Available online at www.sciencedirect.com
SCIENCE DIRECT
Bioorganic & Medicinal Chemistry 14 (2006) 1871–1879
Bioorganic & Medicinal Chemistry

Synthesis, cellular uptake and animal toxicity of a tetra(carboranylphenyl)-tetrabenzoporphyrin

Vijay Gottumukkala,[a] Owendi Ongayi,[a] David G. Baker,[b] Larry G. Lomax[b] and M. Graça H. Vicente[a,*]

[a]*Department of Chemistry, Louisiana State University, Baton Rouge, LA 70803, USA*
[b]*Department of Pathobiological Sciences, School of Veterinary Medicine, Baton Rouge, LA 70803, USA*

Received 30 September 2005; revised 20 October 2005; accepted 20 October 2005
Available online 17 November 2005

Abstract—A water-soluble *nido*-carboranyl-tetrabenzoporphyrin has been synthesized in 43% overall yield, by condensation of butanopyrrole with a carboranylbenzaldehyde, followed by metal insertion, oxidation, demetallation and deboronation reactions. This compound accumulated within human glioblastoma T98G cells to a significant higher extent than a structurally related *nido*-carboranylporphyrin, and localized preferentially in the cell lysosomes. Animal toxicity studies using male and female BALB/c mice revealed that both compounds are non-toxic even at a dose of 160 mg/kg, administered intraperitoneally as a single injection at a concentration of 4 mg/mL. It is concluded that the tetra(carboranylphenyl)-tetrabenzoporphyrin is a promising new sensitizer for the treatment of malignant tumors.

1. Introduction

Boron neutron capture therapy (BNCT) is a binary modality for cancer treatment that involves the irradiation of ^{10}B-rich tumors with low energy (thermal, ~0.025 eV) neutrons, resulting in the release of highly cytotoxic particles, $^{4}He^{2+}$ (α-particle) and $^{7}Li^{3+}$, with approximately 2.4 MeV of kinetic energy.[1,2] These energetic ions are capable of causing severe damage to organic materials and since they display limited paths of travel in tissue (5–9 μm, i.e., about one cell diameter), the toxic effect is highly localized to ^{10}B-containing tissue. Therefore BNCT has the potential for selectively targeting and destroying malignant cells in the presence of normal cells, provided a tumor-selective ^{10}B-delivery drug is available.[3,4] Such localized cancer therapies are particularly attractive for the treatment of high-grade gliomas and metastatic brain tumors, which are usually highly infiltrative of normal brain and where selective tumor destruction could dramatically increase patient life quality and expectancy. Among all BNCT agents proposed in the literature, porphyrins are particularly promising because of their selectivity for tumor cells, persistence within tumors, and easy synthesis with high boron content.[5,6] Furthermore, the photosensitizing properties of porphyrin-type macrocycles allows their application as dual sensitizers in the BNCT and the photodynamic therapy (PDT) treatment of tumors.[7–10] PDT involves the activation of a tumor-localized photosensitizer with red light, which generates singlet oxygen and other cytotoxic species that cause photo-oxidative damage to tumors.[11,12] The PDT treatment of malignant brain tumors using Photofrin®, an FDA-approved derivative of hematoporphyrin IX, has provided moderate palliation of recurrent brain tumors and an increase in patient survival time.[13,14] Boron-containing tetrabenzoporphyrins (TBPs) are potentially promising sensitizers for dual application in BNCT and PDT, because of their stronger absorptions in the red region of the optical spectrum, where light penetration is considerably deeper, compared with porphyrins.[15–18] Furthermore, due to the extension of their π-conjugated systems, TBPs display high chemical stability, red-shifted absorption bands, increased basicity and decreased oxidation potentials compared with the parent porphyrins.[19–21] In addition to their medical applications, TBPs and their metallo-derivatives have been extensively studied as near-IR dyes, O_2-sensors and as nonlinear optical materials.[22–25] Herein we report the total synthesis of a water-soluble *meso*-tetra(carboranylphenyl)-TBP, and compare its uptake by human glioblastoma T98G cells

Keywords: BNCT; PDT; *nido*-Carborane; Tetrabenzoporphyrin.
* Corresponding author. Fax: +1 225 578 3458; e-mail: vicente@lsu.edu

0968-0896/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2005.10.037

in culture and its toxicity in BALB/c mice with a structurally related *nido*-carboranylporphyrin. We have recently reported that these compounds were non-toxic in the dark toward V79 hamster lung fibroblasts, up to 300 μM concentrations.[18]

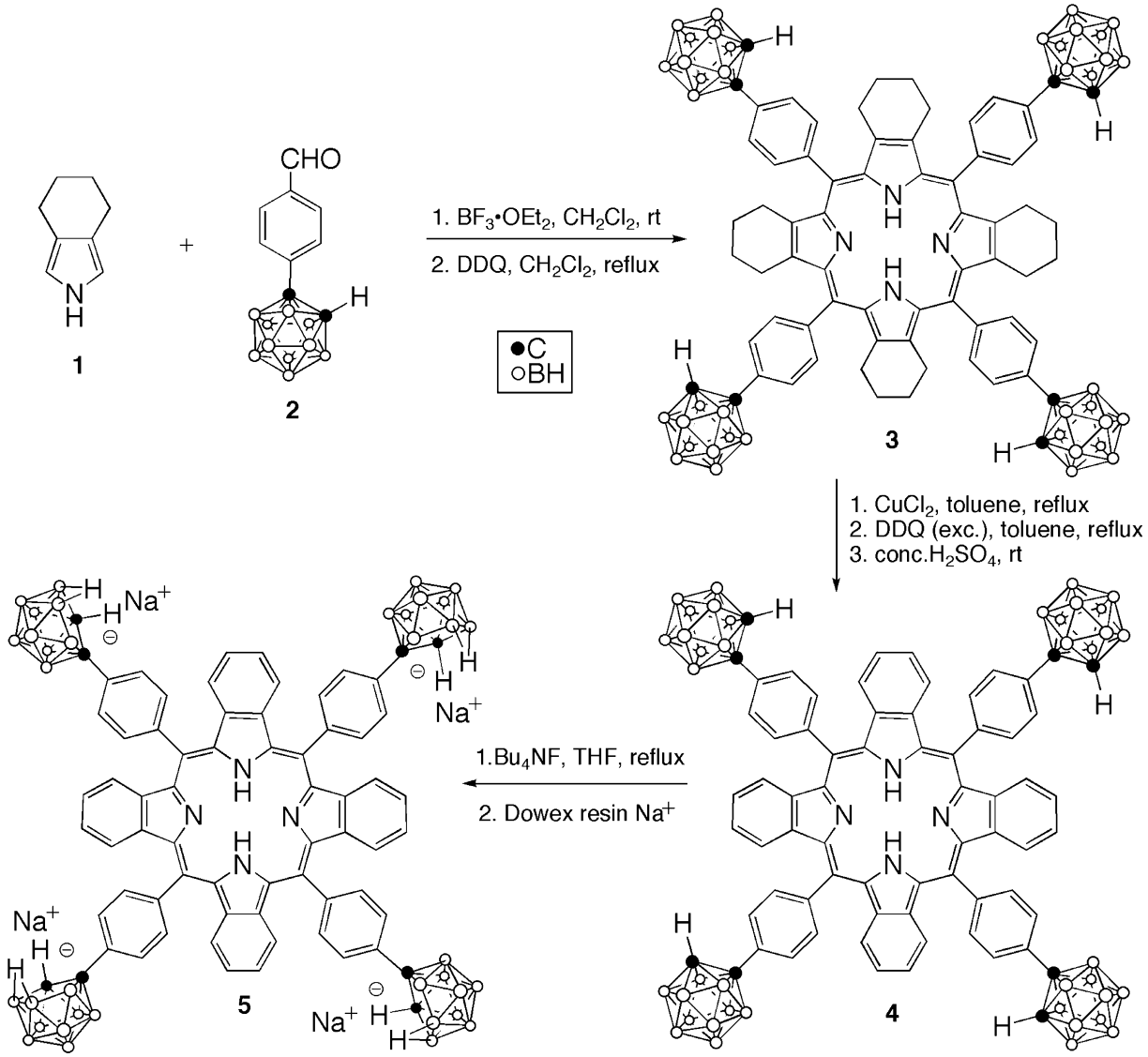
2. Results

2.1. Synthesis

The water-soluble *nido*-carboranyl-TBP **5** was synthesized in 43% overall yield from butanopyrrole **1**[26] and carboranylbenzaldehyde **2**,[9] as shown in Scheme 1. Porphyrin **3** was obtained in 60% yield under Lindsey condensation conditions and metallated with Cu(II) quantitatively, using copper(II) chloride in refluxing toluene.[18] Cu(II) acetate gave lower yield of the metalloporphyrin because of partial deboronation of the carborane cages to the corresponding *nido*-carborane derivatives. Oxidation of the porphyrin Cu(II) complex with excess DDQ followed by demetallation gave *closo*-carboranyl-TBP **4** in 75% overall yield. The water-soluble TBP **5** was obtained in 95% yield by deboronation of the *closo*-carborane cages of **4**, using *tert*-butylammonium fluoride in THF,[27] followed by cation exchange on a Dowex 50WX2-100 resin in the sodium form.[9] In the presence of pyridine/piperidine[9] the deboronation reaction was incomplete, even after 48 h at room temperature.

2.2. Cell culture studies

The concentration-dependent uptake of TBP **5** and carboranylporphyrin **6**[9] (Fig. 1) was investigated in human glioblastoma T98 G cells exposed to concentrations of these compounds up to 200 μM for a period of 3 h. The results obtained are shown in Figure 2. We observed a consistent increase in the uptake of **5** and **6** with increase concentration, and TBP **5** accumulated within cells to a much higher extent (~30% more) than porphyrin **6**, under the same experimental conditions. The uptake of compounds **5** and **6** by T98G cells is also



Scheme 1.

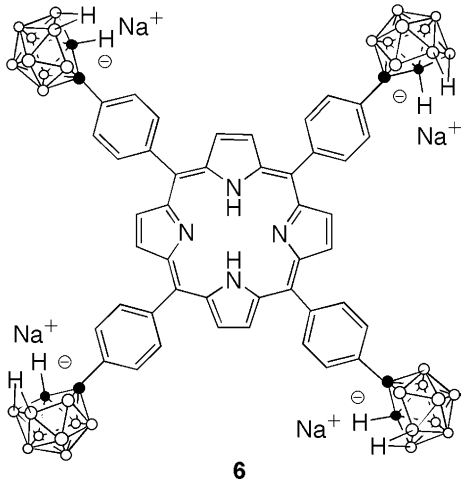


Figure 1. Structure of tetra(*nido*-carboranylphenyl)porphyrin **6**.

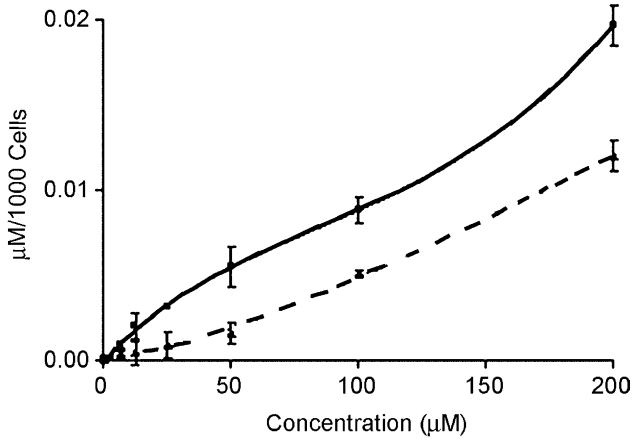


Figure 2. Concentration-dependent uptake of TBP **5** (full line) and porphyrin **6** (dashed line) by human glioma T98G cells, after a 3 h time period.

time-dependent, as shown in Figure 3. Both TBP **5** and porphyrin **6** at a concentration of 10 µM consistently accumulated within cells over time, and TBP **5** was taken up faster and to a much higher extent than porphyrin **6** at all time points studied.

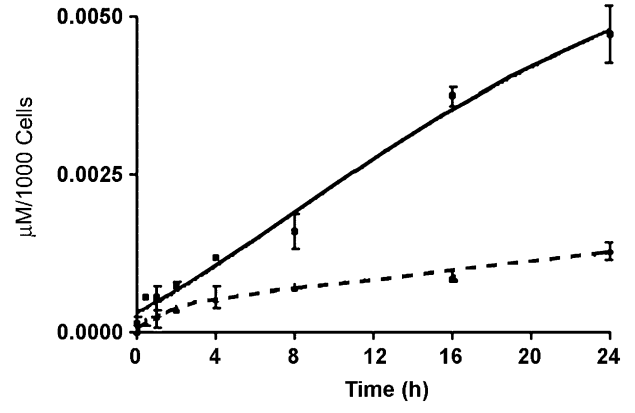


Figure 3. Time-dependent uptake of TBP **5** (full line) and porphyrin **6** (dashed line) at 10 µM by human glioma T98G cells.

The intracellular localization of TBP **5** was evaluated by fluorescence microscopy upon exposure of HEp2 cells to 50 µM of **5** for 24 h, as shown in Figure 4. The HEp2 cells were used rather than T98G for the imaging experiment since the former are smaller and spread nicely on the glass cover slips. The punctate fluorescence pattern observed for TBP **5** (Fig. 4B) is characteristic of localization in the cell lysosomes. Co-localization studies using the lysosome-specific probe Lysosensor confirmed the preferential localization of TBP **5** in the lysosomes (Fig. 4D).

2.3. Animal toxicity

As described in the experimental section, all mice received intraperitoneal (ip) injections of 0.16–0.72 mL. The maximum dosage administered (groups 9 and 10) was 160 mg/kg, and represented maximum saturation of 4 mg/mL. No serum chemistry changes occurred which were attributable to either compound or vehicle administration, as suggested by the clinical and histologic values shown in Tables 1–4. Tables 1 and 2 summarize the clinical data obtained for mice receiving TBP **5** while Tables 3 and 4 summarize the results obtained with porphyrin **6**.

3. Discussion

3.1. Synthesis

The synthesis of water-soluble *nido*-carboranyl-TBP **5**, the first reported boronated tetrabenzoporphyrin, was accomplished in six steps and 43% overall yield, from readily available starting materials (Scheme 1). Condensation of butanopyrrole **1**[26] with carboranylbenzaldehyde **2**[9] gave porphyrin **3**, which was metallated with Cu(II), oxidized to tetrabenzoporphyrin and demetallated to give *closo*-carboranyl-TBP **4**. Metallo-benzoporphyrins have been previously prepared from DDQ oxidation of dihydrobenzo-[28–30] and tetrahydrobenzoporphyrins,[31,32] making this an effective and mild method for the preparation of this type of compound. The direct oxidation of porphyrin **3** under the same conditions gave only traces of tetrabenzoporphyrin **4**; the main products obtained were ring-opened compounds, mainly partially aromatized benzoylbiliverdins.[33] Although TBPs typically display low solubilities due to their planar π-conjugation systems and pronounced tendency for π–π stacking, TBP **4** is soluble in organic solvents, such as dichloromethane and acetone, as a result of its non-planar structure due to the steric crowding about the macrocycle periphery.[18] Conversion of **4** to the water-soluble TBP **5** was accomplished using *tert*-butylammonium fluoride in THF,[27] followed by cation exchange. TBP **5** is highly soluble in polar organic solvents, such as acetone, methanol and DMSO, and sparingly soluble in water.

Porphyrins **3**, **4** and **5** were characterized by MS, UV–vis and ^{1}H NMR. Compounds **4** and **5** display absorption spectra characteristic of tetrabenzoporphyrins, with red-shifted bands compared with porphyrin **3**. The ^{1}H NMR spectra of TBPs **4** and **5** show two distinctive

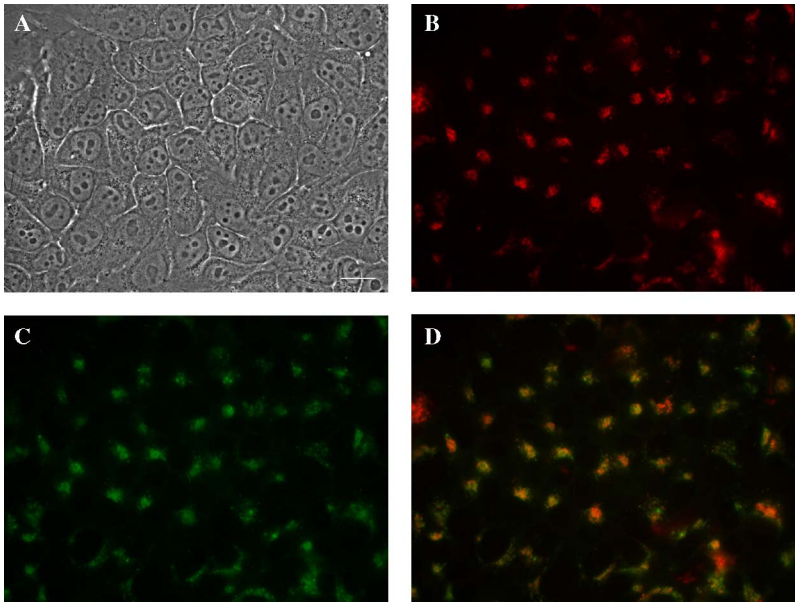


Figure 4. Intracellular localization of TBP **5** in human HEp2 cells. (A) Phase contrast; (B) TBP **5** fluorescence; (C) LysoSensor Green fluorescence; (D) overlay of lysosensor and TBP5 fluorescence. Scale bar: 20 μm.

Table 1. Serum chemistry values for six BALB/c mice administered TBP **5** (groups 1, 3, and 5) or 4% Cremophore (vehicle: group 11), by ip injection

Group	1	3	5	11
Glucose (mg/dl)	153.5[a] (0.5)	219.5[c] (5.5)	229.5[c] (6.5)	188.0[b] (10.0)
AST (U/L)	177.5 (121.5)	455.5 (406.5)	454.5 (411.5)	83.0 (33.0)
ALT (U/L)	151.5 (131.5)	96.5 (73.5)	120.0 (101.0)	31.0 (6.0)
AP (U/L)	189.5[a,c] (0.5)	201.0[b,c] (31.0)	138.0[a] (8.0)	128.5[a] (1.5)
Bilirubin (mg/dL)	0.15 (0.05)	0.20 (0.10)	0.20 (0)	0.15 (0.05)
Total prot. (g/dL)	4.65 (0.15)	4.7 (0)	5.1 (0)	4.5 (0.20)
Albumin (g/dL)	2.85 (0.05)	2.8 (0)	2.75 (0.05)	2.75 (0.15)
Globulin (g/dL)	1.8[a] (0.1)	1.9[a] (0)	2.35[b] (0.05)	2.3[b] (0.1)
BUN (mg/dL)	28.0 (0)	27.0 (3.0)	25.5 (2.5)	29.0 (1.0)

The values represent mean (SEM) serum chemistry levels. For individual analytes measured, rows with superscripts in common are not different from one another ($p > 0.05$).

Table 2. Serum chemistry values for four BALB/c mice administered TBP **5** (groups 7, 9) or 6% Cremophore (vehicle: group 12), by ip injection

Group	7	9	12
Glucose (mg/dL)	224.5 (12.5)	172.0 (19.0)	206.5 (12.5)
AST (U/L)	73.0 (16.0)	498.0 (410.0)	774.0 (149.0)
ALT (U/L)	29.0 (3.0)	206.0 (176.0)	411.5 (123.5)
AP (U/L)	161.0[a] (3.0)	223.5[b] (0.5)	169.0[c] (0)
Bilirubin (mg/dL)	0.2 (0)	0.25 (0.05)	0.2 (0)
Total prot. (g/dL)	5.1[a] (0.10)	4.35[b] (0.05)	4.65[b] (0.05)
Albumin (g/dL)	2.75[a] (0.05)	2.4[b] (0)	2.6[c] (0)
Globulin (g/dL)	2.35[a] (0.05)	1.95[b] (0.05)	2.05[b] (0.05)
BUN (mg/dL)	15.5[a, b] (1.5)	22.5[b,c] (2.5)	33.0[c] (3.0)

The values represent mean (SEM) serum chemistry levels. For individual analytes measured, rows with superscripts in common are not different from one another ($p > 0.05$).

doublets for the *ortho*- and *meta*-phenyl protons around 8 ppm, broad signals for the α and β benzo protons around 7 ppm, a singlet for the CH-carborane protons, and broad signals for the BH and NH protons. In addition, the four protons on the open-face of the carborane cages of TBP **5** appear as a broad singlet centered at −2 ppm. The MS obtained for porphyrins **3**, **4**, and **5** are also in agreement with the proposed structures.

3.2. Cell culture

The concentration- and time-dependent uptake of TBP **5** and the structurally related carboranylporphyrin **6**[9] by human glioblastoma T98 G cells was investigated. We previously showed that **5** and **6** are non-toxic in the dark up to 300 μM concentrations.[18] As seen in Figure 2, the uptake of both **5** and **6** consistently

Table 3. Serum chemistry values for six BALB/c mice administered porphyrin **6** (groups 2, 4, 6) or 4% Cremophore (vehicle: group 11), by ip injection

Group	2	4	6	11
Glucose (mg/dL)	159.5[a] (1.5)	188.0[b] (3.0)	153.5[a] (9.5)	188.0[b] (10.0)
AST (U/L)	146.0 (36.0)	202.0 (144.0)	258.0 (181.0)	83.0 (33.0)
ALT (U/L)	98.5 (25.5)	94.0 (71.0)	340.0 (296.0)	31.0 (6.0)
AP (U/L)	167.5[a] (11.5)	132.0[a,c] (4.0)	148.0[a,c] (12.0)	128.5[b,c] (1.5)
Bilirubin (mg/dL)	0.2 (0.10)	0.25 (0.05)	0.25 (0.05)	0.15 (0.05)
Total Prot. (g/dL)	4.3[a] (0.10)	4.6[a,b] (0)	5.0[b] (0.10)	4.5[b] (0.20)
Albumin (g/dL)	2.7 (0)	2.6 (0)	2.8 (0)	2.75 (0.15)
Globulin (g/dL)	1.6[a] (0.1)	2.0[b] (0)	2.35[c] (0.05)	2.3[c] (0.1)
BUN (mg/dL)	26.5 (2.5)	24.0 (2.0)	27.5 (1.5)	29.0 (1.0)

The values represent mean (SEM) serum chemistry levels. For individual analytes measured, rows with superscripts in common are not different from one another ($p > 0.05$).

Table 4. Serum chemistry values for four BALB/c mice administered porphyrin **6** (groups 8, 10) or 6% Cremophore (vehicle: group 12), by ip injection

Group	8	10	12
Glucose (mg/dL)	239.5 (8.5)	214.5 (29.5)	206.5 (12.5)
AST (U/L)	774.0 (690.0)	981.0 (837.0)	774.0 (149.0)
ALT (U/L)	968.0 (918.0)	1096.0 (1041.0)	411.5 (123.5)
AP (U/L)	160.5[a] (7.5)	223.0[b] (7.0)	169.0[a] (0)
Bilirubin (mg/dL)	0.25 (0.05)	0.25 (0.05)	0.2 (0)
Total prot. (g/dL)	4.9 (0.20)	4.5 (0.20)	4.65 (0.05)
Albumin (g/dL)	2.5 (0.1)	2.5 (0.1)	2.6 (0)
Globulin (g/dL)	2.4[a] (0.1)	2.0[b] (0.1)	2.05[a,b] (0.05)
BUN (mg/dL)	16.0[a] (3.0)	25.5[a,b] (2.5)	33.0[b] (3.0)

The values represent mean (SEM) serum chemistry levels. For individual analytes measured, rows with superscripts in common are not different from one another ($p > 0.05$).

increased with the concentration in an almost linear fashion, and TBP **5** accumulated within cells to a significant higher extent than porphyrin **6**. This may be a result of the higher hydrophobicity of TBP **5** compared with **6**, due to the presence of the four β,β′-fused benzene rings. Our results are in agreement with previous studies showing that the cellular uptake of *nido*-carboranylporphyrins increases with increasing hydrophobic character.[34,35] The cellular uptake of compounds **5** and **6** is also time-dependent (Fig. 3), as it is characteristic of porphyrin compounds. Both TBP **5** and porphyrin **6** consistently accumulated within cells over time, and TBP **5** was taken up faster and to a much higher extent than porphyrin **6** at all time points studied. In fact after 24 h, we found approximately three times more **5** than **6** accumulated within T98G cells. These results are in agreement with reported studies using *nido*-carboranylporphyrins,[34–36] and are probably a consequence of the higher hydrophobicity of TBP **5** compared with porphyrin **6** and consequently a more efficient cellular uptake of the former compound.

The intracellular localization of TBP **5** was evaluated by fluorescence microscopy, as can be seen in Figure 4. The punctate fluorescence pattern observed for TBP **5** (Fig. 4B) is very similar to that observed for Lysosensor (Fig. 4C), and characteristic of localization in the cell lysosomes. The overlay between the TBP **5** and Lysosensor fluorescences (Fig. 4D) further confirms that the two compounds co-localize in the lysosomes. These results are in agreement with previous reports showing that negatively charged carboranylporphyrins preferentially localize subcellularly within the cell lysosomes.[34,36–38]

3.3. Animal toxicity

No signs of toxicity were observed in any of the mice in this experiment. With TBP **5** (Tables 1 and 2) the glucose levels were increased, likely due to excitement and increased corticosteroid release. The serum AST levels were all similar and although generally higher than normal for some mice, they were not considered elevated due to either **5** or vehicle administration, because the increases were inconsistent and the values found for mice in group 7 (receiving 120 mg/kg and 6% Cremophore) had the lowest of the group means. This result suggests that muscle injury or exertion may have contributed to increased AST levels in several of the mice. The serum ALT levels were also similar and although higher than normal in some mice, they were not considered elevated due to either **5** or vehicle administration, because the elevations were not consistent among mice in the same treatment groups. Furthermore, serum ALT is not specific for hepatocellular injury in mice. The serum AP levels differed by group, but were all within the normal range, as were the serum bilirubin levels. The serum TP levels differed by group, but were generally within the normal range. The levels for mice in group 9 were low, likely due to decreased albumin and globulin levels. The serum albumin levels for mice in group 9 were lower than for other mice; this may be due to decreased synthesis resulting from hepatocellular injury, or increased loss through renal or intestinal disease. However, tissue injury was not evident histopathologically. Likewise, the serum globulin levels were lower than expected, or lower than for control mice in groups 1, 3, and 9. These findings likely represented a stress response, rather than compound or vehicle effects. The serum BUN levels differed among groups, but were also within normal limits. For porphyrin **6** (Tables 3 and 4) the glucose levels were increased, likely due to excitement and increased corticosteroid release. The serum AST levels were all similar and although slightly higher than normal, they were not considered elevated due to either **6** or vehicle administration; muscle injury or exertion might have contributed to increased AST levels in several mice. Likewise, the serum ALT levels were all similar and although in some mice higher than normal, these

elevations were not consistent among mice in the same treatment groups and therefore were not attributed to either **6** or vehicle administration. The serum AP and bilirubin levels were within the normal range. The serum TP levels were also within the normal range, and only differed from controls for group 2, which received the lowest dose (20 mg/kg) of **6**. The serum albumin levels were all similar and within normal limits. Likewise, serum globulin levels were only lower than vehicle controls for mice in group 2, which received the lowest porphyrin dose. These results were likely due to stress-related increases in serum corticosteroid levels. The serum BUN levels differed among groups but were also within normal limits. The histopathologic examination revealed no lesions attributable to compound administration. Extramedullary hematopoiesis in the spleen was observed in all mice, and may represent mild levels of stress, typical of mice under manipulative experimental conditions.

The non-toxicity of TBP **5** and porphyrin **6** up to a dose of 160 mg/kg is in agreement with the low toxicity reported for hydrophobic carboranylporphyrins.[39,40] However, compounds **5** and **6** are significantly less toxic than other negatively charged carboranyl-containing porphyrins, such as BOPP, MnBOPP, BTPP, NiNTCP-H, ZnDPE and NiDPE.[40–43] Our results show, as previously observed, that *nido*-carboranylporphyrins can have low mice toxicities, even at high compound doses often required to achieve therapeutic levels of boron in tumors.[40,44,45]

4. Conclusions

The total synthesis of a water-soluble TBP (**5**), with potential application in the BNCT and PDT treatment of tumors, was accomplished in 43% overall yield. In comparison with a structurally related carboranylporphyrin (**6**), TBP **5** showed a significantly higher uptake in human glioblastoma T98G cells in vitro, probably as a result of its higher hydrophobic character due to its four β,β′-porphyrin fused benzene rings. The cellular uptake of TBP **5** and porphyrin **6** were concentration- and time-dependent, systematically increasing with both the concentration and time of exposure to T98G cells. The preferential sites of subcellular localization of TBP **5** are the lysosomes, as has been previously observed for negatively charged carboranylporphyrins. Both TBP **5** and porphyrin **6** were non-toxic to BALB/c mice, even at the high dose of 160 mg/kg administered in a single ip injection. No clinical, biochemical, or histopathological effects were observed which could be attributed to the administration of TBP **5**, porphyrin **6** or the vehicle used. Our results suggest that TBP **5** is a promising new sensitizer for the treatment of malignant tumors by BNCT and/or PDT.

5. Experimental

5.1. Chemistry

Commercially available starting materials were purchased from Sigma–Aldrich and used without further purification. All solvents were purchased from Fisher Scientific (HPLC grade) and either directly used or dried and distilled according to literature procedures. Silica gel 60 (70–230 mesh, Merck) and alumina grade III (70–230 mesh ASTM) were used for column chromatography. Analytical thin-layer chromatography (TLC) was performed on Merck 60 F254 silica gel (precoated sheets, 0.2 mm thick). ^{1}H NMR spectra were obtained using a Bruker DPX 250 MHz or 300 MHz spectrometer; chemical shifts are expressed in ppm relative to TMS (0 ppm). Electronic absorption spectra were measured on a Perkin Elmer Lambda 35 UV–vis spectrophotometer and fluorescence spectra on a Perkin Elmer LS55 instrument. Low resolution MS analyses were conducted at the LSU Mass Spectrometry Facility on a Bruker Prolix III MALDI-TOF mass spectrometer, and the HRMS were conducted at the Ohio State University Mass Spectrometry and Proteomics Facility. Melting points were measured on an Electrothermal MEL-TEMP instrument. HPLC purity analysis was performed on a LCMS-2010 Shimadzu system equipped with an Acclaim 300 C_{18} 300 Å column, 3 μm, 4.6 × 150 mm (Dionex, USA) and Shimadzu SPD-ICA UV–vis detector, using a stepwise gradient from 60% water/40% acetonitrile to 30% water/70% acetonitrile.

5.1.1. 5,10,15,20-Tetra[4-(*o*-carboranyl)phenyl]-2:3,7:8,12:13,17:18-butanoporphyrin (3). 3:4-Butanopyrrole **1** (0.13 g, 1.06 mmol) and of 4-(*o*-carboranyl)benzaldehyde **2** (0.26 g, 1.06 mmol) were dissolved in dried, freshly distilled dichloromethane and the solution was stirred at room temperature under argon for 15 min. The reaction flask was shielded from ambient light and $BF_3 \cdot OEt_2$ (0.02 mL, 0.106 mmol) was added. This solution was stirred for 1 h at room temperature before DDQ (0.36 g, 1.59 mmol) was added and the final mixture refluxed under argon for 1 h to give a dark green solution. After cooling to room temperature, the solvent was evaporated under vacuum and the resulting residue was purified by alumina grade III column chromatography using dichloromethane for elution. Recrystallization from methanol gave purple crystals of the title porphyrin (0.20 g, 60% yield). mp >300 °C; ^{1}H NMR ($CDCl_3$, drop of d-TFA, 300 MHz): δ 8.36 (d, 8H, J = 8.27 Hz, *o*-PhH), 8.03 (d, 8H, J = 8.27 Hz, *m*-PhH), 4.33 (s, 4H, carborane-CH), 3.5–1.3 (br, 40H, BH), 2.55–2.40 (m, 8H, CH_2), 2.00-1.85 (m, 8H, CH_2), 1.80-1.65 (m, 8H, CH_2), 1.20-1.05 (m, 8H, CH_2), −0.47 (s, 4H, NH). UV–vis (CH_2Cl_2): λ_{max} (log ε) 466 (4.52), 617 (3.36), 674 (3.60) nm. HRMS (MALDI) *m*/*z* 1401.1681 ($M+H^+$), calculated for $C_{68}H_{94}N_4B_{40}$ 1401.1682.

5.1.2. 5,10,15,20-Tetra[4-(*o*-carboranyl)phenyl]-tetrabenzoporphyrin (4). To a solution of porphyrin **3** (150 mg, 0.11 mmol) in toluene (20 mL) was added an excess of copper(II) chloride (143 mg, 1.06 mmol). The mixture was refluxed until the reaction was complete, for about 2 h, as evidenced by TLC and UV–vis spectrophotometry. The solvent was evaporated under vacuum, the residue dissolved in dichloromethane (150 mL) and washed once with aqueous saturated $NaHCO_3$ and once with water before being dried over anhydrous Na_2SO_4. After removal of the solvent under vacuum the remaining residue was purified by alumina grade III column chromatography using dichloromethane for elution. The Cu(II)-porphyrin was recovered in quantitative yield upon precipitation using dichloromethane/ethanol. To a solution of this Cu(II)-porphyrin (150 mg,

0.102 mmol) in toluene (20 mL) was added excess DDQ (185 mg, 0.82 mmol) and the final mixture was refluxed for 15 min. During reflux the color of the solution changed from red to deep green. The mixture was then allowed to cool down to room temperature, diluted with chloroform (150 mL) and washed once with aqueous saturated $NaHCO_3$ and once with water. The solvent was removed under vacuum, and the remaining residue was purified by alumina grade III column chromatography using chloroform for elution. Recrystallization from methanol afforded Cu(II)-tetrabenzoporphyrin (110 mg, 75% yield) as dark green crystals. The Cu(II)-tetrabenzoporphyrin (110 mg, 0.076 mmol) was dissolved in concentrated sulfuric acid and stirred at room temperature for 5 min. The solution was poured into water/ice and extracted with chloroform (4×150 mL). The organic layers were collected, dried over anhydrous Na_2SO_4 and the solvent evaporated under vacuum. The resulting residue was recrystallized from methanol to give green crystals of the title compound (105 mg) in quantitative yield from Cu(II)-tetrabenzoporphyrin. mp >300 °C; ^{1}H NMR (acetone-d_6, 250 MHz): δ 8.34 (d, $J = 8.35$ Hz, 8H, *o*-PhH), 8.17 (d, $J = 8.35$ Hz, 8H, *m*-PhH), 7.49–6.93 (br m, 16H, benzoH), 5.55 (s, 4H, carborane-CH), 3.5–1.4 (br, 40H, BH), −1.04 (br s, 2H, NH). UV–vis (CH_2Cl_2): λ_{max} (log ε) 467 (10.10), 553 (3.80), 593 (4.00), 643 (4.37), 698 (3.90) nm. HRMS (MALDI) *m*/*z* 1385.0347 ($M+H^+$), calculated for $C_{68}H_{78}N_4B_{40}$ 1385.0326.

5.1.3. 5,10,15,20-Tetra[4-(*nido*-carboranyl)phenyl]-tetrabenzoporphyrin tetrasodium salt (5). Tetrabenzoporphyrin **4** (25.0 mg, 0.018 mmol) was added to a 1 M solution of $Bu_4NF \cdot 3H_2O$ in THF (1.08 mL, 1.08 mmol) and the solution was refluxed for 6 h. After cooling to room temperature, the solvent was removed under vacuum, the residue dissolved in dichloromethane and washed once with water before being dried over anhydrous Na_2SO_4. The solvent was evaporated under vacuum, the residue redissolved in methanol and purified on a Sephadex LH-20 column, using methanol for elution. The resulting residue was dissolved in 40% aqueous acetone and passed slowly through a Dowex 50WX2-100 resin in the sodium form. The porphyrin fraction was collected, dried under vacuum, redissolved in 60% aqueous acetone and passed twice through the ion-exchange resin. Removal of the solvent under vacuum gave the title tetrabenzoporphyrin (25.8 mg) in quantitative yield as a green powder. mp >300 °C; ^{1}H NMR (acetone-d_6, 300 MHz): δ 8.04 (d, $J = 8.22$ Hz, 8H, *o*-PhH), 7.82 (d, $J = 8.22$ Hz, 8H, *m*-PhH), 7.48–7.28 (br m, 16H, benzoH), 2.69 (s, 4H, carborane-CH), 2.9–1.3 (br, 36H, BH), −1.13 (s, 2H, NH), −1.80 to −2.25 (br s, 4H, BH). UV–vis (CH_2Cl_2): λ_{max} (log ε) 464 (5.2), 542 (3.73), 585 (3.97), 637 (4.27), 694 (3.60) nm. MS (MALDI-TOF) *m*/*z* 1433.619 ($M+H^+$). HPLC (water/acetonitrile): $t_R = 42.15$ min.

5.2. Cell culture

Human glioblastoma T98G cells and cervical carcinoma HEp2 cells were obtained from ATCC. Both cells were maintained in 50% of α-MEM/advanced MEM supplemented with 5% fetal bovine serum (FBS). Phosphate-buffered saline (PBS), FBS and trypsin were purchased from Gibco, Cyquant reagent and Lysosensor from Molecular Probes, and Triton X-100 from Calbiochem. Microscopy was performed on a Zeiss Axiovert 200 M inverted fluorescent microscope fitted with a standard Texas Red and FITC filter sets (Chroma Technology Corp.). The images were aquired with a Zeiss Axiocam MRM CCD camera fitted to the microscope and pseudo-colored with Adobe Photoshop® CS version 8.0. Compounds **5** and **6** were dissolved in DMSO (Sigma–Aldrich) prior to being diluted into cell medium; the final DMSO concentration never exceeded 1%. All medium solutions were filter sterilized (22 μm pore size) prior to use. All data obtained in the FLUOstar plate reader was analyzed using Prism 3.0 graphing software.

5.2.1. Concentration-dependent cellular uptake. Human T98G cells were sub-cultured on 96-well plates at the concentration of 10,000 cells/100 μL α-MEM/advance medium per well, and incubated for 48 h. A 200 μM stock solution of TBP **5** in DMSO was diluted with α-MEM/advance medium and added to the cells to achieve TBP final concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125 and 0 μM. After incubation for 3 h, the medium was removed, the cells washed with PBS, and 100 μL of 0.25% Triton X-100 in PBS added to each well. A TBP standard graph was obtained by diluting a 10 μM stock solution in 0.25% Triton X-100 in PBS to achieve 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0 μM TBP concentrations. The TBP fluorescence was measured on a FLUOstar plate reader using 570 nm excitation and 720 nm emission filters. The standard curve for different cell numbers was obtained by placing 100,000, 80,000, 60,000, 40,000, 20,000, 10,000 and 0 cells in the wells followed by incubation for 3 h. The known cell numbers for the standard graph and the unkown cell numbers from the experiment were determined by first adding 100 μL/well of 5 μM stock solution of Cyquant reagent in PBS and then reading the plate on the FLUOstar plate reader using 480 nm excitation and 520 nm emission filters. The same procedure was followed for determination of the concentration-dependent cellular uptake of porphyrin **6**, using 570 nm excitation and 650 nm emission filters.

5.2.2. Time-dependent cellular uptake. Human T98G cells were sub-cultured and incubated for 48 h as described above. A filter sterilized 10 μM stock solution of TBP **5** in 1% DMSO/medium was added and the cells incubated for 24, 16, 8, 4, 2, 1, 0.5, and 0 h. The medium was removed, the cells washed with PBS and 100 μL of 0.25% Triton X-100 in PBS was added to each well. The known and the unknown concentrations of TBP **5** were determined using the same methodology as described above for the concentration-dependent uptake. The same procedure was also followed for the time-dependent uptake of porphyrin **6**.

5.2.3. Intracellular localization. Human HEp2 cells were sub-cultured on Lab-Tek II chamber cover slips with α-MEM/advance medium for 48 h. TBP **5** was added to each chamber to reach a final concentration of 50 μM. The cells were incubated for 24 h, washed twice with 50 mM Hepes to remove unbound TBP, new medium containing 50 mM Hepes, pH 7.4 was added and the cells

examined immediately by fluorescence microscopy. For the co-localization experiments 100 nM of Lysosensor was added to the TBP-containing cells 30 min before the completion of the incubation time.

5.3. Animal toxicity

The mice were obtained from the breeding colony operated by the Division of Laboratory Animal Medicine, School of Veterinary Medicine, Louisiana State University. The animal studies were conducted under an animal use protocol approved by the LSU Institutional Animal Care and Use Committee, fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International. Twelve groups of two BALB/c mice, 4–6 weeks of age and weighing 12–24 g (mean = 19 g), were used; among these 8 were female and 16 were male. Mice in groups 1, 3, 5, 7, and 9 were administered TBP **5** once via ip injection, at increasing dosages: group 1 (20 mg/kg of a 2 mg/mL solution), group 3 (40 mg/kg of a 2 mg/mL solution), group 5 (80 mg/kg of a 4 mg/mL solution), group 7 (120 mg/kg of a 4 mg/mL solution), and group 9 (160 mg/kg of a 4 mg/mL solution). Mice in groups 2, 4, 6, 8, and 10 were administered porphyrin **6** once via ip injection at increasing dosages: group 2 (20 mg/kg of a 2 mg/mL solution), group 4 (40 mg/kg of a 2 mg/mL solution), group 6 (80 mg/kg of a 4 mg/mL solution), group 8 (120 mg/kg of a 4 mg/mL solution), and group 10 (160 mg/kg of a 4 mg/mL solution). Two groups of mice served as vehicle controls. Mice in group 11 received sterile 4% Cremophor EL (Fluka) in PBS and served as controls for mice receiving 20, 40, and 80 mg/kg compound while mice in group 12 received 6% Cremophor EL and served as controls for mice receiving 120 and 160 mg/kg compound.

For each compound, groups were dosed sequentially and each group evaluated daily for signs of toxicity, including hunched posture, rough hair coat, and decreased responsiveness. Mice were anesthetized with CO_2 48 h after compound administration, and blood collected by cardiocentesis for clinical chemistry evaluation. The serum chemistry performed included glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), bilirubin, total protein (TP), albumin, globulin, and blood urea nitrogen (BUN). The mice were exsanguinated and a necropsy performed. Tissues, including lung, kidney, thymus, heart, harderian gland, spleen, stomach, small intestine, and colon were fixed in 10% neutral buffered formalin. The fixed tissues were processed and examined by a board-certified pathologist.

5.4. Statistical analyses

The clinical chemistry values were compared using the Number Cruncher Statistical System software (NCSS, Kaysville, UT). Variables of interest were statistically evaluated for group effect, using one-way ANOVA. When the overall F statistic was significant ($p < 0.05$), the Fisher's least significant differences test was performed to compare the groups. Significant differences existed when $p > 0.05$.

Acknowledgment

The work described was funded by the National Institutes of Health, Grant No. CA098902.

References and notes

1. Barth, R. F.; Soloway, A. H.; Fairchild, R. G.; Brugger, R. M. *Cancer* **1992**, *70*, 2995–3007.
2. Soloway, A. H.; Tjarks, W.; Barnum, B. A.; Rong, F. G.; Barth, R. F.; Codogni, I. M.; Wilson, J. G. *Chem. Rev.* **1998**, *98*, 1515–1562.
3. Hawthorne, M. F. *Angew. Chem. Int. Ed. Engl.* **1993**, *32*, 950–984.
4. Barth, R. F.; Coderre, J. A.; Vicente, M. G. H.; Blue, T. E. *Clin. Cancer Res.* **2005**, *11*, 3987–4002.
5. Vicente, M. G. H. *Curr. Med. Chem. Anti-Cancer Agents* **2001**, *1*, 175–194.
6. Bregadze, V. I.; Sivaev, I. B.; Gabel, D.; Wöhrle, D. *J. Porphyrins Phthalocyanines* **2001**, *5*, 767–781.
7. Rosenthal, M. A.; Kavar, B.; Hill, J. S.; Morgan, D. J.; Nation, R. L.; Stylli, S. S.; Basser, R. L.; Uren, S.; Geldard, H.; Green, M. D.; Kahl, S. B.; Kaye, A. H. *J. Clin. Oncol.* **2001**, *19*, 519–524.
8. (a) Fabris, C.; Jori, G.; Giuntini, F.; Roncucci, G. *J. Photochem. Photobiol. B* **2001**, *64*, 1–7; (b) Giuntini, F.; Raoul, Y.; Dei, D.; Municchi, M.; Chiti, G.; Fabris, C.; Colautti, P.; Jori, G.; Roncucci, G. *Tetrahedron Lett.* **2005**, *46*, 2979–2982.
9. Vicente, M. G. H.; Nurco, D. J.; Shetty, S. J.; Osterloh, J.; Ventre, E.; Hegde, V.; Deutsch, W. A. *J. Photochem. Photobiol. B* **2002**, *68*, 123–132.
10. Vicente, M. G. H.; Gottumukkala, V.; Wickramasinghe, A.; Anikovsky, M.; Rodgers, M. A. *J. Proc. SPIE* **2004**, *5315*, 33–40.
11. Dougherty, T. J.; Gomer, C. J.; Henderson, B. W.; Jori, G.; Kessel, D.; Korbelik, M.; Moan, J.; Peng, Q. *J. Natl. Cancer Inst.* **1998**, *90*, 889–905.
12. Pandey, R. K.; Zheng, G.. In *The Porphyrin Handbook*; Kadish, K. M., Smith, K. M., Guilard, R., Eds.; Academic Press: Boston, 2000; Vol. 6, pp 157–230.
13. Origitano, T. C.; Caron, M. J.; Reichman, O. H. *Mol. Chem. Neuropathol.* **1994**, *21*, 337–352.
14. Kostron, H.; Obwegeser, A.; Jakober, R. *J. Photochem. Photobiol. B* **1996**, *36*, 157–168.
15. Lavi, A.; Johnson, F. M.; Ehrenberg, B. *Chem. Phys. Lett.* **1994**, *231*, 144–150.
16. Wolford, S. T.; Novicki, D. L.; Kelly, B. *Fundam. Appl. Toxicol.* **1995**, *24*, 52–56.
17. (a) Vallés, M. A.; Gómez, A. M. *Proc. SPIE* **1995**, *2325*, 24–28; (b) Vallés, M. A.; Biolo, R.; Bonnett, R.; Cañete, M.; Gómez, A. M.; Jori, G.; Juarranz, A.; McManus, K. A.; Okolo, K. T.; Soncin, M.; Villanueva, A. *Proc. SPIE* **1996**, *2625*, 11–22.
18. Ongayi, O.; Gottumukkala, V.; Fronczek, F. R.; Vicente, M. G. H. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 1665–1668.
19. Lash, T. D.. In *The Porphyrin Handbook*; Kadish, K. M., Smith, K. M., Guilard, R., Eds.; Academic Press: New York, 2000; Vol. 2, pp 125–199.
20. Rogers, J. E.; Nguyen, K. A.; Hufnagle, D. C.; McLean, D. G.; Su, W.; Gossett, K. M.; Burke, A. R.; Vinogradov, S. A.; Pachter, R.; Fleitz, P. A. *J. Phys. Chem. A* **2003**, *107*, 11331–11339.
21. Vicente, M. G. H.; Smith, K. M. *J. Porphyrins Phthalocyanines* **2004**, *8*, 26–42.
22. Ono, N.; Ito, S.; Wu, C. H.; Chen, C. H.; Wen, T. C. *Chem. Phys.* **2000**, *262*, 467–473.

23. Srinivas, N. K. M. N.; Rao, S. V.; Rao, D. V. G. L. N.; Kimball, B. K.; Nakashima, M.; Decristofano, B. S.; Rao, D. N. *J. Porphyrins Phthalocyanines* **2001**, *5*, 549–554.
24. (a) Vinogradov, S. A.; Wilson, D. F. *J. Chem. Soc. Perkin Trans. 2* **1995**, 103–111; (b) Brunel, M.; Chaput, F.; Vinogradov, S. A.; Campagne, B.; Canva, M.; Boilot, J. P. *Chem. Phys.* **1997**, *218*, 301–307.
25. Drobizhev, M.; Karotki, A.; Kruk, M.; Mamardashvili, N. Z.; Rebane, A. *Chem. Phys. Lett.* **2002**, *361*, 504–512.
26. Barton, D. H. R.; Kervagoret, J.; Zard, S. Z. *Tetrahedron* **1990**, *46*, 7587–7598.
27. Fox, M. A.; Gill, W. R.; Herbertson, P. L.; MacBride, J. A. H.; Wade, K.; Colquhoun, H. M. *Polyhedron* **1996**, *15*, 565–571.
28. Clezy, P. S.; Fookes, C. J. R.; Mirza, A. H. *Aust. J. Chem.* **1977**, *30*, 1337–1347.
29. Vicente, M. G. H.; Tome, A. C.; Walter, A.; Cavaleiro, J. A. S. *Tetrahedron Lett.* **1997**, *38*, 3639–3642.
30. Boudif, A.; Gimenez, S.; Loock, B.; Momenteau, M. *Can. J. Chem.* **1998**, *76*, 1215–1219.
31. Nguyen, L. T.; Senge, M. O.; Smith, K. M. *J. Org. Chem.* **1996**, *61*, 998–1003.
32. Finikova, O. S.; Cheprakov, A. V.; Beletskaya, I. P.; Carroll, P. J.; Vinogradov, S. A. *J. Org. Chem.* **2004**, *69*, 522–535.
33. Ongayi, O.; Fronczek, F. R.; Vicente, M. G. H. *Chem. Commun.* **2003**, 2298–2299.
34. Woodburn, K.; Phadke, A. S.; Morgan, A. R. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 1022–2017.
35. Vicente, M. G. H.; Edwards, B. F.; Shetty, S. J.; Hou, Y.; Boggan, J. E. *Bioorg. Med. Chem.* **2002**, *10*, 481–492.
36. Gottumukkala, V.; Luguya, R.; Fronczek, F. R.; Vicente, M. G. H. *Bioorg. Med. Chem.* **2005**, *13*, 1633–1640.
37. (a) Nguyen, T.; Brownell, G. L.; Holden, S. A.; Kahl, S.; Miura, M.; Teicher, B. A. *Radiat. Res.* **1993**, *133*, 33–40; (b) Nguyen, T.; Brownell, G. L.; Holden, S. A.; Teicher, B. A. *Biochem. Pharm.* **1993**, *45*, 147–155.
38. Callahan, D. E.; Forte, T. M.; Afzal, S. M. J.; Deen, D. F.; Kahl, S. B.; Bjornstad, K. A.; Bauer, W. F.; Blakely, E. A. *Int. J. Rad. Oncol. Biol. Phys.* **1999**, *45*, 761–771.
39. Miura, M.; Micca, P. L.; Fisher, C. D.; Heinrichs, J. C.; Donaldson, J. A.; Finkel, G. C.; Slatkin, D. N. *Int. J. Cancer* **1996**, *68*, 114–119.
40. Miura, M.; Micca, P. L.; Fisher, C. D.; Gordon, C. R.; Heinrichs, J. C.; Slatkin, D. N. *Br. J. Radiol.* **1998**, *71*, 773–781.
41. Kahl, S. B.; Joel, D. D.; Nawrocky, M. M.; Micca, P. L.; Tran, K. P.; Finkel, G. C.; Slatkin, D. N. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 7265–7269.
42. Zhou, R.; Balasubramanian, S. V.; Kahl, S. B.; Straubinger, R. M. *J. Pharm. Sci.* **1999**, *88*, 912–917.
43. Tibbitts, J.; Fike, J. R.; Lamborn, K. R.; Bollen, A. W.; Kahl, S. B. *Photochem. Photobiol.* **1999**, *69*, 587–594.
44. Miura, M.; Micca, P. L.; Heinrichs, J. C.; Gabel, D.; Fairchild, R. G.; Slatkin, D. N. *Biochem. Pharmacol.* **1992**, *43*, 467–476.
45. Vicente, M. G. H.; Wickramasighe, A.; Nurco, D. J.; Wang, H. J. H.; Nawrocky, M. M.; Makar, M. S.; Miura, M. *Bioorg. Med. Chem.* **2003**, *11*, 3101–3108.