0 (CH_2).

Synthesis of 25,27-dihydroxy-26,28-bis(O-methylhydroxamatepropoxy)tert-butyl-calix[4]arene, cone (5)

A suspension of **4a** (122 µmol), 1-hydroxybenzotriazole hydrate (HOBt) (536 µmol), and *N*-(3-dimethylaminopropyl)-*N'*-ethyl-carbodiimine hydrochloride (EDCI) (536 µmol) in CH_2Cl_2 (6 mL) was stirred at ambient temperature for 30 min. The *O*-methylhydroxylamine hydrochloride (536 µmol) was added and the solution was stirred at ambient temperature for 3 days. The solvent was then evaporated at reduced pressure, and the residue was purified by HPLC to give **5**. Yield: 37%, yellow crystals, mp = 157°C, (Found MNa^+: 901.5322, $C_{54}H_{74}O_8{}^{23}Na$ requires 901.5343); IR ν_{max}/cm^{-1} 3290 (OH, NH), 1734 (CO); ^{1}H NMR δ (500 MHz, $CDCl_3$) 7.85 (br s, 1H, NH), 7.81 (br s, 1H, NH), 7.46 (s, 2H, OH), 7.08 (s, 4H, Ar-H meta), 6.83 (m, 4H, Ar-H meta), 4.23 (d, *J* 13.50 Hz, 4H, $ArCH_2Ar$), 4.04 (m, 4H, OCH_2), 3.76 (s, 6H, OCH_3), 3.36 (d, *J* 13.50 Hz, 4H, $ArCH_2Ar$), 2.90 (m, 4H, CH_2), 2.34 (m, 4H, CH_2), 1.30 (s, 18H, CH_3, *t*-butyl), 0.98 (s, 18H, CH_3, *t*-butyl). ^{13}C NMR δ (125 MHz, $CDCl_3$) 171.4 (CONH), 151.0 (Cq, Ar ipso), 150.5 (Cq, Ar ipso), 150.4 (Cq, Ar ipso), 150.2 (Cq, Ar ipso), 147.8 (Cq, Ar para), 147.7 (Cq, Ar para), 142.7 (Cq, Ar para), 142.6 (Cq, Ar para), 132.9 (Cq, Ar ortho), 132.8 (Cq, Ar ortho), 128.9 (Cq, Ar ortho), 128.3 (Cq, Ar ortho), 128.2 (Cq, Ar ortho), 126.0 (CH, Ar meta), 125.9 (CH, Ar meta), 125.7 (CH, Ar meta), 125.6 (CH, Ar meta), 76.1 (OCH_2), 75.9 (OCH_2), 43.6 (OCH_3), 34.3 (Cq, *t*-butyl), 34.2 (Cq, *t*-butyl), 32.1 ($ArCH_2Ar$), 32.0 (CH_3, *t*-butyl), 31.4 (CH_3, *t*-butyl), 30.2 (CH_2), 26.5 (CH_2), 25.9 (CH_2), 25.8 (CH_2).

Synthesis of 25,26,27-trihydroxy-28-(ethoxycarbonylbutoxy)tert-butyl-calix[4]arene, cone (6) and 25-hydroxy-26,27,28-tri(ethoxycarbonylbutoxy) tert-butyl-calix[4]arene, cone (7)

A suspension of *tert*-butyl-calix[4]arene (1.54 mmol) and NaH (7.09 mmol) in dimethylformamide (DMF) (50 mL) was stirred at 55°C for 1 h. Then ethyl 5-bromopentanoate (23.1 mmol) was added and the mixture was stirred for 15 h at the same temperature. The solvent was then evaporated at reduced pressure, and the residue was purified by HPLC to give **3b** and **6–7**.

*25,26,27-Trihydroxy-28-(ethoxycarbonylbutoxy)tert-butyl-calix[4]arene, cone (**6**)* Yield: 7%, white crystals, mp = 210°C, (Found MNa^+: 799.4943, $C_{51}H_{68}O_6{}^{23}Na$ requires 799.4914); IR ν_{max}/cm^{-1} 3297 (OH), 1729 (CO); ^{1}H NMR δ (500 MHz, $CDCl_3$) 10.38 (s, 1H, OH), 10.16 (s, 1H, OH), 9.55 (s, 1H, OH), 7.10 (m, 8H, Ar-H meta), 4.37 (d, *J* 12.71 Hz, 2H, $ArCH_2Ar$), 4.32 (d, *J* 12.71 Hz, 2H, $ArCH_2Ar$), 4.20 (m, 2H, OCH_2, ethyl), 4.19 (m, 2H, OCH_2), 3.47 (m, 4H, $ArCH_2Ar$), 2.56 (m, 2H, CH_2), 2.25 (m, 2H, CH_2), 2.06 (m, 2H, CH_2), 1.33 (m, 3H, CH_3, ethyl), 1.25 (s, 36H, CH_3, *t*-butyl). ^{13}C NMR δ (125 MHz, $CDCl_3$) 173.7 (COO), 149.7 (Cq, Ar ipso), 148.9 (Cq, Ar ipso), 148.6 (Cq, Ar ipso), 148.1 (Cq, Ar ipso), 147.1 (Cq, Ar para), 144.8 (Cq, Ar para), 144.0 (Cq, Ar para), 143.6 (Cq, Ar para), 133.9 (Cq, Ar ortho), 128.7 (Cq, Ar ortho), 128.5 (Cq, Ar ortho), 128.1 (Cq, Ar ortho), 126.7 (CH, Ar meta), 126.3 (CH, Ar meta), 126.1 (CH, Ar meta), 126.0 (CH, Ar meta), 77.1 (OCH_2), 60.8 (OCH_2, ethyl), 34.6 (CH_2), 34.2 (Cq, *t*-butyl), 33.5 ($ArCH_2Ar$), 32.8 ($ArCH_2Ar$), 33.0 (Cq, *t*-butyl), 31.9 (CH_3, *t*-butyl), 31.8 (CH_3, *t*-butyl), 31.6 (CH_3, *t*-butyl), 29.7 (CH_2), 21.9 (CH_2), 14.7 (CH_3, ethyl).

*25-hydroxy-26,27,28-tri(ethoxycarbonylbutoxy)tert-butyl-calix[4]arene, cone (**7**)* Yield: 13%, white crystals, mp = 90°C, (Found MNa^+: 1055.6622, $C_{65}H_{92}O_{10}{}^{23}Na$ requires 1055.6588); IR ν_{max}/cm^{-1} 3305 (OH), 1770 (CO); ^{1}H NMR δ (500 MHz, $CDCl_3$) 8.82 (s, 1H, OH), 7.17 (s, 2H, Ar-H meta), 7.09 (s, 2H, Ar-H meta), 6.55 (s, 2H, Ar-H meta), 6.54 (s, 2H, Ar-H meta), 4.36 (d, *J* 12.60 Hz, 2H, $ArCH_2Ar$), 4.33 (d, *J* 13.08 Hz, 2H, $ArCH_2Ar$), 4.17 (m, 6H, OCH_2, ethyl), 3.96 (t, *J* 8.48 Hz, 2H, OCH_2), 3.85 (t, J 6.48 Hz, 4H, OCH_2), 3.28 (d, *J* 13.08

Hz, 2H, $ArCH_2Ar$), 3.22 (d, *J* 12.60 Hz, 2H, $ArCH_2Ar$), 2.45 (m, 6H, CH_2), 2.35 (m, 2H, CH_2), 1.97 (m, 4H, CH_2), 1.91 (m, 4H, CH_2), 1.76 (m, 2H, CH_2), 1.38 (s, 18H, CH_3, *t*-butyl), 1.28 (m, 9H, CH_3, ethyl), 0.86 (s, 18H, CH_3, *t*-butyl). ^{13}C NMR δ (125 MHz, $CDCl_3$) 174.0 (COO), 173.7 (COO), 154.2 (Cq, Ar ipso), 152.0 (Cq, Ar ipso), 151.0 (Cq, Ar ipso), 146.1 (Cq, Ar para), 145.6 (Cq, Ar para), 142.0 (Cq, Ar para), 136.3 (Cq, Ar ortho), 132.5 (Cq, Ar ortho), 132.1 (Cq, Ar ortho), 130.0 (Cq, Ar ortho), 126.1 (CH, Ar meta), 125.4 (CH, Ar meta), 125.3 (CH, Ar meta), 125.1 (CH, Ar meta), 76.2 (OCH_2), 76.0 (OCH_2), 74.5 (OCH_2), 60.7 (OCH_2, ethyl), 60.6 (OCH_2, ethyl), 34.9 (CH_2), 34.5 (CH_2), 34.0 (Cq, *t*-butyl), 32.2 (CH_3, *t*-butyl), 32.1(CH_3, *t*-butyl), 31.6 ($ArCH_2Ar$), 31.5 ($ArCH_2Ar$), 31.4 (CH_3, *t*-butyl), 30.0 (CH_2), 29.5 (CH_2), 22.1 (CH_2), 22.0 (CH_2), 14.7 (CH_3, ethyl), 14.6 (CH_3, ethyl).

Synthesis of 25,27-dihydroxy-26,28-bis(ethoxycarbonylpropoxy)calix[4]arene, cone (8b), 25,27,26,28-tetra(ethoxycarbonylmetoxy)calix[4]arene, cone (10), 25,27,26,28-tetra(ethoxycarbonylbutoxy) calix[4]arene, 1,3-alternate (11)

A suspension of calix[4]arene (2.35 mmol) and K_2CO_3 (10.34 mmol) was reacted with alkyl bromide (9.87 mmol) in refluxing CH_3CN for 5 days. The solvent was then evaporated at reduced pressure, and the residue was taken up with 10% HCl (150 mL) and extracted with CH_2Cl_2 (200 mL). The organic layer was separated, washed twice with water, dried ($MgSO_4$), and evaporated to dryness. The residue was chromatographed on silica gel with cyclohexane:ethyl acetate (7:3, v/v) to give **8b** and **10–11**.

*25,27-Dihydroxy-26,28-bis(ethoxycarbonylpropoxy) calix[4]arene, cone (**8b**)* Yield: 48%, white crystals, mp = 156°C, (Found MNa^+: 675.2903, $C_{40}H_{44}O_8{}^{23}Na$ requires 675.2934); IR ν_{max}/cm^{-1} 3287 (OH), 1734 (CO); ^{1}H NMR δ (500 MHz, $CDCl_3$) 8.25 (s, 2H, OH), 7.11 (d, *J* 7.56 Hz, 4H, Ar-H meta), 6.96 (d, *J* 7.56 Hz, 4H, Ar-H meta), 6.78 (t, *J* 7.56 Hz, 2H, Ar-H para), 6.71 (t, *J* 7.56 Hz, 2H, Ar-H para), 4.30 (d, *J* 13.00 Hz, 4H, $ArCH_2Ar$), 4.22 (m, 4H, OCH_2, ethyl), 4.11 (t, *J* 5.80 Hz, 4H, OCH_2), 3.44 (d, *J* 13.00 Hz, 4H, $ArCH_2Ar$), 2.98 (t, *J* 7.23 Hz, 4H, CH_2), 2.40 (m, 4H, CH_2), 1.30 (t, *J* 7.28 Hz, 6H, CH_3, ethyl). ^{13}C NMR δ (125 MHz, $CDCl_3$) 173.7 (COO), 153.7 (Cq, Ar ipso), 152.0 (Cq, Ar ipso), 133.7 (Cq, Ar ortho), 129.4 (CH, Ar meta), 128.9 (CH, Ar meta), 128.7 (CH, Ar meta), 128.4 (Cq, Ar ortho), 125.9 (CH, Ar para), 119.5 (CH, Ar para), 75.7 (OCH_2), 61.0 (OCH_2, ethyl), 60.9 (OCH_2, ethyl), 31.8 ($ArCH_2Ar$), 31.1 (CH_2), 25.9 (CH_2), 14.7 (CH_3, ethyl).

*25,27,26,28-Tetra(ethoxycarbonylmethoxy)calix[4]arene, cone (**10**)* Yield: 56%, white crystals, mp = > 225°C, (Found MNa^+: 791.3061, $C_{44}H_{48}O_{12}{}^{23}Na$ requires 791.3043); IR ν_{max}/cm^{-1} 1734 (CO); ^{1}H NMR δ (500 MHz, $CDCl_3$) 7.11 (d, *J* 7.08 Hz, 4H, Ar-H meta), 7.00 (d, *J* 7.08 Hz, 4H, Ar-H meta), 6.85 (t, *J* 7.59 Hz, 4H, Ar-H para), 6.74 (t, *J* 7.59 Hz, 4H, Ar-H para), 4.72 (d, *J* 12.00 Hz, 4H, $ArCH_2Ar$), 4.48 (m, 8H, OCH_2, ethyl), 4.33 (d, *J* 13.20 Hz, 4H, OCH_2), 4.20 (d, *J* 13.20 Hz, 4H, OCH_2), 3.51 (d, *J* 12.00 Hz, 4H, $ArCH_2Ar$), 1.41 (t, *J* 7.05 Hz, 12H, CH_3). ^{13}C NMR δ (125 MHz, $CDCl_3$) 169.6 (COO), 168.6 (COO), 152.5 (Cq, Ar ipso), 151.7 (Cq, Ar ipso), 151.6 (Cq, Ar ipso), 133.4 (Cq, Ar ortho), 133.3 (Cq, Ar ortho), 129.9 (CH, Ar meta), 129.3 (CH, Ar meta), 129.2 (CH, Ar meta), 126.9 (CH, Ar para), 126.8 (CH, Ar para), 120.6 (CH, Ar para), 73.0 (OCH_2), 72.9 (OCH_2), 62.4 (OCH_2, ethyl), 32.2 ($ArCH_2Ar$), 32.0 ($ArCH_2Ar$), 14.6 (CH_3).

*25,27,26,28-Tetra(ethoxycarbonylbutoxy)calix[4]arene, 1,3-alternate (**11**)* Yield: 10%, white crystals, mp = 138°C, (Found MNa^+: 959.4879, $C_{56}H_{72}O_{12}{}^{23}Na$ requires 959.4921); IR ν_{max}/cm^{-1} 1730 (CO); ^{1}H NMR δ (500 MHz, $CDCl_3$) 7.01 (d, *J* 7.53 Hz, 8H, Ar-H meta), 6.74 (t, *J* 7.53 Hz, 4H, Ar-H para), 4.20 (m, 8H, OCH_2, ethyl), 3.54 (t, *J* 7.22 Hz, 8H, OCH_2), 3.70 (s, 8H, $ArCH_2Ar$), 2.33 (t, *J* 7.60 Hz, 8H, CH_2), 1.61 (m, 8H, CH_2), 1.49 (m, 8H, CH_2), 1.26 (t, *J* 7.16 Hz, 12H, CH_3, ethyl). ^{13}C NMR δ (125 MHz, $CDCl_3$) 173.9 (COO), 156.9 (Cq, Ar ipso), 134.1 (Cq, Ar ortho), 130.0 (CH, Ar meta), 122.2 (CH, Ar para), 71.2 (OCH_2), 60.7 (OCH_2, ethyl), 37.5 ($ArCH_2Ar$), 34.7 (CH_2), 29.8 (CH_2), 21.9 (CH_2), 14.7 (CH_3, ethyl).

Pharmacology

Cell cultures

HepaRG cells were cultured as previously described[47]. They were maintained in William's E medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 5×10^{-5} M hydrocortisone hemisuccinate, and 5 µg/mL insulin. Cells were seeded at 2×10^4 cells/cm^2 in 96-well microplates for LDH and SDH measurements.

Cell treatments

The various derivatives were compared to the tridentate hydroxyphenyltriazole ICL670 (Deferasirox, Exjade™)[48]. Stock solutions of each molecule (2.5 mM) were prepared in dimethylsulfoxide (DMSO). The solubility of each derivative in culture medium in the concentration range 0–200 µM was preliminarily verified by turbidimetry measurement. Two controls were done for each experiment: one with the standard culture medium, and the other with culture medium supplemented with DMSO at the concentration used for ICL670 and calix[4]arene tests. DMSO-supplemented controls did not show any difference from standard medium controls. Therefore, only the results obtained with controls without DMSO are reported.

Solubility of the chelators

Solubility of the new calix[4]arenes was estimated in phosphate-buffered saline (PBS) solution and in cell culture medium containing 10% fetal calf serum (FCS). Solutions of the various compounds (200, 100, 50, 25 µM) were prepared in 96-well microplates by diluting the 2.5 mM stock solutions in DMSO in 200 µL of PBS and culture medium. The absorbance (turbidity) of the solutions was measured at 590 nm, out of the absorption range of chromophores.

Comparison of chelator efficiency in aqueous phase

In solution, calcein, a fluoresceinated analog of ethylenediaminetetraacetic acid (EDTA), binds iron(II) and more slowly iron(III) (K_a = 1024 M^{-1}). The fluorescence of this

metallosensor dye is quenched during its interaction with iron and conversely is restored during removal of iron from the calcein–iron complex by various chelators. The rate and extent of fluorescence recovery depend on the chelator concentration, the kinetics and stoichiometry of iron binding, and the relative binding affinity. The fluorescence (λ_{Exc} = 485 nm, λ_{Em} = 520 nm) of calcein (100 nM) in a HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7.3) was measured at room temperature in a microplate fluorescence reader (Packard, Fusion™), equipped with an orbital stirrer. Iron(III) (1 µM) reacted slowly with calcein, and maximal quenching of its fluorescence was observed for a time longer than 6 h. Fluorescence recovery was monitored after 4 h incubation in the presence of various chelator concentrations.

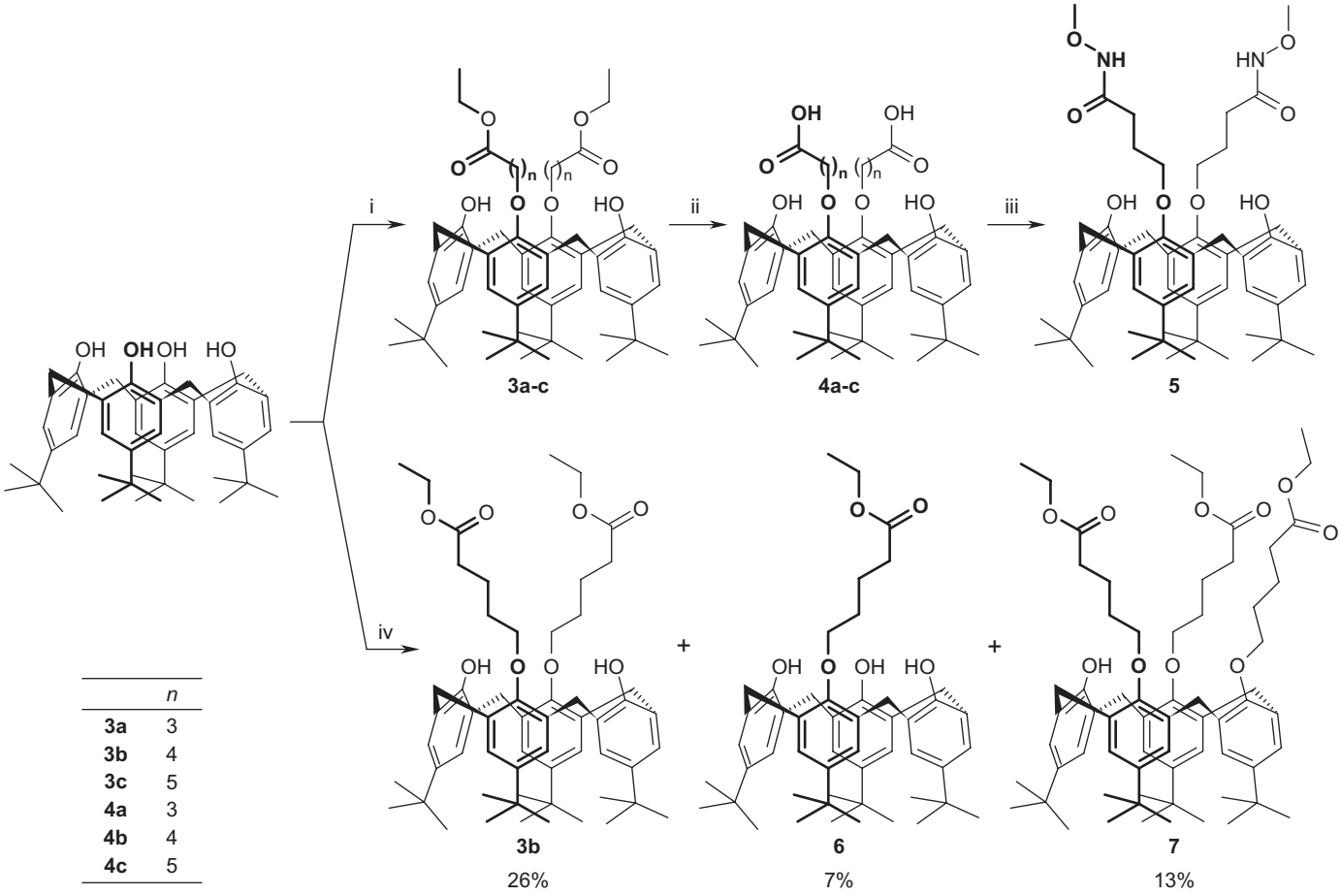
Cytostatic and cytotoxic effects measurement

Chelator exposures were performed 1 day after cell seeding in proliferating HepaRG cells. After 72 h incubation at 37°C, cell supernatant was collected for lactate dehydrogenase measurement (LDH). The HepaRG cells were washed twice with sodium phosphate buffer 50 mM, pH 7, and mitochondrial succinate dehydrogenase activity (SDH) in cell monolayers was measured using the MTT assay. Cytoxicity was evaluated by measuring extracellular LDH activity (cytotoxicity detection kit; LDH, Roche, Penzberg, Germany) and SDH activity by the tetrazolium colorimetric assay (MTT; Sigma, St Louis, MO). Extracellular LDH activity was measured as described by the manufacturer on a 20 µL aliquot of cell-free medium obtained by centrifugation (2500 rpm/min during 5 min). LDH activities were detected by reading the absorbance at 485 nm. Data are the mean of three independent measurements. They are reported as a percentage of extracellular LDH activity with respect to the control value.

SDH activity was detected after 3 h incubation in 100 µL serum-free medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/L). Formazan salts were solubilized in DMSO and absorbance was read at 535 nm. Data are the mean of three independent measurements. They are reported as a percentage of SDH activity with respect to the control value. The concentration inducing a 50% inhibition of cell growth (IC_{50}) was deduced from a four-parameter fit of the dose–effect curves.

Statistical analysis

Results from at least three replicates are expressed as mean ± SD. Statistical analysis was performed using the non-parametric Mann–Whitney test. The significance level was set at 0.01.



	n
3a	3
3b	4
3c	5
4a	3
4b	4
4c	5

Scheme 1. Synthesis of tert-butyl-calix[4]arenes **3–7**. Reagents: (i) K_2CO_3, $Br(CH_2)_nCOOEt$, CH_3CN; (ii) (1) NaOH, EtOH; (2) HCl, H_2O; (iii). HOBt, EDCI, NH_2OCH_3.HCl, CH_2Cl_2; (iv) NaH, $Br(CH_2)_4COOEt$, DMF.

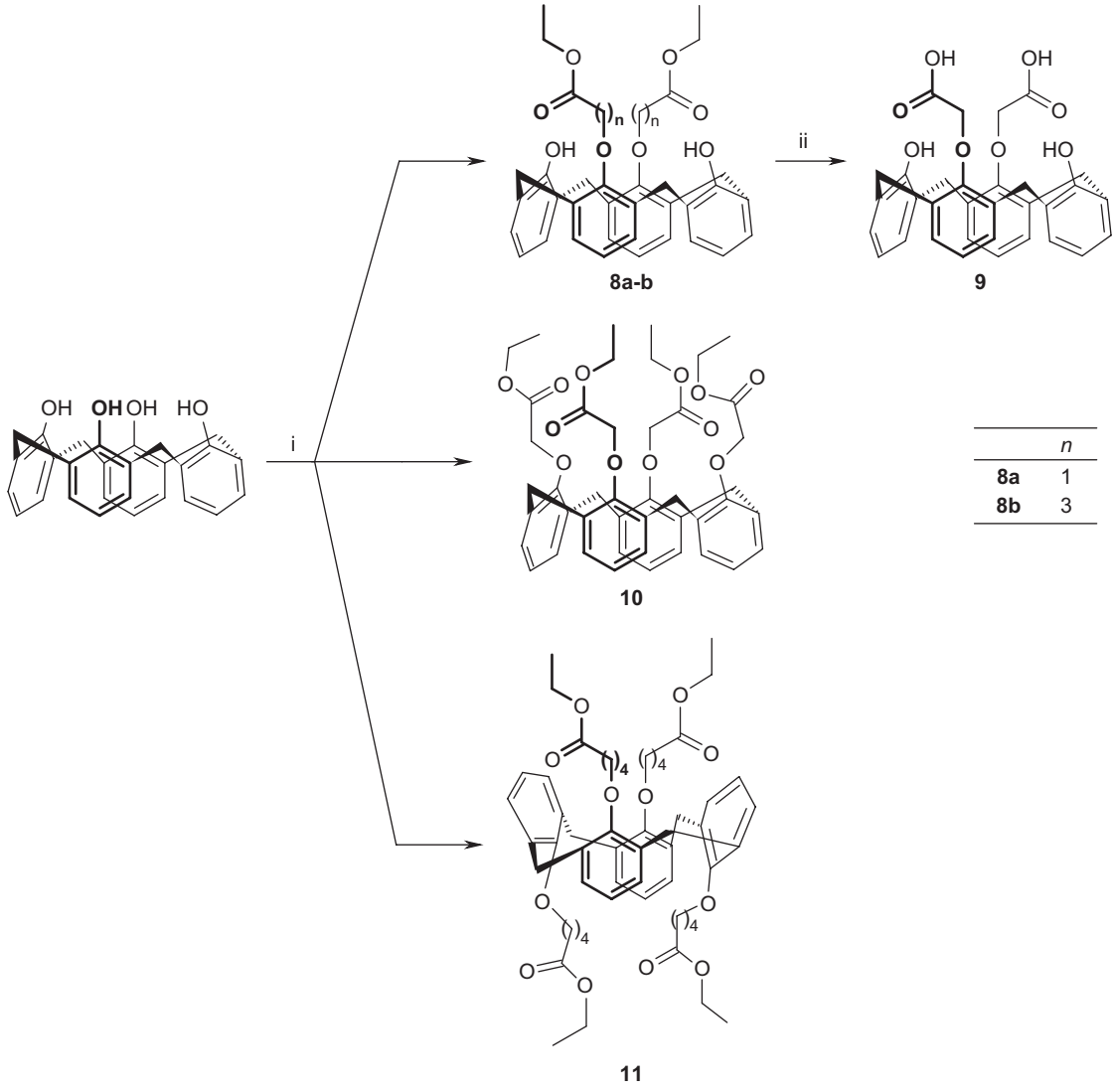
Results and discussion

Chemistry

The reaction of *tert*-butyl-calix[4]arene available commercially with 2.2 eq. of ethyl bromoalkylacetate in the presence of 2.4 eq. of K_2CO_3 as a base in refluxing CH_3CN for 5 days gave the diametrically substituted cone 25,27-diethoxycarbonylalkyloxy *tert*-butyl-calix[4]arenes **3a–c**. Saponification of the diesters **3a–c** with 15% sodium hydroxide in ethanol under reflux gave, after acidification, the corresponding diacids **4a–c**. The *tert*-butyl-calix[4]arene **5** was synthesized from diacid **4a** by coupling with *O*-methylhydroxylamine in CH_2Cl_2 (Scheme 1). The reaction of *tert*-butyl-calix[4]arene with ethyl 5-bromopentanoate with NaH as a base in DMF furnished a mixture of cone mono-, bi-, and tri-alkylated *tert*-butyl-calix[4]arenes **6**, **3b**, and **7** in, respectively, 7%, 26%, and 13% yields (yields obtained after HPLC purification) (Scheme 1).

The cone conformations of the *tert*-butyl-calix[4]arene **3–7** were confirmed by the ^{1}H NMR spectrum. In the case of the dialkylated *tert*-butyl-calix[4]arene **3–5**, the cone conformation is characterized by the characteristic chemical shift ($\delta = 4.37–4.26$ and 3.47–3.22 ppm) and coupling constants ($J = 12.71–13.50$ Hz) of the two types of diastereotopic proton signals of the methylene bridges. For the *tert*-butyl-calix[4]arenes monoalkylated **6** or trialkylated **7**, the typical pattern represented by two 2H doublets at 4.37 and 4.32 ppm for **6** and 4.36 and 4.33 ppm for **7** was due to the axial protons of the bridging methylene ($ArCH_2Ar$). The corresponding equatorial protons were present at 3.28 and 3.22 ppm for **7**, but from **6** were observed at 3.74 ppm in a multiplet signal (4H). The spectrum also showed three sharp signals for the OH groups from **6** at 10.38, 10.16, and 9.55 ppm and one signal from **7** at 8.82 ppm (1H).

The synthesis of the new calix[4]arenes **8–11** was accomplished in one or two step(s) starting from the calix[4]arene commercially available (Scheme 2). Calix[4]arene diester **8a** was prepared in 55% yield by alkylation of the unsubstituted calix[4]arene with ethyl bromoacetate in the



Scheme 2. Synthesis of calix[4]arenes **8–11**. Reagents: (i) K_2CO_3, $Br(CH_2)_nCOOEt$, CH_3CN; (ii) (1) NaOH, EtOH; (2) HCl, H_2O.

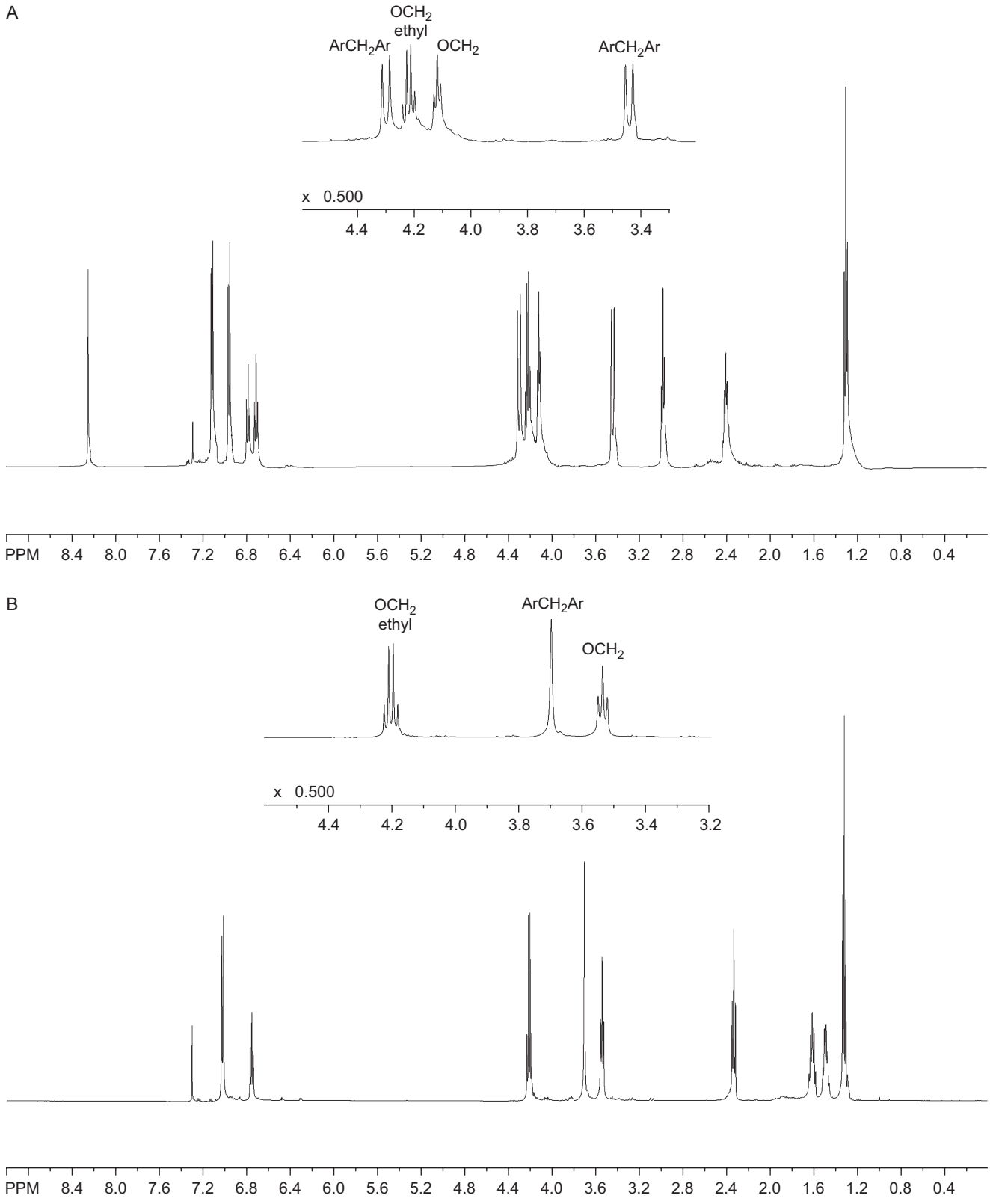


Figure 2. ^{1}H NMR (500 MHz, $CDCl_3$) calix[4]arene spectrum: (A) calix[4]arene **8b** in cone conformation; (B) calix[4]arene **11** in 1,3-alternate conformation.

presence of one equivalent of K_2CO_3 as a base, in refluxing CH_3CN[42–45]. Hydrolysis of **8a** with 15% sodium hydroxide in ethanol under reflux gave, after acidification, the crystalline diacid **9** (95%)[45,49,50].

The compounds **8b**, **10**, and **11** were prepared by alkylation of the calix[4]arene commercially available with 4.2 eq. of alkyl bromide in the presence of 4.4 eq. of K_2CO_3 as a base in refluxing CH_3CN. The alkylation led to the bi- or

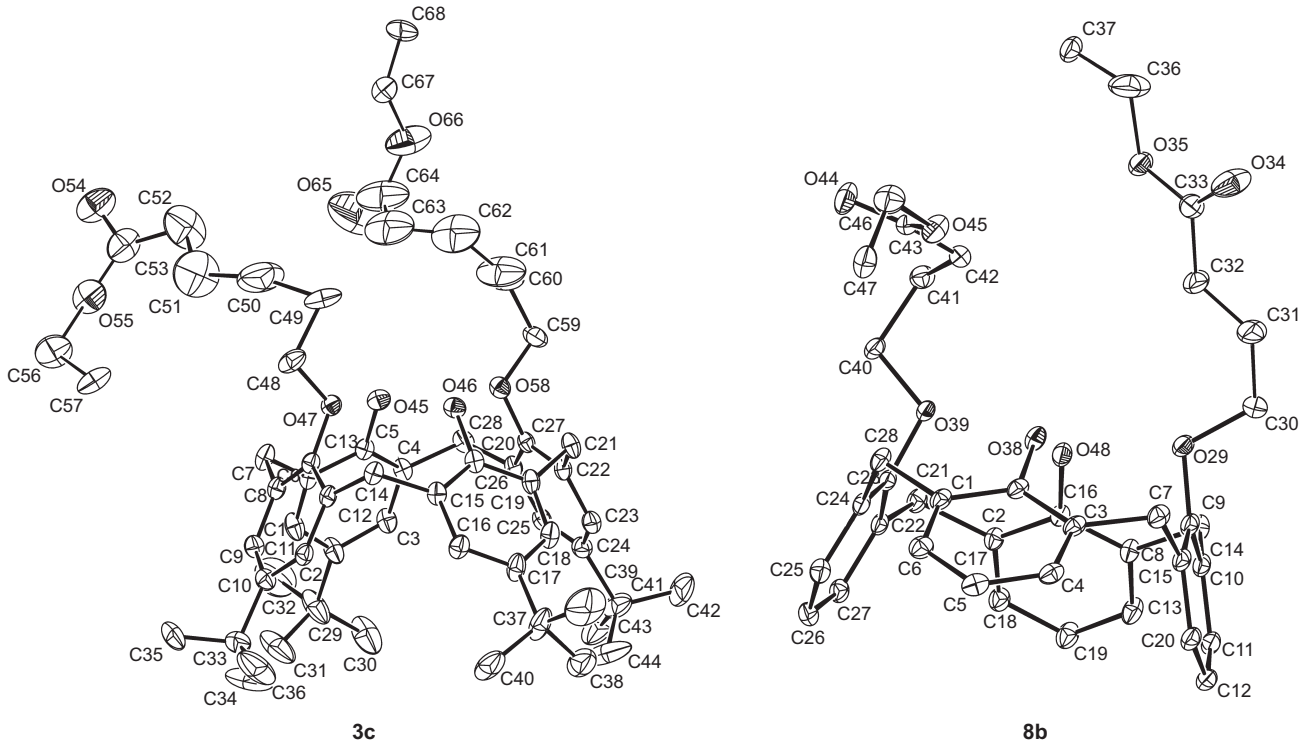


Figure 3. The ORTEP drawing of calix[4]arenes **3c** and **8b**. Displacement ellipsoids are drawn at the 30% probability level, H atoms are omitted for clarity.

tetra-substituted ethoxycarbonylalkyloxycalix[4]arene in cone or 1,3-alternate conformations.

As observed for the dialkylated *tert*-butyl-calix[4]arene **3–5**, the cone conformation calix[4]arene diester **8b** and tetra-alkylated calix[4]arene **10** is characterized by a typical AB pattern, which was observed for the methylene bridge $ArCH_2Ar$ protons (J = 12.00–13.00 Hz) at 4.72–4.30 ppm for the axial protons and 3.51–3.44 ppm for the equatorial protons (^{1}H NMR spectrum, Figure 2). In the ^{13}C NMR spectrum the corresponding carbon absorption was present at 31.8 ppm from diester **8b** and 32.2 and 32.0 ppm from tetra-alkylated **10**. The spectrum of diester **8b** also showed one signal for the two OH groups at 8.25 ppm. The 1,3-alternate conformation of the calix[4]arene **11** was determined from the ^{1}H NMR spectrum, which exhibited a singulet signal at 3.70 ppm that could be attributed to the bridging methylene groups of the calix[4]arene moiety (Figure 2). The signal at 37.5 ppm for the methylene bridge carbons in the ^{13}C NMR spectrum confirmed the structure.

The 3D structures of **3c** and **8b** were established by X-ray crystallography analysis[51] and confirmed the cone conformation in the solid state (Figure 3) as anticipated on the basis of ^{1}H NMR data. Molecules adopted a "pinched-cone" conformation commonly found in calix[4]arenes in cone conformation. With respect to the reference methylene plane C7, C14, C21, and C28, the interplanar angles of C1–C6, C8–C13, C15–C20, and C22–C27 rings in **3c** were 57.76 (25), 63.22 (21), 57.21 (25), and 63.18 (21), respectively. In **8b**, the same angles were noted at 44.24 (8), 74.75 (9), 44.59 (10), and 68.54 (9), respectively. The angles between the aromatic units of the calix[4]arene skeleton through the methylene carbons were 112.2 (5), 111.0 (5), 112.3 (5), and 111.7 (5) for **3c**, and 113.3 (2), 111.4 (3), 112.2 (2), and 111.8(3) for **8b**. Not surprisingly, in both cone calix[4]arenes, the ethoxycarbonylalkyloxy chains adopt various conformations. Moreover, they do not present a regular conformation. In these two structures, the inter-calixarene contacts are of the van der Waals variety and there are no solvent accessible voids in the crystal lattice.

Pharmacology

The low solubility of the methyl and ethyl calix[4]arene esters **3b–c**, **6**, **8b**, and **11** in aqueous solvents and more particularly in cell culture medium prevented us from studying their biological efficiency. A relative and limited structure–solubility study based on the functional groups can be proposed here. Better solubility is always observed in the case of the substituted acid calix[4]arenes. When three methylene units or more were incorporated in the aliphatic chain of mono-, bi-, or tetra-substituted calix[4] arenes, no solubility was observed in the corresponding alkyl acid or ester compounds, whether they were in cone or 1,3-alternate conformation. In the same manner, the presence of *t*-butyl groups on the upper rim increased the lipophilic property of the corresponding compounds (**3b–c**, **7**) and consequently the corresponding water insolubility.

Comparison of chelator efficiency in aqueous phase

An efficient iron chelator such as ICL670 is able to remove and to interact with iron complexed to calcein (totally quenched

fluorescence) and, consequently, release fluorescent free calcein. As shown in Figure 4, ICL670 concentrations higher than 0.1 µM restored the fluorescence of calcein associated with 1 µM $FeCl_3$. The ICL670 concentration inducing 50% of the maximal dequenching of (ED_{50}) was close to 0.4 µM. The chelating efficiency of ICL670, deduced from this calcein assay, can be compared to that of other iron chelators such as *O*-Trensox (ED_{50} = 0.6 µM) or desferal (DFO, ED_{50} = 5 µM). In contrast, various calix[4]arene derivatives were inefficient in restoring the calcein fluorescence in this range of concentrations (Figure 4).

Biological effects in human hepatocarcinoma HepaRG cell cultures

In proliferating HepaRG cells, a dose-dependent decrease of cell viability (MTT assay, Figure 5A) was observed after a 72 h cell treatment in the presence of increasing concentrations of all compounds (Figure 5, 0–200 µM). The relative effect on cell viability of the derivatives was in the following order: **3a** > **4c** = **7** > **4a** > **4b** > **10** > **5** > **9**. This effect was due to the cytotoxicity of the compounds as detected by the concomitant increase in LDH in cell supernatant (Figure 5B).

As shown in Table 1, in the absence of iron, the compound concentrations inducing a 50% inhibition of cell growth (IC_{50}) were close to that of ICL670 (20–50 µM), except for compounds **5** and **9** which were less efficient with an IC_{50} higher than 90 µM. Addition of 20 µM exogenous iron(III) did not change the efficiency of the compounds (same IC_{50} in presence or absence of iron), while it partly reversed the inhibiting effect of ICL670 on cell viability (IC_{50} = 91 µM). Comparison of the relative toxicity of calix[4]arene derivatives at concentration 100 µM, in the presence or absence of exogenous iron, compared to ICL670 is reported in Table 1. At this concentration, the increase in cytotoxity induced by the various compounds remained unchanged in the presence of exogenous iron while it was partly decreased with ICL670.

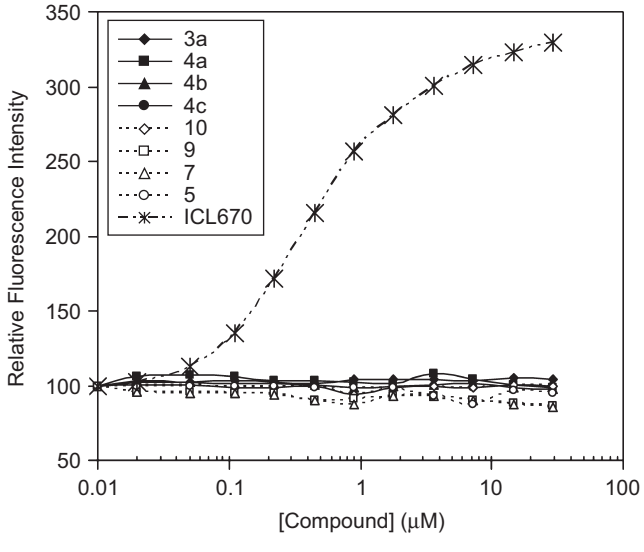


Figure 4. Comparison of iron chelating efficiency using calcein fluorescence measurements in a cell-free system. Fluorescence of 100 nM calcein (λ_{Exc} = 485 nm, λ_{Em} = 520 nm) in HEPES buffer (20 mM HEPES, 150 mM NaCl, pH 7.3) was detected in a microplate fluorescence reader (free calcein). Iron(III) (1 µM) totally quenched the calcein fluorescence and addition of compounds including ICL670 used as a chelator reference led to fluorescence recovery dependent on chelator concentration, and kinetics and stoichiometry of their iron binding affinity.

Conclusion

New calix[4]arene derivatives were shown to reduce the viability of proliferating HepaRG cells. This effect was due to their cytotoxicity leading to membrane damage and associated with LDH leakage in cell supernatant. The efficiency of these compounds was comparable to that of ICL670 used as a reference. The relative antiproliferative efficiency of the

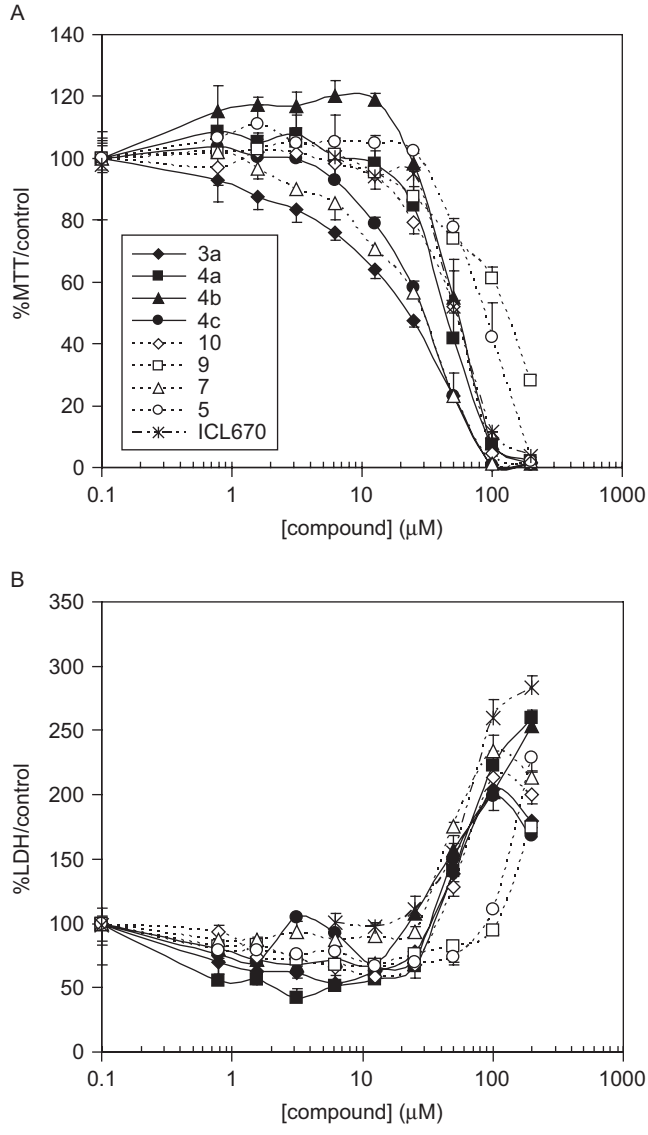


Figure 5. Effect of compounds on cell viability (A, MTT assay) and cytotoxicity (B, LDH release in cell supernatant) in proliferating HepaRG cell cultures. HepaRG cells at D4 were maintained in culture for 72 h with various concentrations of ICL670 (✳) or various compounds. Data expressed as percent of control (absence of compound) are the mean of three independent experiments.

Table 1. Biological effect of a 72 h treatment of HepaRG cells with various calix[4]arene compounds.

Compound	SDH, IC_{50} (μM) without Fe(III)	SDH, IC_{50} (μM) + Fe(III) 20 μM	%LDH/control without Fe(III)	%LDH/control + Fe(III) 20 μM
3a	22 ± 4	23 ± 6	204 ± 16	216 ± 8
4a	41 ± 6	38 ± 11	223 ± 11	234 ± 23
4b	46 ± 7	46 ± 4	202 ± 24	240 ± 7
4c	30 ± 2	27 ± 8	199 ± 2	206 ± 8
5	90 ± 23	70 ± 15	111 ± 3	103 ± 14
7	30 ± 11	27 ± 9	234 ± 11	229 ± 6
9	125 ± 17	120 ± 8	94 ± 23	63 ± 17
10	50 ± 6	50 ± 11	214 ± 11	204 ± 8
ICL670	55 ± 4	91 ± 12	260 ± 14	163 ± 16

Note. LDH leakage in cell supernatant for compound concentration 100 μM was used as an index of membrane damage (cytotoxicity) and SDH activity (MTT assay) expressed by IC_{50} (μM) as a measurement of cell viability. Data expressed as percent of control (absence of compound) are the mean of three independent experiments.

derivatives was in the following order: **3a** > **4c** = **7** > **4a** > **4b** > **10** > **5** > **9**. These first results do not enable us to determine precisely the structure–activity relationship in these series. As deduced from the absence of an effect of exogenous iron, the effect of calix[4]arene on cell viability was not correlated to iron depletion. On the basis of their inability to remove and interact with iron complexed to calcein, we deduced that the compounds were not efficient iron chelators. This effect, independent of iron depletion, remains to be further explored in order to understand the cytostatic effect. Finally, the novel substituted calix[4]arenes could open the way to new valuable medicinal chemistry scaffolding.

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Declaration of interest

The authors have no competing interests as defined by Informa Healthcare journals Publishing Group, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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