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Multi-gram synthesis of a porphyrazine platform for cellular translocation, conjugation to Doxorubicin, and cellular uptake

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ABSTRACT

We report the synthesis of the near infrared (NIR) fluorescent porphyrazine (Pz) 285, with pendant hydroxyl groups, as a non-toxic platform for delivery of conjugated chemotherapeutic agents to tumor cells. Conjugation of Pz 285 to Doxorubicin via an acid labile linker and initial biological studies are reported.

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In recent years, cancer mortality rates have declined, in part because of improved early detection.[1,2] One promising modality for cancer detection is near infrared (NIR) optical imaging[3,4] enabled by use of a tumor specific contrast agent.[5] Such an agent absorbs and emits harmless NIR light that can penetrate soft tissues to considerable depth, allowing non-invasive imaging of tumors, such as those of the breast.[6]

Tetrapyrroles, including porphyrins and phthalocyanines, have been studied as potential NIR contrast agents.[7,8] They exhibit intense absorption and emission at long wavelengths, and they have been extensively studied as photosensitizers for photodynamic therapy (PDT).[9] Our work is focused on heteroatom substituted porphyrazines (Pzs),[10,11] a promising tetrapyrrole sub-class.

We have examined Pzs with peripheral heteroatom (S, O, N) substituents for possible biomedical applications.[12–15] These compounds show promise as classic anti-tumor agents, PDT photosensitizers, and NIR contrast agents. Most recently, we discovered a chiral oxygen atom appended Pz that exhibits intense NIR emission from cells in vitro,[12] mediated by association with low-density lipoprotein (LDL).[16] Most importantly, this Pz exhibits highly preferential tumor uptake in vivo, where its NIR fluorescence from within the tumors can be visualized through the skin.

As a first step toward use of the observed tumor selectivity in generating a multimodal agent, paramagnetic Gd(III) MRI contrast agents, which themselves are not taken up by either tumorigenic or non-tumorigenic cells, were covalently attached to a Pz.[17] Cellular uptake of the conjugates was observed when the MRI contrast agent was conjugated to one side of the Pz, presumably allowing the hydrophobic portion of the Pz to interact with serum proteins while transporting the Gd(III) complex into the cell. Pandey and coworkers have also recently shown that this strategy is viable for producing tumor-selective tetrapyrrole/Gd(III) conjugates.[18]

The favorable properties of these initial Pz conjugates have led us to examine the use of Pzs as translocation vectors for known chemotherapeutics with the expectation that the drug would be preferentially delivered to tumor tissue thereby lessening the damage to healthy tissue. Herein we report the synthesis and cellular uptake of a new Pz platform for both NIR-fluorescence optical imaging on its own and for delivery of chemotherapeutics to tumor cells. Pz **285** (Scheme 1) was designed to conjugate chemotherapeutic agents on one side of the periphery so that they do not interfere with the porphyrazine's ability to interact with serum proteins as the basis of cellular uptake with high tumor selectivity.[19]

The synthesis of **285** (Scheme 1) started with the preparation of 2-(2-chloroethoxy)tetrahydropyran (**1**) by a modified literature procedure.[20] Nucleophilic substitution of chloride **1** with commercially available 1,4-dicyanohydroquinone gave dinitrile (**2**), which was imidated to yield the *iso*-indoline (**3**). Compounds **2** and **3** were isolated as mixtures of diastereoisomers as confirmed by their NMR spectra. Co-cyclization of *iso*-indoline **3** with (5*R*,6*R*)-2,3-dicyano-5,6-dimethoxy-5,6-dimethyl-1,4-diox-2-ene[21] gave a mixture of Mg-Pzs with tetrahydropyran (THP) protected alcohol

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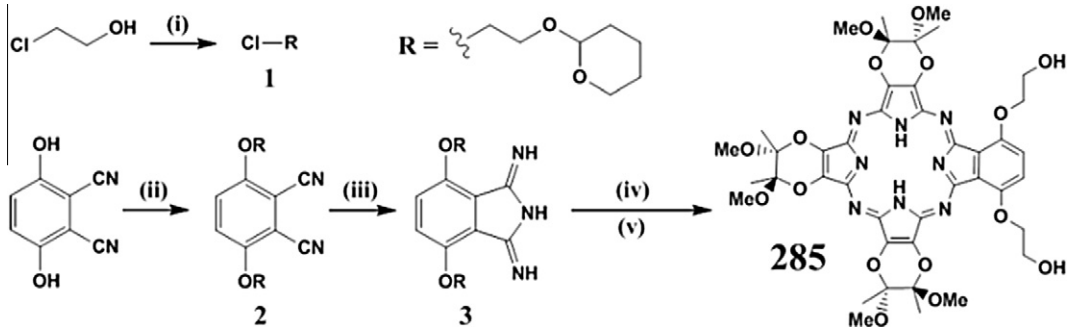


Scheme 1. Synthesis of **285** (i) THP, $TsOH.H_2O$, neat, 0 °C, 2 h; (ii) **(1)**, K_2CO_3, KI, DMF, 60 °C, 48 h; (iii) Na^0, NH_3, ethylene glycol, 140 °C, 6 h; (iv) $Mg(O\text{-}n\text{-}Pr)_2$, (5*R*,6*R*)-2,3-dicyano-5,6-dimethoxy-5,6-dimethyl-1,4-diox-2-ene, 120 °C, 18 h; (v) AcOH, THF, H_2O (4:2:1), 80 °C, 18 h.

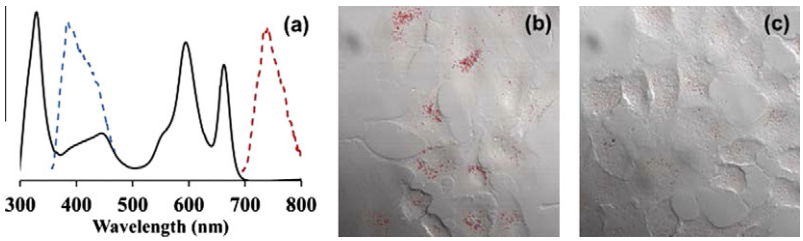
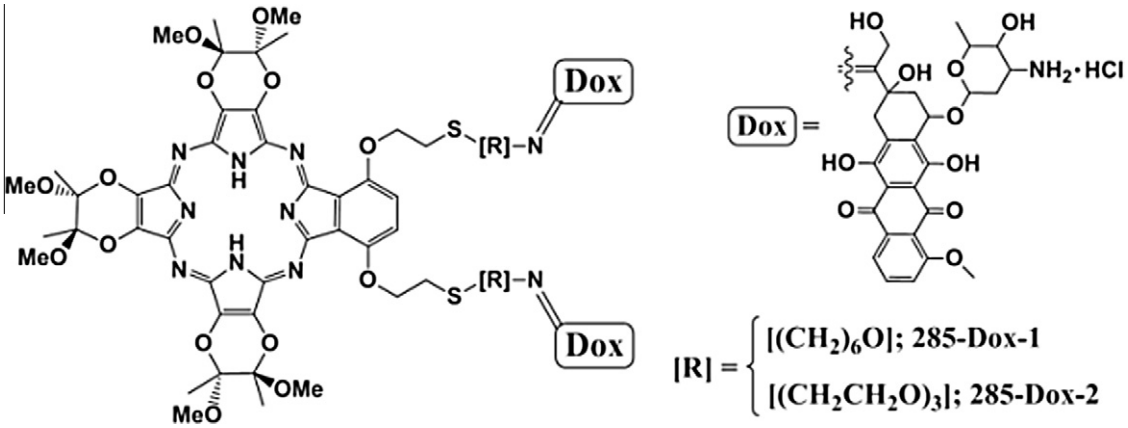


Figure 1. Electronic absorption (black) and emission (blue/red) of pz **285** in MeOH. Spectra were normalized to matching scales. Confocal fluorescence microscopic images of (**b**) A549 human lung carcinoma cells and **(c)** WI38 VA13 human fibroblast cells. Fluorescence from pz **285** (red) is overlaid onto transmitted light images; 63×.

groups. Attempts at macrocyclization without the THP protection led to decomposition and very low yields. The Mg ion and THP groups were removed from the Pz by reaction with acetic acid to produce the enantiomerically pure, chiral **285** in 45% yield (2 steps) after isolation by chromatography. Reaction with such yields was found to be reproducible on a multi-gram scale (5–10 g), thus enabling extensive biological studies. It is important to note that the chirality of **285** is merely a synthetic convenience and there is no expectation or evidence that the chirality would have any effect on biological properties.

Porphyrazine **285** exhibits typical Pz electronic absorption (λ_{max} = 594 nm, 662 nm) and S1 emission (λ_{em} = 740 nm, MeOH) (Fig. 1a).[12] The emission falls within the wavelength range of maximum tissue penetration for NIR optical imaging. Pzs with the core structure of **285** have negligible quantum yields of singlet oxygen generation (Φ_Δ)[12] so phototoxicity is not expected to interfere with optical imaging or cellular translocation applications. Although **285** is rather hydrophobic, it can be administered in biological fluids through use of DMSO (<1%) as solubilizing agent. Cellular uptake of **285** was examined in vitro by fluorescence microscopy using A549 human lung carcinoma cells (Fig. 1b) and WI38 VA13 human fibroblast cells as a non-tumorigenic reference (Fig. 1c). Intracellular fluorescence from **285** is observed in both cell lines relative to vehicle controls, but cellular uptake is much greater for the hyperproliferative tumor cells. Cellular uptake is also observed in the MDA-MB-231 breast tumor cell line (See ESI; Supplementary Fig. S1). Through a series of confocal microscopy experiments, not discussed in detail here, it was determined that **285** enters cells actively by endocytosis and is carried into cells by interactions with the protein serum albumin (See ESI; Supplementary Figs. S2–S6). Albumin binding has been shown with other porphyrinoid systems[22,23] and, in some cases enhances PDT efficacy in vivo.[24] Intracellular endosomes are known to be slightly acidic,[25] prompting the design of Pz – Doxorubicin conjugates connected through



Scheme 2. Structures of Pz-Doxorubicin conjugates **285-Dox-1** and **285-Dox-2** linked with acid-labile oximes.

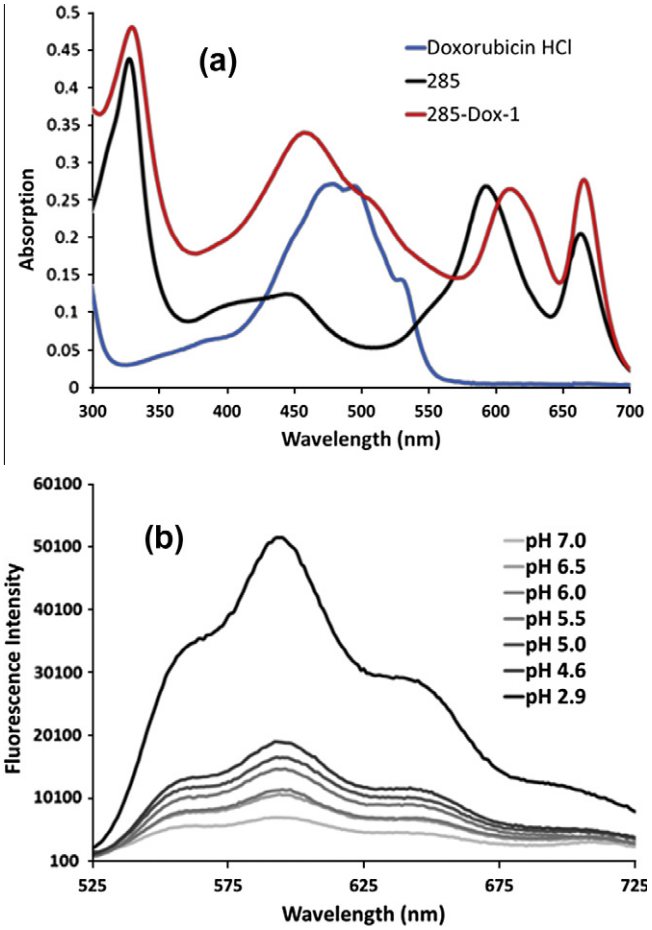


Figure 2. (a) Electronic absorption of Doxorubicin HCl (blue), **285** (black), and **285-Dox-1** (red) in MeOH. (b) Anthracycline fluorescence of **285-Dox-1** increases with decreasing pH indicating oxime cleavage.

an acid-labile oxime linker with the expectation that **285** would carry Doxorubicin into the cell subsequently releasing the drug once exposed to lower pH intracellular compartments.

The free hydroxyl groups of **285** were activated for nucleophilic substitution by arene sulfonylation with 4-toluenesulfonyl and 4-fluorobenzenesulfonyl chlorides to provide the corresponding Pzs **286** and **287** in near quantitative yields (See ESI; Supplementary Scheme S1). Compounds **7** and **8** (See ESI; Supplementary Scheme S2) were synthesized as linkers with terminal thiol and oxyamine moieties and when allowed to react with **286** in excess, under basic conditions, the corresponding Pzs **9** and **10** were obtained (See ESI, Supplementary Scheme S3). Subsequent reaction of **9** and **10** with acid in the presence of Doxorubicin hydrochloride gave Pzs **285-Dox-1** and **285-Dox-2** in 70% and 29% yields, respectively (Scheme 2).

Electronic absorption of Pz-Dox conjugates is as expected with a combination of Pz and anthracycline absorption bands (Fig. 2a). With the oxime linker intact, all fluorescence bands are almost completely quenched, with the remaining green fluorescence from Doxorubicin (λ = 593 nm, H_2O) being the strongest (See ESI; Supplementary Fig. S7). This intramolecular quenching presents an excellent qualitative method to track linker cleavage by monitoring fluorescence intensity of the Doxorubicin fluorescence band. For example, equimolar solutions of **285-Dox-1** (12.5 μM) were prepared in buffer (KP_i/Ac) of varying pH and incubated at 37 °C for seven days. Fluorescence intensity of these solutions increased with acidity (Fig. 2b) indicating, as expected, more extensive cleavage of the oxime linker at lower pH. Comparison of integrated fluorescence intensity (Fig. 2b vs Supplementary Fig S7) indicated that less than 5% oxime cleavage occurred at biologically relevant pH (pH > 4.9). A more quantitative method to monitor oxime cleavage was developed using capillary electrophoresis mass spectrometry (CE-MS) and it was determined that >99% of **285-Dox-2** was intact after 14 days at pH 4.0 (See ESI; Supplementary Fig. S8). This degree of cleavage would not be expected to produce a beneficial cytotoxic effect at biologically relevant pH.

Despite poor cleavage of the oxime linker in solution, cellular uptake of Pz-Dox conjugates was examined. MDA-MB-231 breast tumor cells were treated with both Pz-Dox conjugates and Doxorubicin HCl alone as a positive control and imaged by confocal fluorescence microscopy (Fig. 3).

Doxorubicin HCl treated cells display fluorescence from punctate vesicles throughout the cell and in the nucleus, whereas cells treated with **285-Dox-1** and **285-Dox-2** only display fluorescence from intracellular vesicles. From this we can conclude that the Pz-Dox conjugates successfully enter the cells but the linkers are not cleaved enough to release Doxorubicin for its effective nuclear localization.

The effect of Pz-Dox conjugates on cell viability was examined using the MTS assay (Fig. 4). MDA-MB-231 cells were treated for 5 days with a range of concentrations of Doxorubicin HCl, **285-Dox-1**, and **285-Dox-2** with the maximum dosage corresponding to the highest concentration of Pz-Dox conjugate that is stable in aqueous solution over time. Comparison of cell viability versus vehicle controls shows that, at these dosages, **285-Dox-1** has no effect on cell viability and **285-Dox-2** is ~100-fold less toxic than

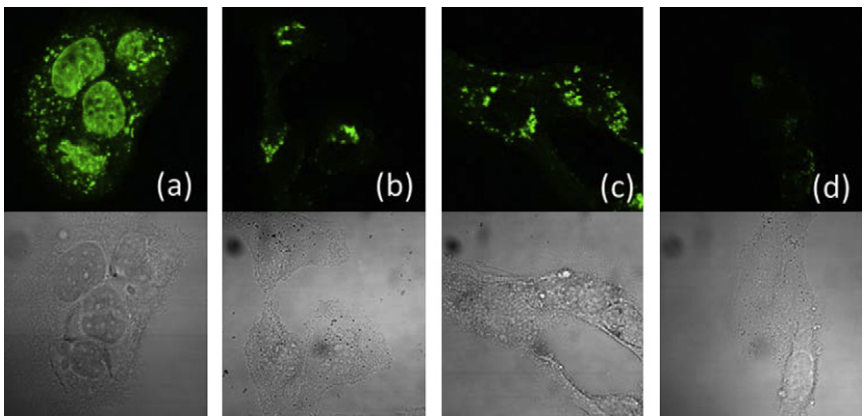


Figure 3. Confocal fluorescence microscopic images of MDA-MB-231 cells treated with (a) 10 μM Doxorubicin HCl, (b) 5 μM **285-Dox-1**, (c) 5 μM **285-Dox-2**, and (d) DMSO vehicle; 488 nm ex., 560–610 nm em., 100× magnification.

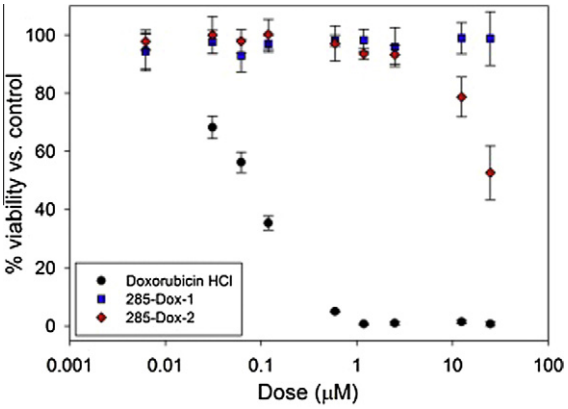


Figure 4. Viability of MDA-MB-231 cells treated for 5 days as % viability of vehicle controls.

Doxorubicin alone. This corresponds well with previously discussed CE-MS oxime cleavage data.

Conclusions

In summary, the unsymmetrical Pz **285** was designed and synthesized to exhibit NIR fluorescence while carrying two pendant hydroxyl groups for conjugation to bioactive agents. A particular advantage of **285** for this role is its vanishingly small quantum yield for singlet oxygen formation, which should eliminate phototoxic side-effects. Cell studies show that Pz **285** undergoes active endocytosis by hyperproliferative tumor cells to a greater extent than by slower growing non-tumorigenic cells, with strong intracellular NIR fluorescence upon uptake. Pz **285** was successfully conjugated to Doxorubicin HCl through an acid-labile oxime linker. Although cleavage of this linker is insufficiently rapid at biologically relevant pH for **285-Dox-1** and **285-Dox-2** to exhibit the desired cytotoxicity, this report lays the foundation for future experiments in which **285** is conjugated to Gd(III) MRI contrast agents, or to Doxorubicin through more favorable linkers.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2012.07.087.

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