Journal of Enzyme Inhibition and Medicinal Chemistry, June 2006; 21(3): 261–270

Taylor & Francis
Taylor & Francis Group

Modulation of cell proliferation in rat liver cell cultures by new calix[4]arenes

VIVIANE SILVA PIRES[1], FRANÇOIS GABORIAU[2], JEAN GUILLON[3], SOPHIE DA NASCIMENTO[1], ALEXANDRA DASSONVILLE[1], GERARD LESCOAT[2], VANESSA DESPLAT[4], JACQUES ROCHETTE[1], CHRISTIAN JARRY[3], & PASCAL SONNET[1]

[1]*EA 3901 - DMAG, UFR de Pharmacie et de Médecine, Université de Picardie Jules Verne, 1 rue des Louvels, 80037 Amiens, France,* [2]*Inserm U522, Régulations des Equilibres Fonctionnels du Foie Normal et Pathologique, Université Rennes 1, Hôpital Pontchaillou, 2 rue Henri Le Guilloux, 35033, Rennes Cedex, France,* [3]*EA 2962 - Pharmacochimie, UFR des Sciences Pharmaceutiques, Université Victor Segalen Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France, and* [4]*EA 3677-Bases Thérapeutiques des Inflammations et Infections, UFR des Sciences Pharmaceutiques, Université Victor Segalen Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France*

(Received 30 September 2005; in final form 18 January 2006)

Abstract

Cell cycle progression is dependent on 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 two aspartic/glutamic acid, ornithine groups or hydrazide function at the lower rim, designed as potential iron chelators. The synthesis only afforded calix[4]arenes in the cone conformation. We report their effect on cell proliferation, in comparison with the new oral chelator ICL670A (4-[3,5-bis-(2-hydroxyphenyl)-1,2,4-triazol-1-yl]-benzoic acid). The antiproliferative effect of these new compounds was studied in the rat hepatoma cell line Fao by measuring mitochondrial succinate dehydrogenase activity. Their cytotoxicity was evaluated by extracellular LDH activity. Preliminary results indicated that among all tested compounds, monohydrazidocalix[4]arene **2** which is not cytotoxic in Fao cells exhibits interesting antiproliferative activity. This effect, independent on iron depletion, remains to be further explored. Moreover, it also shows that new substituted calix[4]arenes could open the way to new valuable medicinal chemistry scaffolding.

Keywords: *Calix[4]arene, hepatocyte, iron chelator, antiproliferative effect, conformation*

Introduction

Iron balance in man is essentially controlled by absorption of dietary iron in the proximal small intestine, because excretory pathways are extremely limited [1]. As a result, an 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 [2–3]. Iron is also necessary for cellular proliferation and we have previously demonstrated that iron overload is associated with increased DNA synthesis and mitotic index in rat hepatocyte cultures stimulated by epidermal growth factor [4]. Moreover, several studies, using different models, show that iron is implicated in tumor cell growth [5–6] and the risk of developing an hepatocellular carcinoma appears to be related to the level and duration of iron overload [7–8]. This is probably why hemochromatosis is frequently complicated by a hepatocellular carcinoma.

Thus, there is a great interest in the search for new iron chelators in order to decrease iron overload in genetic and secondary hemochromatosis. Indeed, iron depletion by different iron chelators has been shown to inhibit proliferation of various cell lines and normal

Correspondence. J. Guillon, EA 2962 – Pharmacochimie, UFR des Sciences Pharmaceutiques, Université Victor Segalen Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France. Tel: 33 5 57 57 10 10. Fax: 33 5 57 57 13 52. E-mail: Jean.Guillon@chimphys.u-bordeaux2.fr

ISSN 1475-6366 print/ISSN 1475-6374 online © 2006 Taylor & Francis
DOI: 10.1080/14756360600700384

activated lymphocytes *in vitro* [9–11]. We have previously demonstrated that iron depletion, induced by deferoxamine (DFO), the hydroxypyridin-4-one CP20 or O-trensox decreases DNA synthesis in both normal and transformed hepatocytes [12–14]. Thus, in view of these data, iron chelators have been proposed as promising antiproliferative agents in the treatment of human cancer.

However, DFO used for the treatment of iron overload, neuroblastoma and other diseases such as malaria [15–16] is poorly absorbed by the gastrointestinal tract; furthermore, continuous exposure to DFO causes a dose- and time-dependent cytotoxicity [17]. 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 [18–20], but this chelator has been shown to induce a severe neutropenia [21]. The orally active tridentate ICL670A is of special interest [21–22], because it induces a cell cycle arrest in S phase associated with a decrease of the polyamine levels which could result from an inhibition of polyamine biosynthesis probably by ODC inactivation [23].

Cyclodextrins have long been used in medicinal and biomimetic chemistry, and other cavity-containing synthetic macrocycles, such as cyclophanes, are popular in host-guest chemistry and enzyme mimics. Thus, calix[4]arenes, cyclic tetramers composed of four phenolic and four methylene moieties [24–26], are emerging as a new class of synthetic hosts enjoying interest in several areas of bioorganic and biomimetic chemistry. Many calixarene derivatives have been proposed and tested as potential bioactive compounds. Calixarene derivatives are also active as antitumoral, antiviral, antimicrobial, anti-thrombotic and antifungal agents [27–28]. A remarkable activity is found in the treatment of infections by enveloped viruses like HIV, herpes simplex and influenza viruses [29].

Thus, taking into account our experience in the field of the synthesis of new compounds of the type calix[4]arenes [30–32] we prepared new calix[4]arene podands **1a–g** bearing two aspartic/glutamic acid or ornithine groups at the lower rim, and a mono hydrazidocalixarene **2** (Figure 1).

Selective introduction of two peptidic groups into calix[4]arenes locked in the cone conformation should improve their hydrophilic character, as well as their chelator behaviour towards iron. The interest of these new molecules consists in their possible therapeutic applications related to secondary iron Fe^{2+} overloads. We present here preliminary results concerning the synthesis, the chelating effects against hydroxyl radical formation and the biological effects in rat hepatoma cell culture of the first representative compounds **1** and **2** of this chemical series.

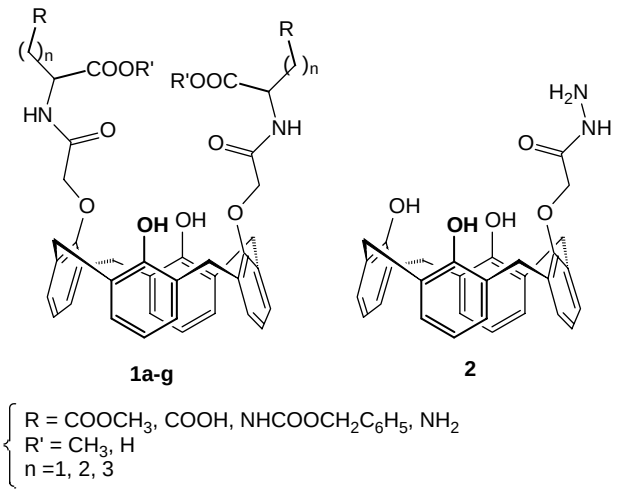


Figure 1. Structures of new cone peptidocalix[4]arenes **1a–g** and mono hydrazidocalix[4]arene **2**.

Materials and methods

Chemistry

Instrumentation. Melting points were determined with an SM-LUX-POL Leitz hot-stage microscope and reported uncorrected. Infrared (IR) spectra were determined in KBr discs on a NICOLET-210 spectrometer. NMR spectra were recorded on a BRUKER AVANCE 500 spectrometer (500 MHz). Chemical shifts refer to tetramethylsilane which was used as an internal reference. Mass spectra were recorded on a Micromass-Waters Q-TOF Ultima spectrometer. The peptides were analyzed on an analytical reverse-phase HPLC Shimadzu instrument with a prosphere 100 C18 5 μm column (4.6 × 150 mm) using a linear gradient of: (A) 0.1% (v/v) TFA in water and (B) 0.1% (v/v) TFA acetonitrile/water mixture (80/20, v/v), at a flow rate of 1 mL/min with UV detection at 220 nm. Peptides were purified by reverse-phase HPLC using a Shimadzu semi-preparative HPLC system on an prosphere 100 C18 5 μm column (10 × 250 mm) by elution with a linear gradient of (A): aqueous 0.1% TFA and (B): 0.1% (v/v) TFA in acetonitrile/water mixture (80/20), at a flow rate of 3 mL/min with UV detection at 220 nm. Elemental analyses were conducted by CNRS, Vernaison, France.

*Synthesis of 25,27-Dihydroxy-26,28-bis(O,O′-dimethyl-aspartyl- or –glutamylcarbonylmethoxy) calix[4]arenes, cone **1a–b** and 25,27-Dihydroxy-26,28-bis[(O-dimethyl-N′-benzyloxycarbonylornithyl)carbonylmethoxy)]calix[4]arene, cone **1c***. To a suspension of aspartic- or glutamyl dimethyl ester or N^6-[(benzyloxy)carbonyl]ornithine methyl ester (194.4 μmol) and 1,3-dicyclohexylcarbodiimide (DCC) (185.15 μmol) in 20 mL of dichloromethane, was added a solution of diacid **4** (92.59 μmol)

and 4-methylmorpholine (185.15 μmol) in 20 mL of dichloromethane. The reaction mixture was stirred at room temperature overnight. After removal of solvents, the residue was purified by HPLC to give **1a–c**.

*25,27-Dihydroxy-26,28-bis(O,O'-dimethylaspartylcarbonylmethoxy)calix[4]arene, cone (**1a**)*. Yield: 71%, white crystals, mp = 193°C, (Found MNa^+: 849.2810, $C_{44}H_{46}N_2O_{14}$ ^{23}Na requires 849.2847); IR ν_{max} (KBr)/cm^{-1} 3430 (OH), 3340 (NH), 1740 (CO); ^1H NMR δ (500 MHz, $CDCl_3$) 9.61 (d, *J* 6.55 Hz, 2H, NH), 8.17 (s, 2H, OH), 7.12 (m, 4H, Ar-H meta), 6.97 (d, *J* 7.20 Hz, 2H, Ar-H meta), 6.91 (d, *J* 7.20 Hz, 2H, Ar-H meta), 6.77 (t, *J* 7.20 Hz, 2H, Ar-H para), 6.68 (t, *J* 7.00 Hz, 2H, Ar-H para), 5.10 (d, *J* 15.00 Hz, 2H, OCH_2CO), 4.98 (m, 2H, CHα Asp), 4.31 (m, 6H, OCH_2CO and $ArCH_2Ar$), 3.75 (s, 6H, OCH_3), 3.57 (d, *J* 13.30 Hz, 2H, $ArCH_2Ar$), 3.37 (d, *J* 13.30 Hz, 2H, $ArCH_2Ar$), 3.25 (s, 6H, OCH_3), 2.90 (m, 4H, CH_2 Asp). ^{13}C NMR δ (125 MHz, $CDCl_3$) 170.6 (CO), 170.2 (CO), 169.1 (CONH), 152.4 (Cq, Ar ipso), 152.1 (Cq, Ar ipso), 132.9 (Cq, Ar ortho), 132.8 (Cq, Ar ortho), 130.2 (CH, Ar meta), 129.0 (CH, Ar meta), 128.5 (CH, Ar meta), 127.9 (Cq, Ar ortho), 126.7 (Cq, Ar ortho), 126.2 (CH, Ar para), 119.9 (CH, Ar para), 75.0 (OCH_2), 52.6 (OCH_3), 51.6 (OCH_3), 49.1 (Cα Asp), 37.0 (CH_2 Asp), 31.9 ($ArCH_2Ar$), 31.6 ($ArCH_2Ar$).

*25,27-Dihydroxy-26,28-bis(O,O'-dimethylglutamylcarbonylmethoxy)calix[4]arene, cone (**1b**)*. Yield: 62%, white crystals, mp = 190°C, (Found MNa^+: 877.3149, $C_{46}H_{50}N_2O_{14}$ ^{23}Na requires 877.3160); IR ν_{max} (KBr)/cm^{-1} 3425 (OH), 3340 (NH), 1739 (CO); ^1H NMR δ (500 MHz, MeOD) 9.65 (br s, 2H, NH), 8.02 (s, 2H, OH), 7.19 (d, *J* 7.60 Hz, 4H, Ar-H meta), 7.00 (d, *J* 7.60 Hz, 2H, Ar-H meta), 6.94 (d, *J* 7.60 Hz, 2H, Ar-H meta), 6.77 (t, *J* 7.60 Hz, 4H, Ar-H para), 5.04 (d, *J* 14.50 Hz, 2H, OCH_2CO), 4.77 (t, *J* 5.80 Hz, 2H, CHα Glu), 4.52 (d, *J* 14.50 Hz, 2H, OCH_2CO), 4.37 (d, 2H, *J* 13.05 Hz, $ArCH_2Ar$), 4.27 (d, 2H, *J* 13.05 Hz, $ArCH_2Ar$), 3.70 (s, 6H, OCH_3), 3.62 (d, *J* 13.05 Hz, 2H, $ArCH_2Ar$), 3.45 (d, *J* 13.05 Hz, 2H, $ArCH_2Ar$), 3.57 (s, 6H, OCH_3), 2.38 (m, 2H, $CH_2\gamma$ Glu), 2.10 (m, 1H, $CH_2\gamma$ Glu), 2.00 (m, 1H, $CH_2\gamma$ Glu), 1.39 (m, 2H, $CH_2\beta$ Glu), 1.11 (m, 2H, $CH_2\beta$ Glu). ^{13}C NMR δ (125 MHz, MeOD) 172.9 (CO), 171.7 (CO), 169.9 (CONH), 151.9 (Cq, Ar ipso), 133.1 (Cq, Ar ortho), 129.7 (CH, Ar meta), 128.9 (CH, Ar meta), 128.6 (CH, Ar meta), 127.5 (Cq, Ar ortho), 127.1 (Cq, Ar ortho), 125.8 (CH, Ar para), 120.0 (CH, Ar para), 74.3 (OCH_2CO), 51.7 (OCH_3), 51.2 (OCH_3), 50.7 (CHα Glu), 33.3 ($CH_2\gamma$ Glu), 31.4 ($ArCH_2Ar$), 30.9 ($ArCH_2Ar$), 27.4 ($CH_2\beta$ Glu), 26.6 ($CH_2\beta$ Glu).

*25,27-Dihydroxy-26,28-bis[(O-dimethyl-N'-benzyloxycarbonylornithyl)carbonylmethoxy)]calix[4]arene, cone (**1c**)*. Yield: 61%, white crystals, mp = 133°C, (Found MNa^+: 1087.4315, $C_{60}H_{64}N_4O_{14}$ ^{23}Na requires 1087.4317); IR ν_{max} (KBr)/cm^{-1} 3540, 3390 (OH), 3335 (NH), 1745 (CO), 1690 (CO). ^1H NMR δ (500 MHz, MeOD) 9.68 (br s, 2H, NH), 9.60 (br s, 2H, NH), 8.13 (s, 2H, OH), 7.71 (m, 10H, Ar'-H), 7.52 (m, 4H, Ar-H meta), 7.26 (m, 2H, Ar-H meta), 7.22 (d, *J* 7.00 Hz, 2H, Ar-H meta), 7.09 (t, *J* 7.50 Hz, 2H, Ar-H para), 7.02 (t, *J* 7.00 Hz, 2H, Ar-H para), 5.42 (d, *J* 12.20 Hz, 4H, OCH_2CO), 5.18 (s, 4H, OCH_2Ar'), 5.13 (m, 2H, CHα), 4.69 (d, *J* 13.00 Hz, 2H, $ArCH_2Ar$), 4.58 (d, *J* 13.00 Hz, 2H, $ArCH_2Ar$), 4.09 (s, 3H, OCH_3), 3.96 (d, *J* 13.00 Hz, 2H, $ArCH_2Ar$), 3.81 (d, *J* 13.00 Hz, 2H, $ArCH_2Ar$), 3.70 (s, 3H, OCH_3), 3.33 (m, 4H, $CH_2\gamma$ Orn), 2.23 (m, 2H, $CH_2\delta$ Orn), 2.09 (m, 2H, $CH_2\delta$ Orn), 1.71 (m, 2H, $CH_2\beta$ Orn), 1.55 (m, 2H, $CH_2\beta$ Orn). ^{13}C NMR δ (125 MHz, MeOD) 172.0 (CO), 169.7 (CONH), 157.5 ($COOCH_2Ar'$), 151.9 (Cq, Ar ipso), 137.0 (Cq, Ar'), 132.9 (Cq, Ar ortho), 129.8 (CH, Ar meta), 129.1 (CH, Ar meta), 128.7 (CH, Ar meta), 128.5 (Cq, Ar ortho), 128.0 (Cq, Ar ortho), 127.5 (CH, Ar'), 127.4 (CH, Ar'), 126.9 (CH, Ar'), 125.7 (CH, Ar para), 119.9 (CH, Ar para), 74.3 (OCH_2CO), 65.9 (OCH_2Ar'), 51.6 (OCH_3), 48.3 (CHα Orn), 39.9 ($CH_2\gamma$ Orn), 31.5 ($CH_2\delta$ Orn), 30.9 ($ArCH_2Ar$), 30.0 ($ArCH_2Ar$), 25.3 ($CH_2\beta$ Orn), 24.6 ($CH_2\beta$ Orn).

*Synthesis of 25,27-Dihydroxy-26,28-bis(aspartyl-or–glutamylcarbonylmethoxy)calix[4]arenes, cone **1d–e** and 25,27-Dihydroxy-26,28-bis[(N'-benzyloxycarbonyl ornithyl)carbonylmethoxy)]calix[4]arene, cone **1f***. The methyl ester **1a–c** (50 μmol) was dissolved in a mixture of water (5 mL) and methanol (10 mL). NaOH (2.5 equiv.) was added, and the solution was stirred at room temperature for 20 h. The solution was neutralized using excess HCl and the product was extracted in EtOAc (3 × 10 mL). The organic phase was washed with water, dried ($MgSO_4$) and evaporated to furnish a solid which was purificated by HPLC to give **1d–f**.

*25,27-Dihydroxy-26,28-bis(aspartylcarbonylmethoxy) calix[4]arene, cone (**1d**)*. Yield: 75%, white crystals, mp = 190°C, (Found MNa^+: 793.2194, $C_{40}H_{38}N_2O_{14}$ ^{23}Na requires 793.2220); IR ν_{max} (KBr)/cm^{-1} 3430 (COOH and OH), 3335 (NH), 1740 (CO); ^1H NMR δ (500 MHz, MeOD) 9.75 (br s, 2H, NH), 7.95 (s, 2H, OH), 7.15 (m, 4H, Ar-H meta), 6.88 (d, *J* 7.10 Hz, 2H, Ar-H meta), 6.71 (d, *J* 6.80 Hz, 2H, Ar-H meta), 6.63 (m, 4H, Ar-H para), 5.12 (d, *J* 15.25 Hz, 2H, OCH_2CO), 4.47 (m, 2H, CHα Asp), 4.31 (d, *J* 15.25 Hz, 2H, OCH_2CO), 3.58 (d, *J* 13.10 Hz, 4H, $ArCH_2Ar$), 3.46 (d, *J* 13.10 Hz, 4H, $ArCH_2Ar$), 2.99 (m, 4H, $CH_2\beta$ Asp). ^{13}C NMR δ (125 MHz, MeOD) 172.2

(COOH), 171.9 (COOH), 170.2 (CONH), 152.1 (Cq, Ar ipso), 152.0 (Cq, Ar ipso), 133.1 (Cq, Ar ortho), 129.5 (CH, Ar meta), 129.0 (CH, Ar meta), 128.7 (CH, Ar meta), 128.5 (CH, Ar meta), 127.8 (Cq, Ar ortho), 127.3 (Cq, Ar ortho), 125.6 (CH, Ar para), 119.6 (CH, Ar para), 74.2 (OCH_2CO), 49.0 (CHα Asp), 35.8 ($CH_2\beta$ Asp), 31.2 ($ArCH_2Ar$), 31.0 ($ArCH_2Ar$).

*25,27-Dihydroxy-26,28-bis(glutamylcarbonylmethoxy) calix[4]arene, cone (**1e**)*. Yield: 69%, white crystals, mp = 180°C, (Found MNa^+: 821.2544, $C_{42}H_{42}N_2O_{14}$ ^{23}Na requires 821.2534); IR ν_{max} (KBr)/cm^{-1} 3430 (COOH and OH), 3319 (NH), 1740 (CO); ^1H NMR δ (500 MHz, MeOD) J, Hz: 9.85 (d, J 6.45 Hz, 2H, NH), 7.54 (m, 4H, Ar-H meta), 7.31 (m, 2H, Ar-H meta), 7.32 (m, 2H, Ar-H meta), 7.18 (t, *J* 7.55 Hz, 2H, Ar-H para), 7.11 (m, 2H, Ar-H para), 5.05 (d, *J* 14.40 Hz, 2H, OCH_2CO), 4.89-4.65 (m, 2H, CHα Glu), 3.95 (d, *J* 14.40 Hz, 2H, OCH_2CO), 2.75 (m, 2H, $CH_2\gamma$ Glu), 2.59 (m, 1H, $CH_2\gamma$ Glu), 2.47 (m, 1H, $CH_2\gamma$ Glu), 2.21 (d, *J* 12.75 Hz, 4H, $ArCH_2Ar$), 2.08 (d, *J* 12.75 Hz, 4H, $ArCH_2Ar$), 1.69 (m, 2H, $CH_2\beta$, Glu), 1.56 (m, 2H, $CH_2\beta$, Glu). ^{13}C NMR δ (125 MHz, MeOD) 174.5 (COOH), 172.6 (COOH), 170.2 (CONH), 151.9 (Cq, Ar ipso), 133.0 (Cq, Ar ortho), 132.9 (Cq, Ar ortho), 129.5 (CH, Ar meta), 129.0 (CH, Ar meta), 128.9 (CH, Ar meta), 128.6 (CH, Ar meta), 127.5 (Cq, Ar ortho), 127.2 (Cq, Ar ortho), 125.7 (CH, Ar para), 119.9 (CH, Ar para), 119.4 (CH, Ar para), 74.3 (OCH_2CO), 51.7 (CHα Glu), 33.3 ($CH_2\gamma$ Glu), 31.3 ($ArCH_2Ar$), 31.0 ($ArCH_2Ar$), 29.4 ($CH_2\beta$ Glu), 27.6 ($CH_2\beta$ Glu).

*25,27-Dihydroxy-26,28-bis[(N′ -benzyloxycarbonyl ornithyl)carbonylmethoxy)]calix[4] arene, cone (**1f**)*. Yield: 71%, white crystals, mp = 141°C, (Found MNa^+: 1059.3972, $C_{58}H_{60}N_4O_{14}$ ^{23}Na requires 1059.4004); IR ν_{max} (KBr)/cm^{-1} 3545, 3390 (COOH and OH), 3335 (NH), 1740 (CO), 1690 (CO); ^1H NMR δ (500 MHz, $CDCl_3$) 9.61 (br s, 2H, NH), 8.05 (s, 2H, OH), 7.27 (m, 10H, Ar'-H), 7.18 (m, 4H, Ar-H meta), 7.00 (m, 2H, Ar-H meta), 6.85 (d, *J* 7.10 Hz, 2H, Ar-H meta), 6.80 (t, *J* 7.10 Hz, 2H, Ar-H para), 6.70 (t, *J* 7.30 Hz, 2H, Ar-H para), 5.11 (d, *J* 13.30 Hz, 2H, OCH_2CO), 5.01 (s, 4H, OCH_2Ar'), 4.80 (m, 2H, CHα), 4.61 (d, *J* 13.30 Hz, 1H, OCH_2CO), 4.25 (d, *J* 13.30 Hz, 1H, OCH_2CO), 4.10 (d, *J* 12.70 Hz, 4H, $ArCH_2Ar$), 3.60 (d, *J* 12.70 Hz, 4H, $ArCH_2Ar$), 2.95 (m, 4H, $CH_2\gamma$ Orn), 1.82 (m, 2H, $CH_2\delta$ Orn), 1.62 (m, 2H, $CH_2\delta$ Orn), 1.38 (m, 2H, $CH_2\beta$ Orn), 1.20 (m, 2H, $CH_2\beta$ Orn). ^{13}C NMR δ (125 MHz, $CDCl_3$) 172.9 (CO), 170.3 (CONH), 156.4 ($COOCH_2Ar'$), 152.0 (Cq, Ar ipso), 152.2 (Cq, Ar ipso), 136.7 (Cq, Ar'), 132.6 (Cq, Ar ortho), 130.2 (CH, Ar meta), 129.2 (CH, Ar meta), 128.9 (CH, Ar meta), 128.7 (Cq, Ar ortho), 128.4 (CH, Ar'), 128.0 (CH, Ar'), 127.5 (CH, Ar'), 126.6 (CH, Ar para), 126.2 (CH, Ar para), 120.1 (CH, Ar para), 74.7 (OCH_2CO), 66.5 (OCH_2Ar'), 49.6 (CHα Orn), 40.4 ($CH_2\gamma$ Orn), 32.1 ($CH_2\delta$ Orn), 31.5 ($ArCH_2Ar$), 30.2 ($ArCH_2Ar$), 25.5 ($CH_2\beta$ Orn), 24.8 ($CH_2\beta$ Orn).

*25,27-Dihydroxy-26,28-bis[(ornithyl)carbonyl-methoxy)]calix[4] arene, cone (**1g**)*. To a solution of **1f** (25 mg, 24.13 μmol) dissolved in MeOH (10 mL) was added 10% Pd/C (0.20 g). The reaction mixture was then stirred at room temperature under an atmosphere of hydrogen for 24 h. The solution was filtered through Celite, and the solvent was evaporated to give **1g**. The product was characterized by ^1H NMR and used without further purification. Yield: 81%, white crystals, mp = 143°C, (Found MH^+: 769.3409, $C_{42}H_{49}N_4O_{10}$ requires 769.3449); IR ν_{max} (KBr)/cm^{-1} 3545 (COOH and OH), 3330 (NH), 3265 (NH_2), 1740 (CO); ^1H NMR δ (500 MHz, MeOD) 7.20 (m, 4H, Ar-H meta), 7.01 (d, *J* 7.70 Hz, 2H, Ar-H meta), 6.97 (d, *J* 7.70 Hz, 2H, Ar-H meta), 6.81 (t, *J* 7.70 Hz, 2H, Ar-H para), 6.75 (t, *J* 7.30 Hz, 2H, Ar-H para), 5.06 (d, *J* 14.30 Hz, 1H, OCH_2CO), 4.72 (m, 2H, CHα), 4.34 (d, *J* 14.30 Hz, 2H, OCH_2CO), 4.23 (d, *J* 14.30 Hz, 1H, OCH_2CO), 3.62 (d, *J* 13.35 Hz, 2H, $ArCH_2Ar$), 3.49 (d, *J* 13.35 Hz, 4H, $ArCH_2Ar$), 3.30 (m, 4H, $ArCH_2Ar$ and NH_2), 2.86 (m, 4H, $CH_2\gamma$ Orn), 1.86 (m, 2H, $CH_2\delta$ Orn), 1.72 (m, 2H, $CH_2\delta$, Orn), 1.36 (m, 2H, $CH_2\beta$, Orn), 1.18 (m, 2H, $CH_2\beta$, Orn). ^{13}C NMR δ (125 MHz, MeOD) 172.7 (COOH), 170.3 (CONH), 152.0 (Cq, Ar ipso), 133.0 (Cq, Ar ortho), 132.9 (Cq, Ar ortho), 129.8 (CH, Ar meta), 128.9 (CH, Ar meta), 128.7 (CH, Ar meta), 128.5 (CH, Ar meta), 127.6 (Cq, Ar ortho), 127.0 (Cq, Ar ortho), 125.7 (CH, Ar para), 119.8 (CH, Ar para), 74.3 (OCH_2CO), 48.3 (CHα Orn), 38.9 ($CH_2\gamma$ Orn), 31.3 ($CH_2\delta$ Orn), 30.7 ($ArCH_2Ar$), 29.8 ($ArCH_2Ar$), 25.3 ($CH_2\beta$ Orn), 23.4 ($CH_2\beta$ Orn).

*25,26,27-Trihydroxy-28-(hydrazinocarbonylmethoxy) calix[4] arene, cone (**2**)*. To a solution of 25,27-diethoxycarbonylmethoxycalix[4]arene **3** (0.7 mmol) in 30 mL of ethanol, was added an excess of hydrazine hydrate (98%). The mixture was refluxed for 5 h. The precipitate formed was filtered, washed successively with ethanol and diethyl ether, and dried to give **2**. Yield: 82%, yellow crystals, mp = 185°C, (Found MNa^+: 519.1902, $C_{30}H_{28}N_2O_5$ ^{23}Na requires 519.1896); IR ν_{max} (KBr)/cm^{-1} 3310 and 3240 (NH and NH_2), 1670 (CO); ^1H NMR δ (500 MHz, d_6-DMSO) 10.28 (s, 2H, OH), 8.93 (s, 1H, OH), 8.21 (bs, 1H, NH), 7.02 (d, *J* 7.35 Hz, 1H, Ar-H meta), 7.00 (d, *J* 7.35 Hz, 1H, Ar-H meta), 6.86 (d, 4H, *J* 7.35 Hz, Ar-H meta), 6.76 (d, 2H, *J* 7.35 Hz, Ar-H meta), 6.60 (t, *J* 7.35 Hz, 1H, Ar-H para), 6.40 (t, *J* 7.35 Hz, 2H, Ar-H para), 6.07 (t, *J* 7.35 Hz, 1H, Ar-H para), 4.88 (br s, 2H, NH_2), 4.33 (d, *J* 12.5 Hz, 2H, $ArCH_2Ar$), 4.31 (s, 2H, OCH_2CO), 4.11 (d, *J* 11.95 Hz, 2H, $ArCH_2Ar$),

3.17 (d, J 11.95 Hz, 2H, $ArCH_2Ar$), 3.13 (d, J 12.5 Hz, 2H, $ArCH_2Ar$).

Pharmacology

Chemicals. Unless stated otherwise, all chemicals were purchased from Sigma Chemical Co. (La Verpillière, France) or Merck (Darmstadt, Germany). All chemicals were of first-grade purity and were used without further purification. Ferric ammonium citrate, $FeSO_4$, $CuSO_4$ or $ZnSO_4$ solutions were prepared in sterile water. The final concentration of Fe, Cu or Zn in the stock solutions was 1 mM. These solutions were diluted in the culture medium to obtain a final medium iron, copper or zinc concentration of 20 μM.

Cell cultures. The rat hepatoma cell line Fao used in this study was obtained by Deschatrette and Weis [33] and maintained by subculture in the following medium-50% Ham F12 medium and 50% NCTC (National Collection of Type Cultures) 135 medium (Eurobio, Les Ulis, France)-containing per mL: fetal calf serum (FCS, 0.1 mL), glutamine (0.002 mmol), penicillin (50 IU), streptomycin (50 μg), $NaHCO_3$ (2.2 mg). For the experiments, the Fao cells were seeded in 96-well microplates (Becton Dickinson, Oxnard, CA) at a density of 6×10^3 cells per well and maintained for 72 h in the same medium as above. Cell cultures were maintained during 72 h in the control condition (neither metal nor chelators present), in presence of metal alone, compounds alone or compounds plus divalent cations.

Solubility of the chelators. Solubility of the new calix[4]arenes **1** and **2** was estimated in pure water and in cell culture medium containing 10% FCS. Solutions of the various compounds (200 μM) were prepared by diluting the 10 mM stock solutions in DMSO in 2 mL of water and culture medium contained in a quartz cuvette. The absorbance (turbidity) of the solutions was measured at 450 nm, outside the absorption range of chromophores (280 nm). Results were expressed as percentage of absorbance (turbidity) with respect to the values in the absence of compound.

Inhibition of homovanillic acid autoxidation. The main advantages of the homovanillic acid (HVA) autoxidation assay were previously shown to be its versatility to investigate in a single run the antioxidant capacity of compounds including the scavenging and the inhibitory effects of radical formation, as well as its relevance to the reactive hydroxyl radical [34]. Autoxidation of HVA gives rise to fluorescent dimers. Their relative fluorescence intensity ($\lambda_{exc} = 315$ nm; $\lambda_{em} = 425$ nm) follows a linear kinetic pattern (for less than 50 min). This Fenton-like reaction was transiently stopped by various reactive oxygen species scavengers (delay) while metal chelating agents such as DFO, EDTA and polyamines only reduced its rate. The final reaction mixture for the HVA assay contained 3.10^{-4} M HVA in 0.1 M borate buffer (pH 9.0). A final volume of 200 μL was incubated at 37°C under gentle stirring in each well of a 96 wells microplate. Fluorescence was measured every min for 30 min, using a Gemini model fluorescence microplate reader (Molecular Devices, France). Autoxidation rate in the absence (R_0) or in the presence of various compounds concentrations (R_a) was calculated from the slope of these fluorescence kinetics and results were expressed as percentage of control (R_0). Each kinetic determination was performed in triplicate.

Cytostatic and cytotoxic effects measurements. Cytoxicity was evaluated by measuring extracellular lactate dehydrogenase activity (cytotoxicity detection kit–LDH, Roche, Penzberg, Germany) and mitochondrial succinate dehydrogenase activity (SDH) 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 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.

Statistical analysis. Results from at least three replicates were expressed as means ± SD. Statistical analysis was performed using the non-parametric Mann-Whitney test. The significant level was set at 0.01.

Results and discussion

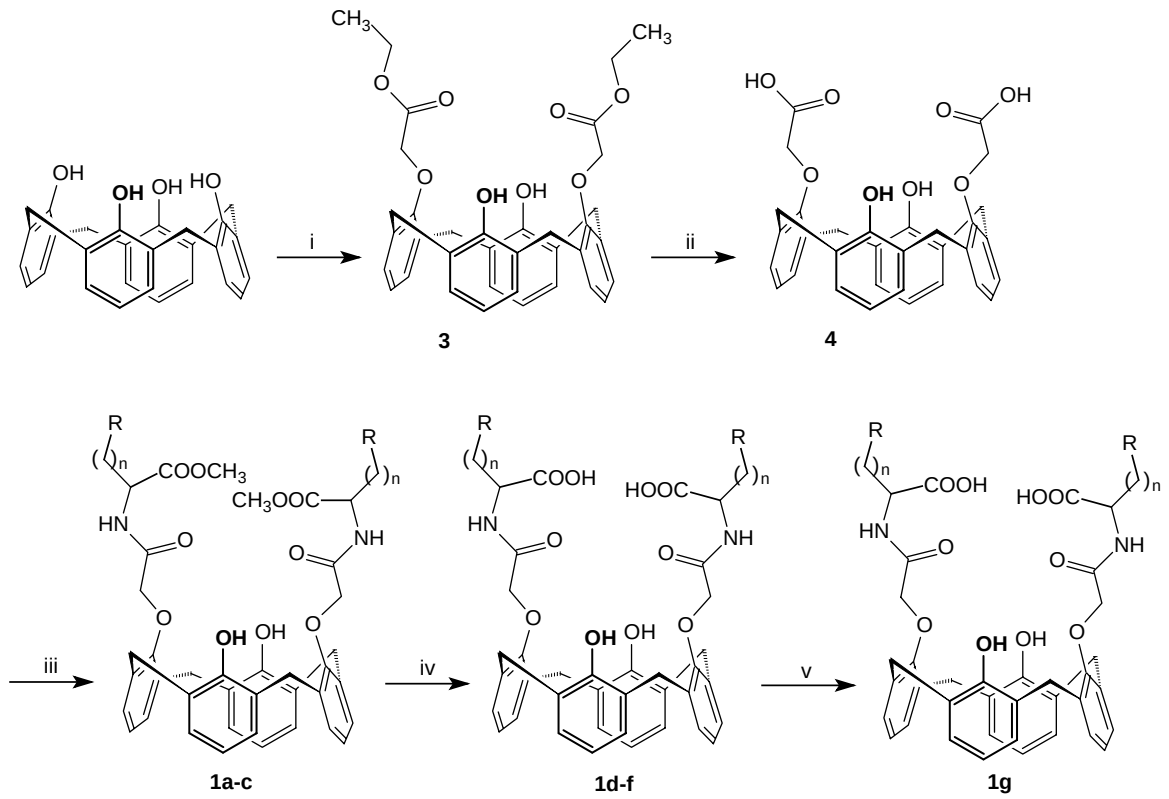
Chemistry

The synthesis of the new peptidocalix[4]arenes **1** has been accomplished in three or four steps starting from

commercially available calix[4]arene (Scheme 1). Calix[4]arene diester **3** was prepared in a 55% yield by alkylation of the unsubstituted calix[4]arene with ethyl bromoacetate in the presence of 1 equivalent of K_2CO_3 as a base, in refluxing CH_3CN [35–38]. The alkylation led selectively to the diametrically substituted 25,27-diethoxycarbonylmethoxycalix[4]arene **3** in the cone conformation, as indicated by the ^1H NMR spectrum. A typical AB pattern was observed for the methylene bridge $ArCH_2Ar$ protons (J 13.15 Hz) at 4.51 and 3.42 ppm. Hydrolysis of **3** with 15% sodium hydroxide in ethanol under reflux gave, after acidification, the crystalline diacid **4** (95%) [39–40]. Condensation between aspartic/glutamic dimethyl ester or N^6-[(benzyloxy)carbonyl]ornithine methyl ester and diacid **4** in the presence of 1,3-dicyclohexylcarbodiimide (DCC) and 4-methylmorpholine (NMM) afforded the calixarenes **1a–c** in the cone conformation [41]. Mild hydrolysis of the ester functions of **1a–c** then resulted in the carboxylated cone calix[4]arenes **1d–f**. Deprotection of the amino group at the *N*-6 position of the ornithine moiety in **1f** yielded the peptidocalix[4]arene **1g**.

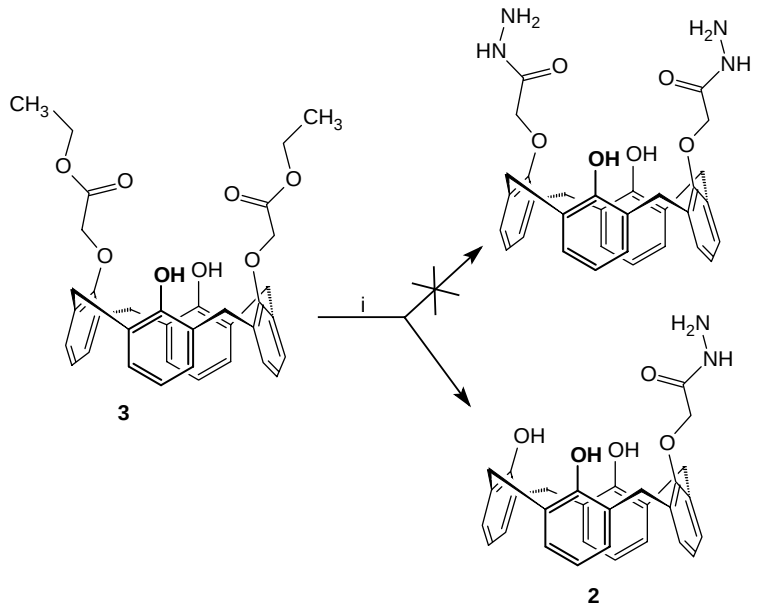
Treatment of the ester **3** in refluxing ethanol with hydrazine hydrate led to the mono hydrazinocarbonylmethoxycalix[4]arene **2** in the cone conformation and surprisingly not to the dihydrazido compound (Scheme 2).

The calix[4]arene **2** had a cone structure as indicated by the ^1H NMR spectrum. A typical pattern represented by two 2H doublets at 3.17 and 3.13 ppm for the equatorial protons and two doublets at 4.33 and 4.11 ppm for the axial protons of the bridging methylene ($ArCH_2Ar$) was observed [30]. The spectrum also showed two sharp signals for the three OH groups, one at 8.93 ppm (1H) and one at 10.28 ppm (2H) [30]. Moreover, the spectrum exhibited only one singlet (2H) around 4.31 ppm



	R	n
a	$COOCH_3$	1
b	$COOCH_3$	2
c	$NHCOOCH_2C_6H_5$	3
d	COOH	1
e	COOH	2
f	$NHCOOCH_2C_6H_5$	3
g	NH_2	3

Scheme 1. Synthesis of compounds **1a–g**. Reagents: (i) K_2CO_3, $BrCH_2COOEt$, CH_3CN; (ii) 1) NaOH, EtOH; 2) HCl, H_2O (iii). R-$(CH_2)_n$-$CHNH_2$-$COOCH_3$, DCC, NMM, CH_2Cl_2; (iv) 1) NaOH, MeOH, H_2O; 2) HCl, MeOH, H_2O (v) H_2, Pd/C, MeOH.



Scheme 2. Synthesis of compounds **2**. Reagents: (i) H_2N-NH_2, H_2O, EtOH.

which corresponded to the OCH_2CO methylene. These observations confirmed the cone conformation of derivative **2**.

Pharmacology

Solubility. Initially, the poor solubility of some compounds was deduced from phase contrast microscopy analysis of aggregates for various compound concentrations (0, 25, 50, 100 and 200 μM). Solubility of the various compounds was estimated by turbidimetry measurement (absorption at 450 nm). As shown in Table I, at a concentration of 200 μM, the different calix[4]arene esters **1a–c** and **3** were poorly soluble in aqueous media such as the culture medium used for Fao cell cultures. These insoluble compounds were not further tested in cell culture.

Table I. Relative solubility of the various compounds **1a–g**, **2**–**4** and ICL670A.

	Relative solubility (%turbidity/solvent)	
Compound	cell culture medium	Water
1a	373	377
1b	386	570
1c	645	353
1d	172	313
1e	102	133
1f	nd[a]	nd[a]
1g	109	199
2	106	225
3	479	1029
4	110	481
ICL670A	103	105

[a]nd: not determined.

Scavenging and chelating effects against hydroxyl radical formation: the HVA test. Hydroxyl radical formation mediated by transition metals such as iron(II), is hypothesized to be an important physiological condition, and is thought to be associated with oxidative damage *in vivo*, rather than hydrogen peroxide itself. The effect of the various tested compounds concentrations on HVA autoxidation rate is presented in Figure 2. Neither was able to delay HVA autoxidation like typical scavengers.

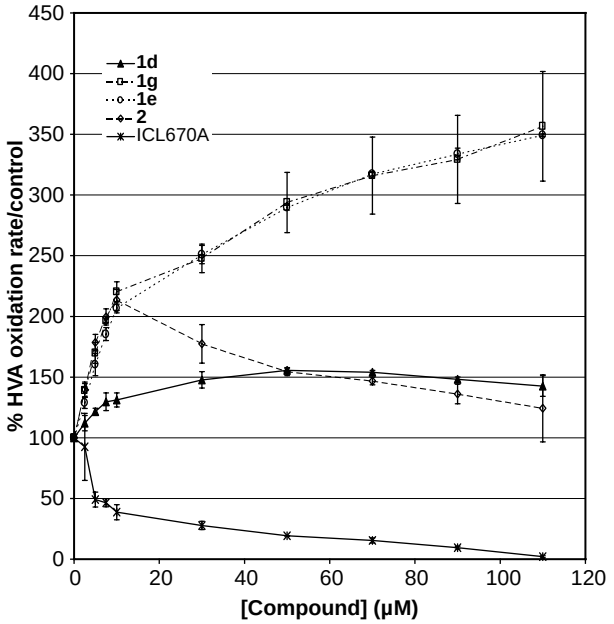


Figure 2. Dose-effect modulation of the homovanillic acid autoxidation rate (Fenton-like reaction) induced by the various compounds **1d**, **1e**, **1g**, and ICL670A. Data expressed as a percent of the control (absence of compound) are the mean ± SD of three independent experiments.

Some of them, calixarenes **1e** and **1g** appeared to be prooxidants inducing an increase in HVA autoxidation rate for a concentration lower than 30 μM followed in the case of **1d** and **2** by a decrease in the oxidation rate. In this HVA test, micromolar concentrations of our reference iron chelator ICL670A, induced an inhibition of HVA autoxidation rate (without delaying the oxidation kinetic).

Biological effects in rat hepatoma cell culture. A dose-dependent decrease of cell viability (MTT assay) was observed after a 72 h cell treatment in the presence of increasing concentrations (0 up to 100 μM) of all compounds (Figure 3).

Due to the low solubility of the compounds as well as their relatively low antiproliferative efficiency, it was not possible to estimate the concentration inducing a 50% inhibition of cell growth (IC_{50}), except for ICL670A (12 μM), 2 (24 μM), 1b (63 μM) and 1d (92 μM). The comparison of the relative antiproliferative activities of calixarene derivatives and ICL670A at the concentration 25 μM are reported in Table II. At this concentration, the decrease in cell viability induced by the various compounds remained low compared to ICL670A (12.7%), except for compounds 2 and 1b (51.3 and 70.5%, respectively). The inhibitory effect on cell viability of the derivatives was in the following order: 2 > 1b > 4 > 1d > 1e > 1g. As shown in Table II, the absence of LDH leakage in Fao cells supernatant treated 72 hours with the compounds indicated an absence of cytotoxicity except for ICL670A which exhibited weak membrane damage (130% LDH with respect to the control).

The addition of 20 μM of iron sulfate simultaneously with 25 μM of ICL670A partially inhibited the antiproliferative effect of this iron chelator (Table III) while the addition of 20 μM of copper or zinc, under the same conditions, was without effect. In contrast, the addition of 20 μM of iron, copper or zinc sulfate simultaneously with 25 μM of **2** did not protect the Fao cells from the cytostatic effect of this compound. The antiproliferative effect of calixarene **2** was not correlated to iron, copper or zinc depletion.

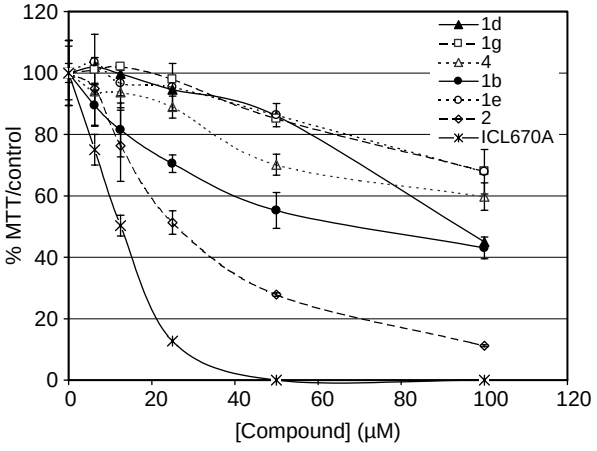


Figure 3. Dose-effect decrease in viability of Fao cells (SDH activity measured from the MTT assay) induced by the various compounds **1b**, **1d**, **1e**, **1g**, **2**, **4** and ICL670A after a 72 h cell treatment. Data expressed as a percent of the control (absence of compound) are the mean ± SD of three independent experiments.

Table II. Biological effect of a 72 h treatment of Fao cells with 25 μM of the calixarene compounds **1a–g** and **2**–**4** and the reference compound ICL670A. LDH leakage in cell supernatant was used as an index of membrane damages (cytotoxicity) and SDH (MTT assay) as a measurement of cell viability.

Compound	Toxicity[a] %LDHext/cont ± SD	Cell viability[a] %SDH/cont ± SD
1a	nd[b]	nd[b]
1b	105.8 ± 4.1	70.5 ± 2.8
1c	nd[b]	nd[b]
1d	98.2 ± 3.1	94.6 ± 5.0
1e	98.6 ± 1.8	95.4 ± 7.7
1f	nd[b]	nd[b]
1g	92.7 ± 3.2	97.9 ± 8.3
2	85.4 ± 1.6	51.3 ± 3.8
3	nd[b]	nd[b]
4	100.0 ± 4.6	88.9 ± 3.6
ICL670A	130.6 ± 1.6	12.7 ± 0.7

[a]Data expressed as a percent of the control (absence of compound) are the mean ± SD of three independent experiments.
[b]nd: not determined.

Conclusion

The low solubility of the methyl and ethyl calix[4]arene esters **1a–c** and **3** in aqueous solvents and more particulary in cell culture medium, prevented us studying their biological efficiency. We observed that the soluble compounds, *i.e.* calixarenes **1d**, **1g**, **1e**, **2** and **4** were not cytotoxic at concentrations lower than 100 μM.

The antiproliferative effect of various new substituted calix[4]arenes remained low compared to

Table III. Biological effect of addition of 20 μM of iron, copper or zinc sulfate simultaneously with 25 μM of **2** or ICL670A in the rat hepatoma cell line Fao (72 h treatment). LDH leakage in cell supernatant was used as an index of membrane damages (cytotoxicity) and SDH (MTT assay) as a measurement of cell viability.

Compound	Toxicity[a] %LDHext/cont ± SD	Cell viability[a] %SDH/cont ± SD
2	85.4 ± 1.6	51.3 ± 3.8
2 + Fe	91.0 ± 5.8	53.5 ± 2.2
2 + Cu	79.0 ± 20.6	50.1 ± 5.8
2 + Zn	89.8 ± 5.9	50.1 ± 2.6
ICL670A	130.6 ± 1.6	12.7 ± 0.7
ICL670A + Fe	138.4 ± 4.2	88.8 ± 5.6
ICL670A + Cu	122.2 ± 6.5	7.7 ± 1.5
ICL670A + Zn	93.4 ± 2.6	9.3 ± 1.8

[a]Data expressed as a percent of the control (absence of compound) are the mean ± SD of three independent experiments.

ICL670A, except for compounds **2** and **1b**. The relative antiproliferative efficiency of the derivatives was in the following order: **2** > **1b** > **4** > **1d** > **1e** > **1g**. These initial results do not enable us to determine precisely the structure-activity relationships in these series. In the case of calix[4]arene **2**, its antiproliferative effect was not reversed by addition of exogenous iron such as observed with the iron chelator ICL670A. On the basis of their ability to inhibit metal-catalyzed oxidation reaction such as the HVA autoxidation, we deduced that the compounds were not efficient iron nor copper chelators. Some of them were prooxidants in the HVA autoxidation test. All together, our results indicated that among all tested compounds, the mono hydrazidocalixarene **2** which is not cytotoxic in Fao cells exhibits an interesting antiproliferative activity. This effect, independent on iron depletion, remains to be further explored in order to understand its cytostatic effect. One possibility will be to study the mono hydrazidocalixarene **2** effect on the expression cyclins, like cyclinD for example. Finally, the new substituted calix[4]arenes could open the way to new valuable medicinal chemistry scaffolding.

Acknowledgements

The authors thank Novartis Pharma Laboratories (Basel, Switzerland) for supplying ICL670A. This work was partly supported by ACI « CAPFER: nouveaux outils chimiques et analytiques pour la détection et le dosage du fer; application à l'étude de son métabolisme »; n° 03 2 135.

References

[1] McCance RA, Widdowson EM. Absorption and excretion of iron. Lancet 1937;ii:680–684.

[2] Deugnier Y, Charalambous P, Le Quilleuc D, Turlin B, Searle J, Brissot P, Powell LW, Halliday JW. Preneoplastic significance of hepatic iron-free foci in genetic hemochromatosis: a study of 185 patients. Hepatology 1993;18: 1363–1369.

[3] Hsing AW, McLaughlin JK, Olsen JH, Mellemkjar L, Wacholder S, Fraumeni JF. Cancer risk following primary hemochromatosis: a population-based cohort study in Denmark. Int J Cancer 1995;60:160–162.

[4] Chenoufi N, Loréal O, Drénou B, Cariou S, Hubert N, Leroyer P, Brissot P, Lescoat G. Iron may induce DNA synthesis and repair in rat hepatocytes stimulated by EGF/pyruvate. J Hepatol 1995;26:650–658.

[5] Hann HWL, Stahlhut MW, Blumberg BS. Iron nutrition and tumor growth: decreased tumor growth in iron deficient mice. Cancer Res 1988;48:4168–4170.

[6] Thompson HJ, Kennedy K, Witt M, Juzefyk J. Effect of dietary iron deficiency or excess on the induction of mammary carcinogenenis by 1-methyl-1-nitrosourea. Carcinogenesis 1991;12:111–114.

[7] Tarao K, Shimizu A, Ohkawa S, Harada M, Ito Y, Tamai S, Kuni Y, Okamoto N, Inoue T, Kanisawa M. Development of hepatocellular carcinoma associated with increases in DNA synthesis in the surrounding cirrhosis. Gastroenterol 1992;103:595–600.

[8] Ballardini G, Groff P, Zoli M, Bianchi G, Giostra F, Francesconi R, Lenzi M, Zauli D, Cassani F, Bianchi F. Increased risk of hepatocellular carcinoma development in patients with cirrhosis and with high hepatocellular proliferation. J Hepatol 1994;20:218–222.

[9] Soyano A, Chinea M, Romano EL. The effect of desferrioxamine on the proliferative response of rat lymphocytes stimulated with various mitogens in vitro. Immunopharmacol 1984;8:163–169.

[10] Polson RJ, Jenkins R, Lombard M, Williams AC, Roberts S, Nouri-Aria K, Williams R, Bomford A. Mechanisms of inhibition of mononuclear cell activation by the iron-chelating agent desferrioxamine. Immunology 1990;71:176–181.

[11] Richardson DR, Milnes K. The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents II: the mechanism of action of ligands derived from salicylaldehyde benzoyl hydrazone and 2-hydroxy-1-naphthylaldehyde benzoyl hydrazone. Blood 1997;89:3025–3038.

[12] Chenoufi N, Baffet G, Drénou B, Cariou S, Desille M, Clément B, Brissot P, Lescoat G, Loréal O. Deferoxamine arrests in vitro the proliferation of porcine hepatocyte in G1 phase of the cell cycle. Liver 1997;18:60–67.

[13] Chenoufi N, Drénou B, Loréal O, Pigeon C, Brissot P, Lescoat G. Antiproliferative effect of deferiprone on the HepG2 cell line. Biochem Pharmacol 1998;56:431–437.

[14] Rakba N, Loyer P, Gilot D, Delcros JG, Glaise D, Baret P, Pierre JL, Brissot P, Lescoat G. Antiproliferative and apoptotic effects of O-Trensox, a new synthetic iron chelator, on differentiated human hepatoma cell lines. Carcinogenesis 2000;21:943–951.

[15] Brodie C, Siriwardana G, Lucas J, Schleicher R, Terada N, Szepesi A, Gelfand E, Seligman P. Neuroblastoma sensitivity to growth inhibition by desferrioxamine: evidence for a block in G1 phase of the cell cycle. Cancer Res 1993;53:3968–3975.

[16] Glickstein H, Breuer W, Loyevsky M, Konijn AM, Shanzer A, Cabantchik ZI. Differential cytotoxicity of iron chelators on malaria-infected cells versus mammalian cells. Blood 1996; 87:4871–4878.

[17] Valle P, Timeus F, Piglione M, Rosso P, di Montezemolo LC, Crescenzio N, Marranca D, Ramenghi U. Effect of different exposures to desferrioxamine on neuroblastoma cell lines. Pediatr Hematol Oncol 1995;12:439–446.

[18] Olivieri NF, Koren G, Matsui D, Liu PP, Blendis L, Cameron R, McClelland RA, Templeton DM. Reduction of tissue iron stores and normalization of serum ferritin during treatment with the oral iron chelator L1 in thalassemia intermedia. Blood 1992;79:2741–2748.

[19] Al-Refaie FN, Wonke B, Hoffbrand AV, Wickens DG, Nortey P, Kontoghiorges GJ. Efficacy and possible adverse effects of the oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one (L1) in thalassemia major. Blood 1992;80:593–599.

[20] Collins AF, Fassos FF, Stobie S, Lewis N, Shaw D, Fry M, Templeton DM, McClelland RA, Koren G, Olivieri NF. Iron-balance and dose-response studies of the oral iron chelator 1,2-dimethyl-3-hydroxypyrid-4-one (L1) in iron-loaded patients with sickle cell disease. Blood 1994; 83:2329–2333.

[21] Porter JB, Davis BA. Monitoring chelation therapy to achieve optimal outcome in the treatment of thalassaemia. Best Pract Res Clin Haematol 2002;15:329–368.

[22] Caris C, Baret P, Beguin C, Serratrice G, Pierre JL, Laulhere JP. Metabolization of iron by plant cells using O-Trensox, a high-affinity abiotic iron-chelating agent. Biochem J 1995;312:879–885.

[23] Unpublished results.

[24] Gutsche CD. Calixarenes revisited, Monographs in Supramolecular Chemistry. In: Stoddart JF, editor. London, UK: The Royal Society of Chemistry; 1998.
[25] Vicens J, Böhmer V. Calixarenes, A Versatile Class of Macrocyclic Compounds; Topics in Inclusion Science. In: Vicens J, Böhmer V, editors. Dordrecht, The Netherlands: Kluwer Academic Press; 1991.
[26] Böhmer V. Calixarenes, macrocycles with (almost) unlimited possibilities. Angew Chem Int Ed Engl 1995;34:713–745.
[27] Sansone F, Segura M, Ungaro R. Calixarenes in Bioorganic and Biomimetic Chemistry. In: Asfari Z, Böhmer V, Harrowfield J, Vicens J, editors. Calixarenes 2001. Dordrecht, The Netherlands: Kluwer Academic Publishers; 2001. p 496–512.
[28] Lamartine R, Tsukada M, Shirata A. Antimicrobial activity of calixarenes. C R Chimie 2002;5:1–7.
[29] Da Silva E, Ficheux D, Coleman AW. Anti-thrombotic activity of water-soluble calix[n]arenes. J Inclusion Phenom Macrocyclic Chem 2005;52:201–206.
[30] Guillon J, Léger JM, Sonnet P, Jarry C, Robba M. Synthesis of cone, partial-cone and 1,3-alternate 25,27-bis[1-(2-ethyl) hexyl]- and 25,27-bis[1-(2-*tert*-butoxy)ethyl]calix[4]arene-crown-6 conformers as potential selective cesium extractants. J Org Chem 2000;65:8283–8289.
[31] Guillon J, Sonnet P, Malval JP, Massip S, Gosse I, Léger JM, Lapouyade R, Rochette J, Monti JP, Jarry C. Synthesis and cesium binding affinity of new 25,27-bis(alkyloxy)calix[4] arene-crown-6 conformers in relation to the alkyl pendent moiety. Supramol Chem 2002;14:437–450.
[32] Guillon J, Léger JM, Dapremont C, Denis LA, Sonnet P, Massip S, Jarry C. First synthesis of 1,3-alternate 25,27-dialkyloxy-5,17-diarylcalix[4]arenes-crown-6 as new cesium selective extracants by Suzuki cross-coupling reaction. Supramol Chem 2004;16:319–329.
[33] Deschatrette J, Weiss MC. Characterization of differentiated clones from a rat hepatoma. Biochimie 1974;56:1603–1611.
[34] Gaboriau F, Delcros JG, Moulinoux JP. A simple assay for the measurement of plasma antioxidant status using spontaneous autoxidation of homovanillic acid. J Pharmacol Toxicol Methods 2002;47:33–43.
[35] Aoki I, Sakaki T, Shinkai S. A new metal sensory system based on intramolecular fluorescence quenching on the ionophoric calix[4]arene ring. J Chem Soc, Chem Commun 1992;:730–732.
[36] Rudkevich DM, Verboom W, Reinhoudt DN. Calix[4]arene salenes: A bifunctional receptor for NaH_2PO_4. J Org Chem 1994;59:3683–3686.
[37] Kubo Y, Tokit S, Kojima Y, Osano YT, Matsuzaki T. A new family of indoaniline-derived calix[4]arenes: synthesis and optical recognition properties as a chromogenic receptor. J Org Chem 1996;61:3758–3765.
[38] Arena G, Casnati A, Mirone L, Sciotto D, Ungaro R. A new water-soluble calix[4]arene ditopic receptor rigidified by microsolvation: Acid-base and inclusion properties. Tetrahedron Lett 1997;38:1999–2002.
[39] Collins EM, McKervey A, Harris SJ. Molecular receptors with calix[4]arene substructure. Synthesis of derivatives with mixed ligating functional groups. J Chem Soc, Perkin Trans 1 1989:372–374.
[40] Collins EM, McKervey A, Madigan E, Moran MB, Owens M, Ferguson G, Harris SJ. Chemically modified calix[4]arenes. Regioselective synthesis of 1,3-(distal) derivatives and related compounds. X-ray crystal structure of a diphenol-dinitrile. J Chem Soc, Perkin Trans 1 1991:3137–3142.
[41] Franceschini N, Da Nascimento S, Sonnet P, Guillaume D. Efficient enantioselective synthesis of 2-substituted thiomorpholin-3-ones. Tetrahedron: Asymmetry 2003;14:3401–3405.