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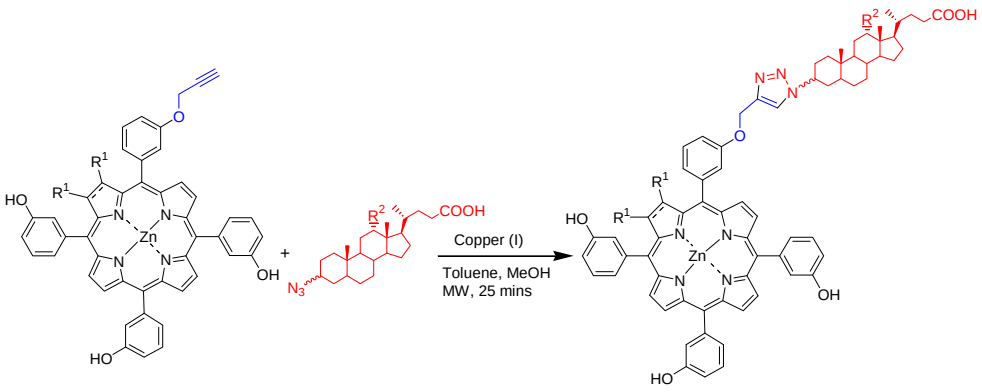

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Lead Structures for Applications in Photodynamic Therapy. 5. Synthesis of Foscan® Bile Acid Conjugates to Target Esophagel Cancer Cells

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ABSTRACT

Porphyrins and chlorins such as Foscan® have a natural proclivity to accumulate in cancer cells. This trait has made them good candidates for photosensitizers and as imaging agents in phototherapy. In order to improve on cellular selectivity to lower post-treatment photosensitivity bile acid porphyrin bioconjugates have been prepared and investigated in esophageal cancer cells. Bile acids which are known to selectively bind to, or be readily taken up by cancer cells were chosen as targeting moieties. Synthesis of the conjugates was achieved *via* selective nucleophilic monofunctionalization of 5,10,15,20-tetrahydroxyphenylporphyrins with propargyl bromide followed by Cu(I) mediated cycloaddition with bile acid azides in good yields. The compounds were readily taken up by esophageal cancer cells but showed no PDT activity.

The search for improved photosensitizers (PS) has been ongoing since the advent of photodynamic therapy. Up until now porphyrin and chlorin based systems such as *m*-THPP (5,10,15,20-tetrakis(3-hydroxyphenyl)porphyrin) and Foscan® (*m*-THPC, 5,10,15,20-tetrakis(3-hydroxyphenyl)chlorin) have been benchmark standards in this regard.[1] However, there are still many problems associated with this class of compound, most important of which is post-treatment photosensitivity due to their less than perfect cellular selectivity.

PDT has been primarily investigated as a treatment for tumors and neoplasias of the skin,[2] breast,[3] esophageal[4] and prostate.[5] Interest currently lies in the advancement of efficient and specific carrier delivery platforms for systemic PDT, be it as bioconjugates,[6] by encapsulating them in liposomes[7] or even connecting them to nanoparticles.[8] These modifications focus on designing systems to impart greater selectivity and specificity on the photosensitizer in order to enhance cellular uptake. All are novel means to functionalize the PS; however, no one method has yet stood out above the rest or even against Foscan® itself.

Bile acids (BAs) such as lithocholic acid (LCA) and deoxycholic acid (DCA) have been shown to induce oxidative stress and generate reactive oxygen species, which can induce DNA damage leading to mutations. BAs have also been shown to activate a number of mitogenic and apoptotic signaling pathways.[9,10] These include the epidermal growth factor receptor and the Raf/Mek/Erk pathway, the activator protein -1 (AP-1) and NF-κB transcription factors, the protein kinase C (PKC) family and endoplasmic reticulum (ER) stress pathways, all of which are known to be deregulated during tumorigenesis.[11]

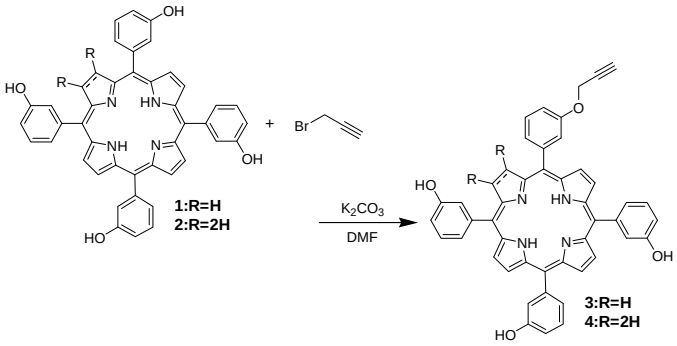
Chronic esophageal exposure to bile acids in patients with gastro-esophageal reflux disease is associated with the development of Barrett's metaplasia and associated molecular markers of inflammation which have been shown to support transformation, initiation and progression of tumor development. Another feature of Barrett's is the development of bile acid transporters such as the apical sodium dependent bile acid transporter (ASBT) along with associated intracellular transporters and a homologue of ilial Ost alpha/beta.[12]

Properly positioned, the BAs would be expected to endow the porphyrin with substrate activity towards ASBT which is present in Barrett's and cancer tissue but not in the normal esophageal squamous epithelium. This approach is expected to increase the selectivity for aberrant cells over the normal type because of this specific distribution of transporter activity. Additionally, the selected BAs, which are activators of cell surface death receptors, could endow the conjugates with additional efficacy in stimulating cell death induction. Previous work carried out by Kralova *et al*. found bile acid porphyrins to show effective cellular uptake and successful ablation of tumors *via* apoptosis and necrosis through PDT.[13]

Tetrasubstituted porphyrins are the workhorses of porphyrin chemistry due to the relative ease of their synthesis. Nevertheless, most biomedical applications require unsymmetrically substituted porphyrins, *e.g.* for increased membrane passage. Often used mixed condensation reactions can lead to a statistical

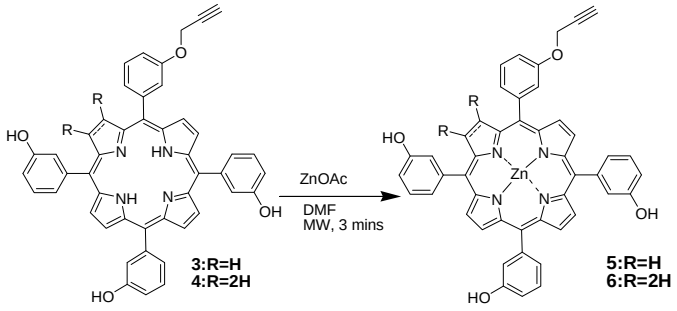
mixture of porphyrins occasionally result in low yields and tedious column chromatography to obtain the desired compound. *m*-THPP **1** is a readily available tetrasubstituted porphyrin and PS. By selectively modifying the porphyrin scaffold through S_N2 reactions, one could install a number of biologically pertinent moieties around the porphyrin periphery in a controlled fashion.

Taking compound **1** and reacting it with 3 eq. of propargyl bromide for 2 h, in the presence of a base, one can selectively mono-functionalize this porphyrin in 48% yield (Scheme 1). Propargyl bromide was selected as the functional group to install because the alkyne moiety present provides the opportunity of metal-mediated post functionalization reactions, *e.g.*, through 'click' chemistry.



Scheme 1. Mono functionalization of **1** and **2**.

The reaction can be controlled by monitoring the equivalents and length of reaction time, thus resulting in the desired product whether it be mono-, di-, tri- or tetrasubstituted porphyrins. These groups act as a synthetic handle upon which one can add a plethora of biologically interesting compounds. Likewise, metallation is unproblematic and was achieved through microwave irradiation over 3 minutes with excess ZnOAc in DMF in quantitative yield (Scheme 2).[14]



Scheme 2. Metalation of **3** and **4**.

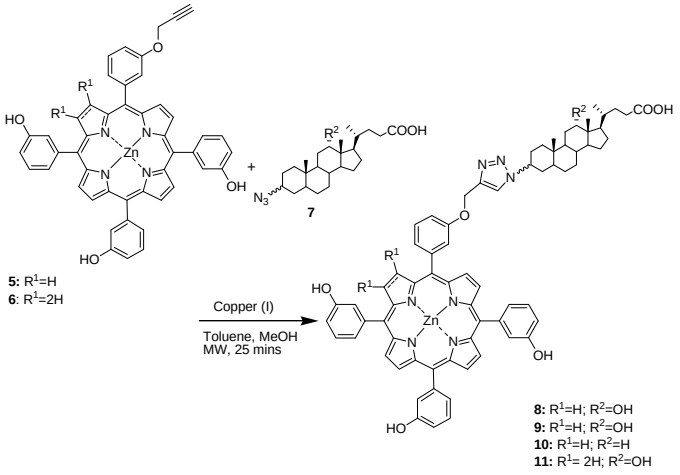
As one of the industry's gold standard, the credentials of any subsequent PS are measured against *m*-THPC (Foscan®, **2**). Thus, a Foscan® bile acid conjugate was an obvious synthetic target. However, many of the standard chemical reactions, *e.g.*, metallation reactions, carried out on porphyrins become cumbersome when translated to their chlorin counterpart. Metallation of a porphyrin usually requires elevated temperatures; unfortunately these conditions may oxidize the chlorin to the parent porphyrin molecule. As a result of these limitations, investigations into functionalization reactions of Foscan® have remained relatively dormant in recent years.

However, from the optimization of the porphyrin functionalization, these methodologies were implemented on the chlorin scaffold with similar yields being attained. Microwave assisted metallation of Foscan® **2** to yield **6** works quantitatively with confirmation by UV-vis and NMR. This is the first example of the successful metallation of Foscan® and shows that the brief irradiation provided by a microwave is sufficient to insert the metal into the chlorin's core without subsequent oxidation.

Synthetic modifications were made to the bile pigments using known protection and deprotection chemistry to yield compounds 3*β*-DCA(**7a),** 3*α*-DCA**(7b)** and 3*β*-LCA**(7c)**. All reactions were high yielding and capable of gram scale synthesis.[15]

The azide group was chosen due to its ease of installation, the opportunity to screen both the 3-α and β functionality and finally to leave the acid fragment of the compound intact as it is theorized to be the group recognized by the transporter that provides cellular uptake. These azides used in conjunction with synthetically available alkyne porphyrins and chlorins make them the perfect candidate for the robust and high yielding microwave assisted 1,3-dipolar cycloaddition reaction. A modified Huisgen cycloaddition reaction[16] is a 1,3-dipolar cycloaddition between an azide and, in this case, a terminal alkyne to give a 1,2,3-triazole. As shown in Scheme 3, the azide **7** reacts with alkyne **5** to afford the 1,4 regioisomer of a 1,2,3-triazole **8** under microwave conditions. Although azides are not the most reactive 1,3-dipoles available, they are preferred in this case both for their relative lack of side reactions and their stability under typical synthetic conditions.[17]

Test reactions were carried out using previously optimized conditions for porphyrin click reactions.[6b] The hydroxy groups present interfered with the copper(I) catalytic cycle as no reaction was occurring, even at elevated temperature. Tetrakis(acetonitrile)copper(I) hexafluorophosphate has been shown to work in 1,3 cycloaddition reactions utilizing similar substrates as the ligands present help stabilize the catalyst and generally improve the yields of the reactions.[18] Using this catalyst, a library of four highly soluble conjugates was synthesized in high yields (Scheme 3, Table 1).[19]



Scheme 3. Synthesis of bile acid porphyrin conjugates.

Table 1. Porphyrin bile conjugates synthesized *via* Scheme 4.

Macrocycle	Bile acid	Product	Yield %
5	**7a**	**8**	63
5	**7b**	**9**	65
5	**7c**	**10**	63
6	**7a**	**11**	61

Compounds **8-11** underwent biological screening to assess their localization and cytotoxic properties using esophageal carcinoma OE33, esophagus adenocarcinoma, and well-differentiated SKGT-4 human cell lines. All four compounds are successfully taken up into the cell and appear to localize in the

ER and Golgi apparatus, similar to the accumulation patterns seen with Foscan®; however further co-localization studies are needed to definitively confirm this hypothesis (Fig. 1).

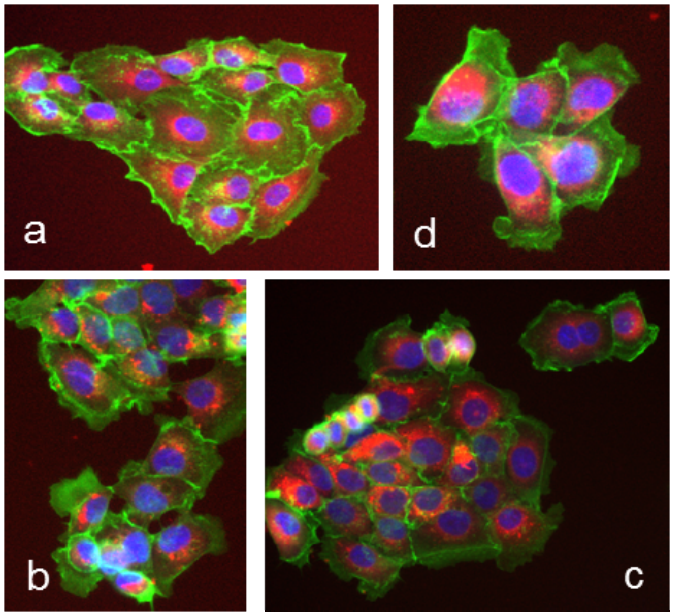


Figure 1. OE33 and SKGT-4 cells stained with compounds **8-11** (red), nuclear dye - Hoechst (blue), F-actin dye - Phalloidin (green): a) **8** 30μM, SGT-4; b) **9** OE33 40μM; c) **10** OE33 40μM; d) **11** OE33 50μM.

The standard test of the efficacy of a PS *in vitro* is measured using the colorimetric MTT or MTS assays. Compounds **8-11** were analyzed with an MTS assay after 48 h incubation and an illumination period of two min.[20-22] Unfortunately, none of the bile porphyrins exhibited any phototoxic effect, which may be due to a number of factors, *e.g.*, the triazole ring may be inhibiting the production of $^1O_2$ by the PS, or intracellular quenching may be occurring due to intracellular aggregation of the PS. However, as porphyrins are selectively retained in tumor tissue, the compounds may show promise as imaging agents for phototherapy as they were all readily taken up by the different cell lines and exhibit strong fluorescence, which is ideal for imaging. To resolve the issue of poor cytotoxicity, a larger library of bile porphyrins is being synthesized, utilizing a variety of different linkers and also varying the location of conjugation.

We have successfully synthesized porphyrin bile acid adducts through the copper(I) catalyzed click reaction. By controlling the number of equivalents, one can control the degree of substitution and obtain mono-functionalized porphyrin and chlorin derivatives in high yields and few synthetic steps. Foscan®, as a molecule, is able to tolerate microwave irradiation and S_N2 chemistry with no subsequent oxidation to the parent porphyrin. This result opens up the range of possible modifications one can make to this already approved drug in the continued search for improved photosensitizers. Note, that co-administration of certain bile acids has also been shown to improve the overall PDT effect.[23]

This library of highly soluble PS were readily up taken and accumulated within the cytoplasm of the cell. Future work will focus on improving the cytotoxicity of these conjugates through the use of different linkers and bile acids, while, also identifying their mechanism of uptake and sites of localization.

Acknowledgments

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19. **8** was synthesized in the optimum yield using the following procedure: To a 5 mL Pyrex microwave vessel, **7a** (14 mg), **5** (16 mg), $Cu(MeCN)_4PF_6$ (20 %, 2 mg) were added to the flask containing Toluene (0.6 mL) and MeOH (0.6 mL). The reaction mixture was heated to 125 °C for 25 min under microwave irradiation. The solvent was removed *in vacuo*, and the product purified by flash chromatography to yield a purple solid **8** (15.4 mg, 63%). Analytical data: Mp: >300 °C; 1H NMR (400 MHz, $(CD_3)_2SO_2$): 8.77 (d, $^3J_{H\text{-}H}$ =4Hz, 6H, H_β), 8.71 (d, $^3J_{H\text{-}H}$ =4Hz, 2H, H_β), 8.29 (s, 1H, triazole), 7.69 (s, 1H, -Ar), 7.62 (s, 1H, -Ar), 7.53 (m, 10H, -Ar), 7.52 (m, 4H, -Ar), 5.47 (s, 2H, CH_2), 3.98 (m, 1H, 3 β-H); ^{13}C NMR (100 MHz, $CDCl_3$):175.4 (C=O, 24-C), 157.1, 156.9, 142.9, 143.4, 141.5, 139.8, 131.7, 129.2, 128.3, 126.2, 122.3, 120.4, 121.3 (triazole), 115.5, 71.5, 58.7, 47.9, 46.6, 46.5, 37.5, 35.8, 35.4, 34.6, 32.8, 31.3, 31.2, 30.9, 30.1, 29.1, 27.5, 26.6, 26.2, 24.5, 23.9, 23.8, 17.4, 12.9; UV/Vis (CH_2Cl_2) λ_{max} (log ε): 421 (6.83), 553 (5.40), 593 (4.78); HRMS (MALDI) calcd for $[M]^+$ $C_{71}H_{69}N_7O_6Zn$ 1194.4601, found 1194.4624.
9 (15.7 mg, 65%). Analytical data: Mp: >300 °C; 1H NMR (400 MHz, $(CD_3)_2SO_2$): 8.77 (d, $^3J_{H\text{-}H}$ =4Hz, 6H, H_β), 8.71 (d, $^3J_{H\text{-}H}$ =4Hz, 2H, H_β), 8.28 (s, 1H, triazole), 7.69 (s, 1H, -Ar), 7.62 (s, 1H, -Ar), 7.53 (m, 10H, -Ar), 7.52 (m, 4H, -Ar), 5.47 (s, 2H, CH_2), 3.98 (m, 1H, 3 β-H); ^{13}C NMR (100 MHz, $CDCl_3$): 175.4 (C=O, 24-C), 157.1, 156.9, 142.9, 143.4, 141.5, 139.8, 131.7, 129.2, 128.3, 126.2, 122.3, 120.4, 121.0 (triazole), 115.5, 71.5, 58.7, 47.9, 46.6, 46.5, 37.5, 35.8, 35.4, 34.6, 32.8, 31.3, 31.2, 30.9, 30.1, 29.1, 27.5, 26.6, 26.2, 24.5, 23.9, 23.8, 17.4, 12.9; UV/Vis (CH_2Cl_2) λ_{max} (log ε): 421 (6.83), 553 (5.40), 593 (4.78); HRMS (MALDI) calcd for $[M]^+$ $C_{71}H_{71}N_7O_6Zn$ 1197.4706, found 1197.4666.
10 (13.3 mg, 63%). Analytical data: Mp: >300 °C; 1H NMR (400 MHz, $(CD_3)_2SO_2$): 8.77 (d, $^3J_{H\text{-}H}$ =4Hz, 6H, H_β), 8.71 (d, $^3J_{H\text{-}H}$ =4Hz, 2H, H_β), 8.29 (s, 1H, triazole), 7.69 (s, 1H, -Ar), 7.62 (s, 1H, -Ar), 7.53 (m, 10H, -Ar), 7.52 (m, 4H, -Ar), 5.47 (s, 2H, CH_2), 3.98 (m, 1H, 3 β-H); ^{13}C NMR (100 MHz, $CDCl_3$): 174.3 (C=O, 24-C), 167.6, 156.9, 155.8,

151.5, 143.4, 142.4, 139.8, 131.7, 129.2, 128.0, 127.8, 124.9, 123.0, 121.9, 121.0 (triazole), 119.2, 114.9, 114.7, 112.1, 71.5, 58.7, 47.9, 46.6, 46.5, 37.5, 35.8, 35.4, 34.6, 32.8, 31.3, 31.2, 30.9, 30.1, 29.1, 27.5, 26.6, 26.2, 24.5, 23.9, 23.8, 17.4, 12.9; UV/Vis (CH_2Cl_2) λ_{max} (log ε): 421 (), 5.53 (4.92), 593 (4.39); HRMS (MALDI) calcd for $[M]^+$ $C_{71}H_{69}N_7O_7Zn$ 1195.4550, found 1195.4514.

11 (14.7mg, 61%). Analytical data: Mp: >300 °C; 1H NMR (400 MHz, $(CD_3)_2SO_2$): 8.79 (d, $^3J_{H\text{-}H}$ =5Hz, 4, H_β), 8.75 (d, $^3J_{H\text{-}H}$ =5Hz, 1H, H_β), 8.74 (d, $^3J_{H\text{-}H}$ =5Hz, 1H, H_β), 8.29 (s, 1H, triazole), 7.79 (s, 1H, -Ar), 7.66 (s, 1H, -Ar), 7.54 (m, 10H, -Ar), 7.52 (m, 4H, -Ar), 5.47 (s, 2H, CH_2), 4.16(s, 4H), 3.98 (m, 1H, 3 β-H); ^{13}C NMR (100 MHz, $CDCl_3$): 175.2 (C=O, 24-C), 167.6, 156.9, 155.8, 151.5, 143.4, 142.4, 139.8, 131.7, 129.2, 128.0, 127.8, 124.9, 123.0, 121.9, 121.0 (triazole), 119.2, 114.9, 114.7, 112.1, 71.5, 58.7, 47.9, 46.6, 46.5, 37.5, 35.8, 35.4, 34.6, 32.8, 31.3, 31.2, 30.9, 30.1, 29.1, 27.5, 26.6, 26.2, 24.5, 23.9, 23.8, 17.4, 12.9; UV/Vis (CH_2Cl_2) λ_{max} (log ε): 423 (6.70), 524 (5.33), 609 (4.71); HRMS (MALDI) calcd for $[M]^+$ $C_{71}H_{69}N_7O_7Zn$ 1195.4550, found 1195.4514.

20. *General procedure for cell cultures and cell proliferation assay (MTS):* Cell lines were seeded at a concentration of 8×10^4 cells per ml into sterile 96-well plates, left to attach overnight and treated. To previously prepared 96-well assay plates containing cells in 100 µL of culture medium, the test compounds at different concentrations and appropriate controls were added. After incubation for 24 h the medium was removed and changed for fresh one, dark controls were left in the dark for next 24 h. To assess the phototoxicity, the rest of the plates were illuminated for 2 min and incubated for 24 h. Finally, 20 µL of MTS dye solution was added to each well of the dark controls and illuminated plates and these were incubated for 3h and the absorbance was recorded at 470nm using a 96-well plate reader. Cell lines were seeded at a concentration of 3×10^4 cells per ml into sterile 96-well plates leaving them for 24 h to attach. For imaging experiments, the cell culture medium was removed, replaced with freshly prepared solutions of the porphyrins **8-11** of various concentrations in the medium and incubated at 37 °C under 5% CO_2 for 24 h. After that the medium was removed and fixed with 4% PFA in medium and then washed with PBS. Fluorescent images were collected and analyzed by high content screening and imaging technique (IN Cell 1000 instrument, GE Healthcare).[21]
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22. *Biological evaluation:* Intracellular screening for the compounds **8-11** has been carried out in OE33 and SKGT-4 cells. Stock solutions of the bile porphyrins (0.5 mM) were prepared in ethanol. Intracellular experiments were carried out by high-content screening using IN Cell 1000 and *in vitro* images were taken at different concentrations 10 µM to 50 µM. Living cells were incubated first with the materials **8-11** for 24 h in the dark and then fixed. Next, fixed adenocarcinoma cells were co-stained using nuclear dye Hoechst and the bicyclic peptide Phalloidin as cytoskeleton stain (F-actin). The images were collected using three independent channels for Hoechst, Phalloidin and the compounds **8-11** with excitation/emission filters of 345 nm/435 nm (blue), 475 nm/535 nm (green) and 620 nm/700 nm (red), respectively.
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