Photodynamic Efficiency of Diethylene Glycol-Linked Glycoconjugated Porphyrins in Human Retinoblastoma Cells

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Photodynamic therapy (PDT) is emerging as a new strategy for the conservative treatment of hereditary retinoblastoma. The glycoconjugated porphyrins TPP(*p*-Deg-O-α-GalOH)$_3$, TPP(*p*-Deg-O-*β*-GalOH)$_3$, TPP-(*p*-Deg-O-α-ManOH)$_3$, and their S-analogues were synthesized to obtain efficient photosensitizers with some retinoblastoma cell affinity. In these systems, a sugar motif and porphyrin core were linked by a diethylene glycol spacer (Deg). Cellular uptake, localization, and photoactivity have been examined in human retinoblastoma cells (Y79). After preincubation with corresponding glycosylated albumin, the uptake of TPP(*p*-Deg-O-*β*-GalOH)$_3$ and TPP(*p*-Deg-O-α-ManOH)$_3$ was 40−45% inhibited, indicating a possible cell-sugar-receptor saturation. High photoactivity was observed for the two α-galacto/manno porphyrins **8** and **10** (LD_{50} = 0.05 and 0.35 μM, respectively) at 514 nm and low fluence (1 J/cm^2). Analysis by MALDI-TOF mass spectrometry only indicated a small metabolic cleavage of the O-glycoconjugates and a good stability of the S-glycoside porphyrins. On the basis of these in vitro data, TPP(*p*-Deg-O-α-GalOH)$_3$ and TPP(*p*-Deg-O-α-ManOH)$_3$ were selected for in vivo studies.

Introduction

Retinoblastoma is the most common malignant intraocular tumor in children. About 40% of cases result from a RB1 gene mutation and are associated with a high risk of second cancers. Recent developments of therapeutic modalities for the conservative treatment of retinoblastoma, based on a synergy between carboplatin chemotherapy and tumor hyperthermia, have meaningfully diminished the necessity for external beam radiation, with a consequent reduction in severe side effects. But the short and long-term risk associated with carboplatin chemotherapy still remains.[1]

Given this context, the development of an alternative treatment involving photodynamic therapy (PDT) is of particular interest. This technique is based on the administration of a sensitizer devoid of mutagenic properties, followed by the exposure of the pathological area to visible light.[2]

So far, the use of PDT to treat retinoblastoma in man has been limited to a small series of patients[3] and to the use of Photofrin. In this study, ocular side effects were observed. However, no definite conclusion could be drawn from the data because high drug and light doses were required to compensate for the relatively poor photoactivity of Photofrin.

Continued research to develop new sensitizers with higher photoactivity and increased therapeutic index has resulted in a number of second generation sensitizers becoming available.[4,5] In our laboratories, efforts have been focused on the preparation and evaluation in vitro of the phototoxicity of a broad series of neutral glycoconjugated tetrapyrrolic macrocycles. Generally, it has been shown that triglycoconjugated sensitizers are more phototoxic than either the parent tetrapyrrolic or the symmetrical tetraglycoconjugated derivatives.[6] In these systems, glycoconjugation modifies the amphiphilicity of macrocycles and can favor their interaction with the cell surface membrane of tumor cells.[7] Concerning this latter property, indications are that glycosylation provides the possibility for specific interaction of the resulting conjugate with lectin type receptors. These receptors are overexpressed in certain malignant cells.[8,9]

Glycoconjugation can thus be a potentially effective strategy for targeting photosensitizers toward tumor cells. For example, a considerable increase in the photosensitizing efficiency in vitro of purpurinimides conjugated with either galactose or lactose compared to that of the free analogue has been reported.[10] Galectin-binding studies also demonstrated a higher affinity of a benzochlorin-lactose conjugate relative to that of the corresponding glucoconjugated benzochlorin, resulting in greater photoactivity.[11] Recently, the photobiological activity of glycoconjugated chlorins in HeLa cells has been shown to depend on the nature of the sugar residue.[12]

Also, Griegel and co-workers[13] established that human retinoblastoma cells express sugar receptors that exhibit a preferential affinity for galactose and mannose residues. Following this direction, we have engaged a multidisciplinary program to study the photoefficiency in vitro and in vivo of glycoconjugates of tetrapyrrolic macrocycle based sensitizers for a potential PDT treatment of retinoblastoma.

In the present study, the biological and photobiological properties of five novel diethylene glycol (Deg)-linked O- and S-galacto/mannoconjugated *m*-tetraphenyl porphyrins (TPPs) have been explored in vitro on a human retinoblastoma cell line (Y79). The photoefficiency of these mannosylated and galactosylated derivatives **6**, **8**, **10**, **13**, and **15** were compared with those of the parent unconjugated diethylene glycol porphyrin **2**

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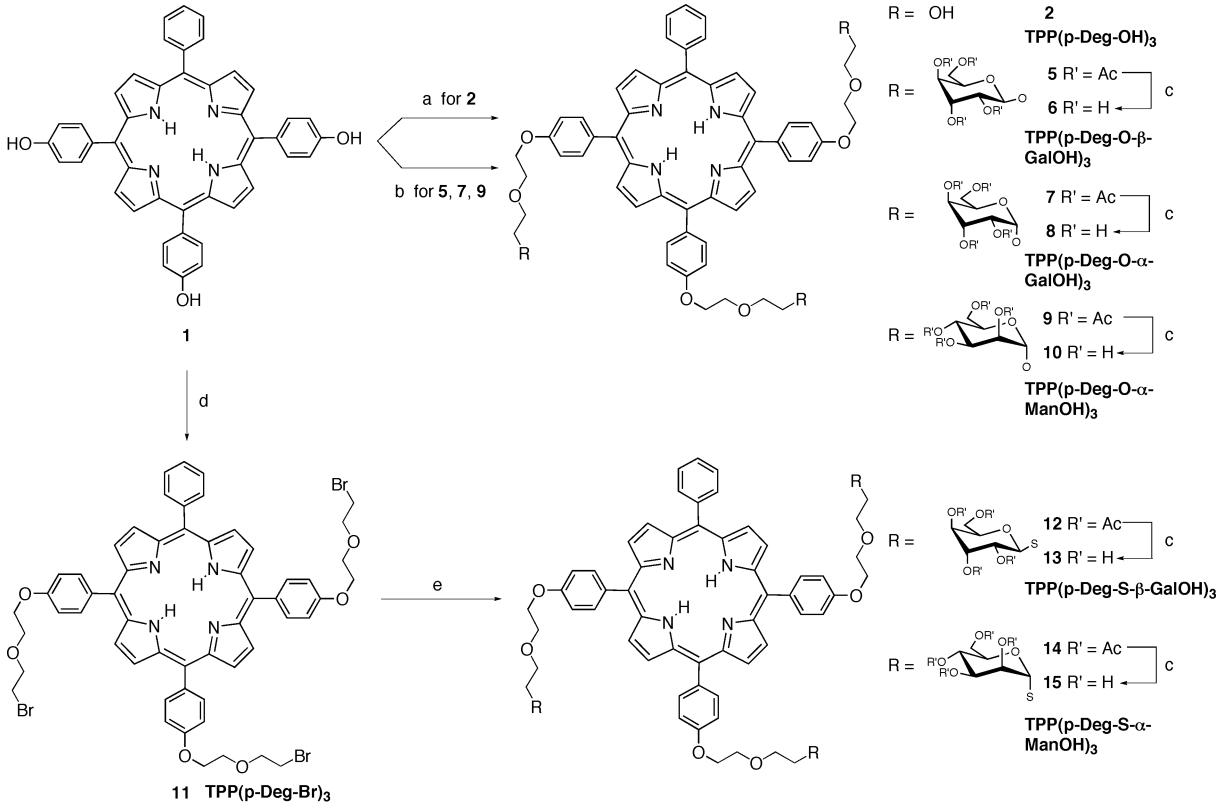


a) 2-(2-chloro-ethoxy)-ethanol , KI, K_2CO_3, dry DMF, 100°C, 4 h; b) α-**3**, β-**3** or **4**, K_2CO_3, dry DMF, 60°C, 15 h; c) NaOMe (cat.), MeOH, d) 1-bromo-2-(2-bromo-ethoxy)-ethane, K_2CO_3, dry DMF, 50°C, 4 h; e) 1-thio-2,3,4,6-tetraacetyl-β-D-galactose or 1-thio-2,3,4,6-tetraacetyl-α-D-mannose, K_2CO_3, dry acetone, 20°C, 15 h.

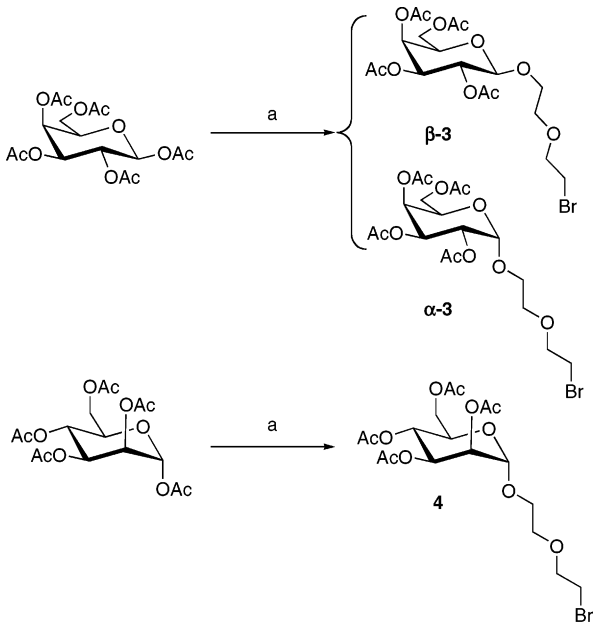
Figure 1. Structure and synthesis of glycoconjugated photosensitizers.

and the corresponding monoethylene glycol (Meg)-linked mannosylated porphyrin **16**.

Results

Chemistry. The reference diethylene glycol porphyrin **2** (TPP(*p*-Deg-OH)$_3$) was prepared in 51% yield by Williamson type condensation of 5,10,15-*m*-tri(*p*-phenol)-20-phenylporphyrin (TPP(*p*-OH)$_3$) (**1**) with 2-(2-chloroethoxy)ethanol at 100 °C (Figure 1). In a similar way, the O-glycosylated diethylene glycol porphyrins **6** (TPP(*p*-Deg-O-*β*-GalOH)$_3$), **8** (TPP(*p*-Deg-O-α-GalOH)$_3$), and **10** (TPP(*p*-Deg-O-α-ManOH)$_3$) were obtained by reacting **1** with the requisite bromo-substituted glycosides α-**3**, *β*-**3**, and **4**, followed by O-deprotection (NaOMe cat, MeOH[14]) of intermediates **5**, **7**, and **9**. Because the difficulty in chromatographic separation of bromo-substituted galactosides α-**3** and *β*-**3**, compounds **7** and **8** contain approximately 10% of *β*-linked galactosyl derivatives **5** and **6**. Galactosides **3** [*β*/α; 1/1.4) and α-mannoside **4** were prepared by the reaction of peracetyl galactose and mannose, respectively, with 2-(2-bromoethoxy)ethanol in the presence of BF_3-etherate (Figure 2).[15]

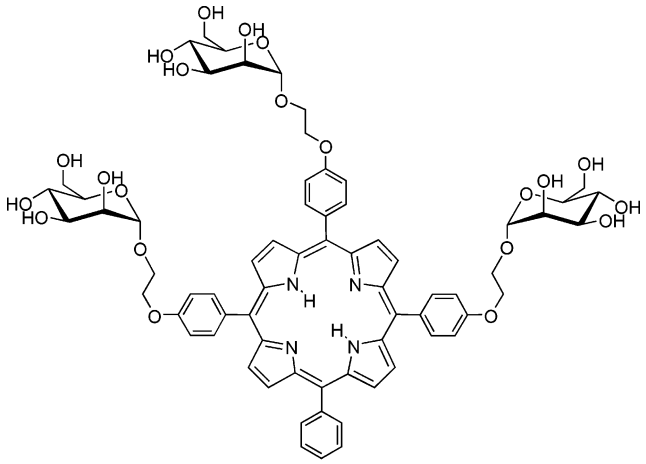
To obtain the corresponding thiosugar-porphyrin conjugates TPP(*p*-Deg-S-*β*-GalOH)$_3$ (**13**) and TPP(*p*-Deg-S-α-ManOH)$_3$ (**15**), bromo-substituted porphyrin glycol **11**, obtained by a Williamson-type reaction of **1** with 1-bromo-2-(2-bromo-ethoxy)-ethane (91% yield), was condensed with 1-thio-tetraacetate-*β*-D-galactose and 1-thio-tetraacetate-α-D-mannose (prepared from the corresponding pseudothioureas just prior to use[16,17]), the derived intermediates **12** and **14** being quantitatively O-deacetylated under methanolysis conditions.[14] The second reference compound **16** (TPP(*p*-Meg-O-α-ManOH)$_3$) containing a monoethylene glycol linker was prepared according to Momenteau et al. (Figure 3).[6]



a) 2-(2-bromoethoxy)ethanol, $BF_3{\cdot}Et_2O$, dry CH_2Cl_2, 0°C to RT

Figure 2. Synthesis of sugar precursors.

As expected, the presence of the sugar-substituted diethylene glycol ether motif on the three meso- and para-phenol substituents of compounds **5**−**10** and **12**−**15** did not alter the UV−visible spectra of the porphyrin systems (Table 1). The fluorescence emission of compounds **5**−**10** and **12**−**15** exhibits a maximum at 632 nm and a weaker band around 720 nm with a low relative fluorescence quantum yield (around 0.03) in all cases.



TPP(p-Meg-O-α-ManOH)$_3$ 16

Figure 3. Structure of mono ethylene glycol glycoconjugated photosensitizer **16**.

Table 1: Physical Characteristics of Glycosylated Porphyrins

compd	UV−visible spectrum λ_{max}, nm (ϵ)	C_1H ^1H NMR chemical shift J in Hz	Maldi-TOF found (calcd)
5	421 (429), 517.5 (18.5), 554 (12.8),593 (8.2), 649.5 (8.5)[a]	4.67 (d, $J = 7.9$)[c]	
6	417 (392.1), 515.5 (17.3), 551.5 (12.4), 592 (7.7), 648.5 (8)[b]	4.9 (d, $J = 7.9$)[d]	1413,60 (1413,55)
7	421 (439.2), 517.5 (18.2), 554 (12.5), 593 (7.8), 649.5 (8.8)[a]	5.28 (d, $J = 3.5$)[c]	
8	417 (411.8), 515.5 (17.7), 552 (12.4), 592 (7.6), 648.5 (7.9)[b]	5.5 (d, $J = 3.6$)[d]	1413.49 (1413.55)
9	419 (429.5), 516 (16.8), 553 (10.5), 592 (5.5), 647.5 (5.3)[a]	4.99 (s)[c]	
10	417.5 (359.2), 516 (15.3), 551.5 (10.5), 592 (6.3), 648 (6)[b]	5.51 (s)[d]	1413,61 (1413,55)
12	421 (403.8), 517.5 (16.2), 554 (10.6), 593 (6.1), 649 (6)[a]	4.67 (d, $J = 9.9$)[c]	
13	424.5 (370.3), 519 (17.1), 556 (12.75), 595.5 (7.7), 652 (7.9)[b]	5.06 (d, $J = 9.4$)[d]	1461.44 (1461.48)
14	421 (380), 517.5 (14.4), 552 (9.6), 593 (4.6), 649.5 (4.6)[a]	5.47 (d, $J = 1.3$)[c]	
15	417 (275.7), 515 (11.6), 552.5 (7.7), 592.5 (4), 647.5 (3.9)[b]	6.09 (d, $J = 0.9$)[d]	1461.60 (1461.48)

[a] CH_2Cl_2. [b] MeOH/Pyridine (24:1). [c] $CDCl_3$. [d] $Pyridine_{d5}$.

^1H and ^{13}C NMR, homonuclear correlation (COSY), and heteronuclear multiple coherence (HMQC) spectra were also obtained for all compounds. The overall appearance of the ^1H- and ^{13}C NMR spectra for protected and glycoconjugated compounds **5**−**15** was very similar to that for the *p*-glycoconjugated porphyrins previously studied.[6] In particular, the anomeric proton resonance (Table 1) appeared as a well-defined doublet with $J = 8-9$ Hz (characteristic of a β anomeric configuration) for galactosyl derivatives, ***β*-3**, **5**, **6**, **12**, and **13**, and as a narrow doublet with $J < 2$ Hz (α anomeric configuration) for the mannosylated (**4**, **9**, **10**, **14**, and **15**) and galactosylated compounds (α-**3**, **7**, and **8**). However, the S-CH_2 protons were non equivalent, appearing as two distinct multiplets (cf. **12**, 3.12 and 2.94 ppm).

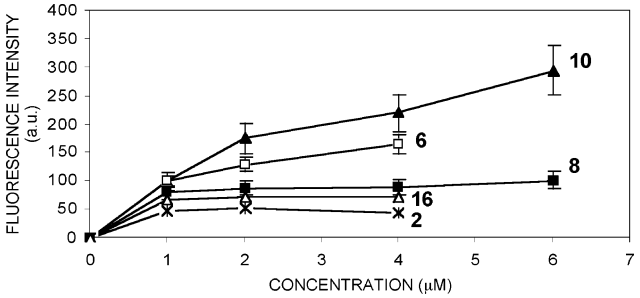


Figure 4. Concentration dependence of cellular uptake (incubation time 4 h) as determined on the basis of cellular fluorescence intensity measured by flow cytometry. The error bars represent the standard error of the mean (SEM) of at least three experiments. [TPP(*p*-Deg-O-α-ManOH)$_3$] (**10**) (▲), [TPP(*p*-Deg-O-*β*-GalOH)$_3$] (**6**) (○), [TPP(*p*-Deg-O-α-GalOH)$_3$] (**8**) (■), [TPP(*p*-Meg-O-α-ManOH)$_3$] (**16**) (△), and [TPP(*p*-Deg-OH)$_3$] (**2**) (×).

Table 2. Ratio of Glycoconjugated Photosensitizer to Unglycosylated TPP(*p*-DegOH)$_3$ (**2**) Uptake in the Y79 Cell Line (4 μM, 4 h Incubation)[a]

compd	Y79	B16	HT29	log PC
TPP(*p*-DegOH)$_3$ (**2**)	1	1	1	>2
TPP(*p*-Deg-S-α-ManOH)$_3$ (**15**)	1.4	1.3	1.2	0.46
TPP(*p*-Meg-O-α-ManOH)$_3$ (**16**)	1.6	1.8	1.7	0.78
TPP(*p*-Deg-O-α-GalOH)$_3$ (**8**)[b]	2	x	1.8	0.5
TPP(*p*-Deg-S-*β*-GalOH)$_3$ (**13**)	3.5	2.7	3	−0.07
TPP(*p*-Deg-O-*β*-GalOH)$_3$ (**6**)	3.7	4	4.4	0.23
TPP(*p*-Deg-O-α-ManOH)$_3$ (**10**)	4.9	6.5	5	0.02

[a] Partition coefficients (log PC) have been determined in 2-octanol/PBS.[6] [b] Compound containing approximately 10% of the *β*-isomer.

For all glycoconjugated derivatives, the protonated porphyrin macrocycle (MH^+) was observed in the positive ion MALDI-TOF spectra (Table 1). For TPP(*p*-Deg-O-*β*-GalOH)$_3$ (**6**), TPP(*p*-Deg-O-α-GalOH)$_3$ (**8**), and TPP(*p*-Deg-O-α-ManOH)$_3$ (**10**), signals of low intensity could also be detected at *m*/*z* 1251.58 and 1163.51 (corresponding to the loss of 162 and 250 mass units, respectively).

Biology

Cellular Uptake. The amount of photosensitizer taken up by the Y79 cells was determined by fluorescence intensity measurements using flow cytometry. The fluorescence intensity was found to increase with incubation time without reaching a saturation value (data not shown). Figure 4 shows the concentration dependence of photosensitizer uptake by Y79 cells. In separate cellular extraction experiments, the intracellular concentrations of treated cells (4 h; incubation concentration of 4 μM) were in the range of $0.78 \pm 0.05\ 10^{-6}$ to $0.26 \pm 0.03\ 10^{-6}$ nmol/cell for TPP(*p*-Deg-O-α-ManOH)$_3$ (**10**) (higher uptake) and TPP(*p*-Deg-OH)$_3$ (**2**) (lower uptake), respectively. These values correlated well with the corresponding fluorescence intensities. In as far as the fluorescence quantum yield for each porphyrin derivative determined in solution is effectively the same (about 0.03), such a correlation shows that any eventual variation resulting from the cellular environment would be similar for all of the compounds studied. The intracellular fluorescence intensity can thus be assumed to reflect the internalized drug concentration.

The values in Figure 4 together with a comparison of the ratio of glycoconjugated photosensitizer to unconjugated reference TPP(*p*-DegOH)$_3$ (**2**) uptakes (4 h**,** 4 μM) (Table 2) indicate that compound **2** is poorly internalized by Y79 cells relative to the glycosylated compounds. The extent of uptake of the conjugates is dependent upon the nature of the sugar component,

its anomeric configuration, and the linker. For the *p*-Deg-O-α-galactosylated **8**, the *p*-Deg-S-α-mannosylated **15**, and the *p*-Meg-O-α-mannosylated **16** conjugates, only a small increase in uptake was observed. The considerably higher values observed for O- and S-*β*-galactosyl compounds **6** and **13** (3.7/2 = 1.85) underscore the importance of the anomeric configuration in the galactose series and further indicate that there is little difference in uptake between **6** and, in principle, the metabolically more stable S-analogue. Most encouraging was the high uptake of the *p*-Deg-O-α-mannosyl conjugate **10** relative to that of both unconjugated compound **2** and corresponding *p*-Deg-S-α-mannosylated analogue **15**.

It is important to note that the cellular uptake of glycoconjugated porphyrins was found to depend on the presence of glucose in the culture medium. Prior to incubation with the photosensitizer, the COON's culture medium was replaced by a DMEM medium with or without glucose. The absence of glucose did not modify the cellular uptake in the case of TPP(p-Deg-OH)$_3$ (**2**). In contrast, the cellular uptake of TPP(p-Deg-O-α-GalOH)$_3$ (**8**) and TPP(p-Deg-O-α-ManOH)$_3$ (**10**) was inhibited by 50%. Glucose, thus, appears to be necessary for the cell uptake of these glycoconjugated porphyrins, suggesting that internalization partly occurs via an active mechanism that is energy dependent involving a transporter.

The photosensitizer uptake was also examined in murine melanocyte cells (B16) and human adenocarcinoma cells (HT29). As seen from Table 2, the same correlation between the uptake and the structure was found in these systems

Glycosylation and Membrane-Specific Receptor Affinity. The binding affinity of the glycoconjugated photosensitizers for sugar specific receptors on Y79 cells has been investigated by examining a possible competition effect between the glycoconjugated porphyrins and the corresponding glycosylated albumin.[18] Thus, when the cells were preincubated with mannosylated albumin (2.6 mg/mL, 30 min incubation) followed by the addition of *p*-Deg-O-α-mannosylated porphyrin **10** (final concentration of 2 μM) and incubation for an additional 60 min, the mannosylated porphyrin uptake was inhibited by 40−45%. A similar inhibition effect was observed for *p*-Deg-O-*β*-galactosylated porphyrin **6** when preincubation was performed with the requisite *β*-galactosylated albumin. In each case, the contribution resulting from the interaction of the glycosylated porphyrin with the glycosylated albumin was taken into account (Experimental Section). A corresponding inhibitory effect (approximately 40%) was found for the O-α-mannosylated and *β*-galactosylated porphyrin conjugates in competition experiments involving murine melanoma cells (B16). This inhibitory effect was, however, significantly lower (around 20%) for human adenocarcinoma cells HT29. Note that the uptake for reference compound **2** and Meg-linked O-α-mannosylated porphyrin conjugate **16** was not affected by a preincubation with glycoconjugated albumins in the different cell lines studied.

Cellular Phototoxicity. Phototoxicity of the photosensitizer was determined in Y79 cells by cell survival fraction measurements after incubation and exposure to 514 nm light with a fluence of 1 J/cm^2 (Figure 5**)**. Toxicity in darkness was found to be negligible in all cases, with a survival fraction close to 100% (2 μM, 4 h incubation). It is important to mention that under the same experimental conditions (data not shown), Foscan (*m*THPC) was cytotoxic in the dark for Y79 cells with a 50% cell survival fraction for treated cells (2 μM, 4 h incubation), whereas this compound does not exhibit any dark cytotoxicity in other cell lines such as HT29.[19] As seen in Table 3, Y79 cells appeared to be highly sensitive to the PDT treatment

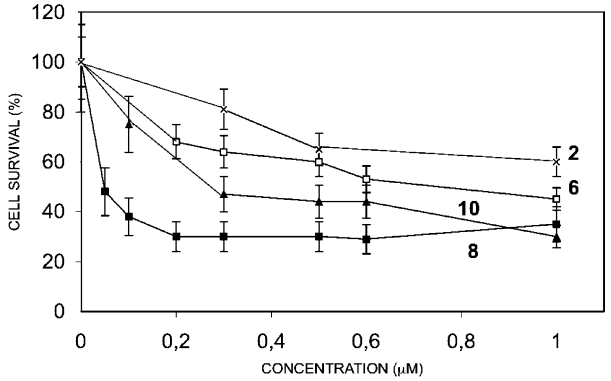


Figure 5. Phototoxicity concentration dependence of glycoconjugated dyes in Y79 cell line after incubation (4 h) and exposure to 514 nm light with a fluence of 1 J/cm^2. The error bars represent the standard error of the mean (SEM) of at least three experiments. [TPP(*p*-Deg-O-α-ManOH)$_3$] (**10**) (▲), [TPP(*p*-Deg-O-*β*-GalOH)$_3$] (**6**) (○) , [TPP(*p*-Deg-O-α-GalOH)$_3$] (8) (■), and [TPP(*p*-Deg-OH)$_3$] (**2**) (×).

Table 3. Dose of Porphyrin Derivatives (μM) Necessary to Observe 50% of Cell Survival (LD_{50}) after Incubation (4 h) and Exposure to 514 nm Light

	LD_{50}		
compd	Y79[a]	HT29[b]	B16[b]
TPP(*p*-DegOH)$_3$ (**2**)	>2	nd	nd
TPP(*p*-Deg-S-α-ManOH)$_3$ (**15**)	0.65	2	>2
TPP(*p*-Meg-O-α-ManOH)$_3$ (**16**)	0.6	0.85	0.44
TPP(*p*-Deg-O-α-GalOH)$_3$ (**8**)	0.05	0.1	0.82
TPP(*p*-Deg-S-*β*-GalOH)$_3$ (**13**)	0.6	2	0.82
TPP(*p*-Deg-O-*β*-GalOH)$_3$ (**6**)	0.6	0.68	0.25
TPP(*p*-Deg-O-α-ManOH)$_3$ (**10**)	0.35	0.43	0.35

[a] Fluence of 1 J/cm^2. [b] Fluence of 5 J/cm^2.

as indicated by the low LD_{50} values determined with a fluence of 1 J/cm^2, (in the range of 0.05−0.65 μM).

As a comparison, the phototoxicity has also been evaluated in HT29 and B16 cells (Table 3). The LD_{50} values determined in these two cell lines were significantly higher, despite a light fluence that was 5-fold higher (5 J/cm^2).

Concerning the correlation between photobiological efficiency and cellular uptake, for TPP(*p*-Deg-O-α-ManOH)$_3$ (**10**), the observed high cellular uptake appears to translate directly to a high photobiological activity (LD_{50} = 0.35 μM). Similarly, the low uptake rate for TPP(*p*-Deg-OH)$_3$ (**2**) may account for its poor photoactivity. Interestingly, however, α-galactosylated porphyrin **8** (containing 10% of *β*-isomer), which is only two times more internalized than the unconjugated dye, is highly phototoxic (LD_{50} = 0.05 μM).

Subcellular Localization. The subcellular localization of the photosensitizers was examined by fluorescence imaging microscopy. The cellular nucleus remained dark in all cases. For Y79 cells treated with unglycoconjugated porphyrin **2** (6 μM), the fluorescence was diffuse, being distributed throughout the cytoplasmic area independent of the incubation time. For cells treated with one of the glycoconjugated porphyrins for less than 3 h, a diffuse fluorescence pattern with some additional bright spots close to the nucleus was observed (Figure 6A). This distribution was similar to that observed when a co-staining was performed with DiOC6, a marker of the endoplasmic reticulum and mitochondria to a lesser extent. However, the fluorescence pattern was modified for longer incubation times and became very similar to that observed with co-staining with Nile Red (Figure 6B), indicating a preferential localization in the membranes of all cytoplasmic organelles. It should be pointed out that because Y79 cells are small round suspended cells (about

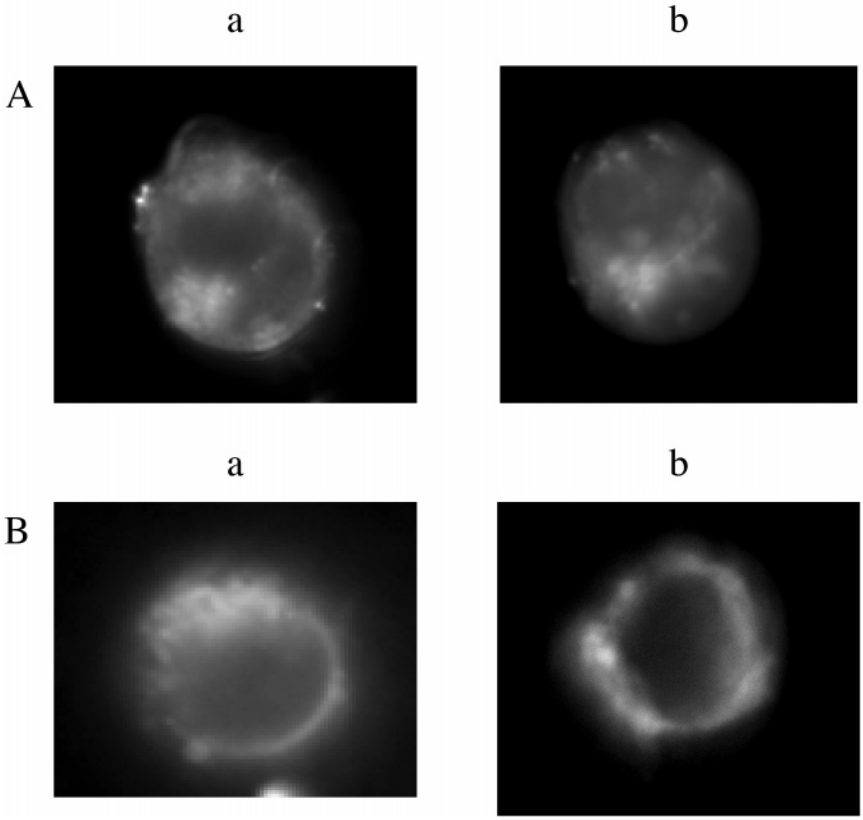


Figure 6. Y79 cell line colocalization fluorescence pattern. (A): (a) [TPP(*p*-Deg-O-*β*-GalOH)$_3$] (6 μM, 30 min incubation); (b) DiOC6, an endoplasmic reticulum marker (4 μM, 30 min incubation). (B): (a) [TPP(*p*-Deg-O-*β*-GalOH)$_3$] (6 μM, 6 h incubation); (b) Nile Red, a membrane marker (0.32 μM, 15 min incubation). A filter set (BP 425 DF45; FT 450 DCLP; LP 600 ALP) was used for the sensitizer, and a FITC filter set (BP450-FT510, LP 520) was used with fluorescent probes.

10 μm in diameter) with a large nucleus and a small cytoplasm a relationship between the subcellular localization and the photosensitizer structure remains difficult to establish.

Cellular Metabolism. An important issue when considering an in vivo use of glycoconjugated drugs is the possible cleavage of glycoside bonds by endogenous glycosidases.[20] This will result in the modification of the amphiphilic properties, biodistribution, blood clearance, and drug-cell interactions. Furthermore, in the case of a PDT treatment, the metabolism of glycoconjugated photosensitizers may also alter their photobiological activity. The metabolism of the series of O- and S-glycoconjugated porphyrins in Y79 cells was thus examined by analyzing cellular extracts using MALDI-TOF mass spectrometry.

The MALDI-TOF mass spectra of the extracts of Y79 cells treated with TPP(*p*-Deg-S-*β*-GalOH)$_3$ (**13**) and TPP(*p*-Deg-S-α-ManOH)$_3$ (**15**) displayed, as starting compounds (Supporting Information), a major peak at *m*/*z* 1462 corresponding to the protonated glycoconjugated molecule (MH^+) and two minor peaks at *m*/*z* 1239 and 1315. These minor peaks were also present in the spectrum of an extract obtained from a solution of glycoconjugated porphryins in cell culture medium alone, indicating clearly that as anticipated the S-glycoconjugated porphyrins are metabolically stable.

For the O-glycoconjugated porphyrins, the mass spectral based metabolism study proved to be considerably more complicated. As illustrated for the MALDI-TOF spectrum of TPP(*p*-Deg-O-α-ManOH)$_3$ (**10**) (Figure 7a) obtained from cellular extracts after incubation by Y79 cells, in addition to the signal of the protonated intact glycoconjugated molecule (MH^+, *m*/*z* 1413), other signals were observed at *m*/*z* 1251, 1207, and 1163, corresponding to one sugar unit, one sugar and a monoethylene glycol (C_2H_4O) fragment, and one sugar and a diethylene glycol ($C_4H_8O_2$) fragment, respectively. These peaks were also observed to be present in the spectra for O-glycoconjugated compounds **6** and **8**. A natural reflex is to attribute these peaks to fragments derived from the parent MH^+ ion. However, MS-MS experiments performed on the parent molecular ion (*m*/*z* 1413) and the ion corresponding to a diglycosylated molecule (*m*/*z* 1251) did not reveal any gas phase fragmentation. Furthermore, the ESI mass spectral analysis also showed the presence of signals at *m*/*z* 1251 and 1163 (data not shown). It was also possible, but not certain (especially for the peak at *m*/*z* 1207), that these peaks arise from trace impurities carried through the synthesis of the glycoconjugated porphyrins (*m*/*z* 1163 could correspond to the presence of traces of diglycoconjugated compounds). The origin of these extraneous peaks remains obscure at this time.

To obtain a qualitative estimation of the metabolic stability of the Deg-linked O-glycoconjugated porphyrins, an experiment was initially carried out in which TPP(*p*-Deg-O-α-ManOH)$_3$ (**10**) was incubated with α-mannosidase (from almond) for 24 h. In the MALDI-TOF spectrum of the extracted material (Figure 7c), the MH^+ signal at *m*/*z* 1413 was lower in intensity than the peak at *m*/*z* 1251. This peak was similarly markedly stronger than the peaks at *m*/*z* 1207 and 1163, demonstrating that enzyme-induced cleavage of one of the sugar motifs had occurred. Lower intensity peaks were also observed at *m*/*z* 1089 and 927, corresponding to the loss of second and third mannose units, respectively.

By subsequently comparing the MALDI-TOF mass spectrum for the starting synthetic material (Figure 7b) to that for the extract obtained after the incubation of **10** with Y79 cells (Figure 7a), it was determined that the ratio of the *m*/*z* 1413 to *m*/*z*

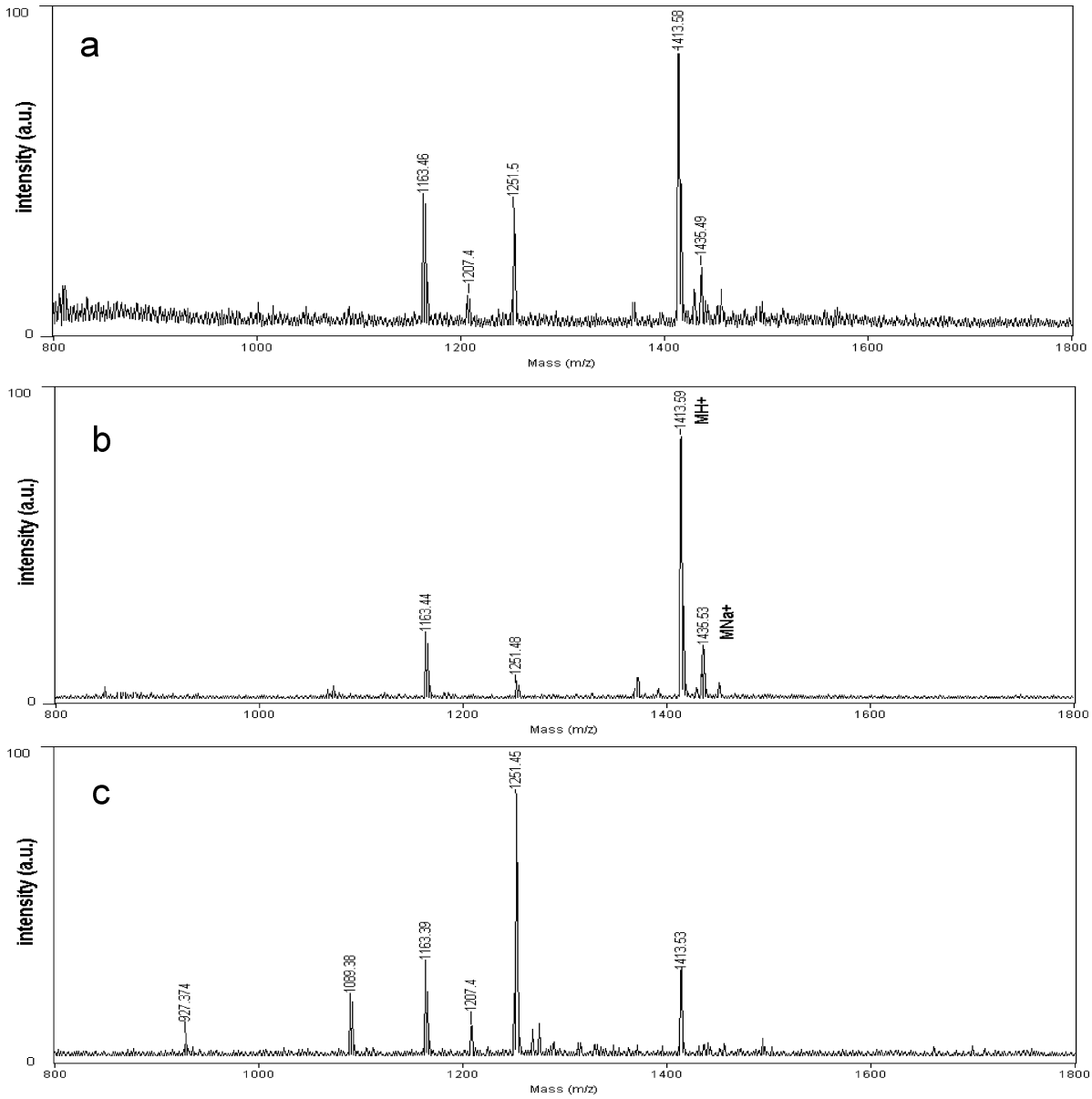


Figure 7. MALDI-TOF mass spectra of TPP(*p*-Deg-O-α-ManOH)$_3$ (**10**). (a) Cellular extract of treated Y79 cells (6 μM, 10 h incubation); (b) starting compound; (c) incubation with α-mannosidase (0.8 IU, Na acetate buffer pH 5, Zn Cl_2; 0.3 mM, 37 °C, 24 h).

1251 peak heights had changed. This served as an indication that metabolic cleavage of a single sugar unit in **10** had occurred to a small extent. Similar results were obtained for O-α-Gal porphyrin **8**. Interestingly, in the case of cells treated with O-*β*-Gal porphyrin **6**, a minor signal at *m*/*z* 1089 corresponding to the loss of two sugar units was also observed. Similar features were observed for the cellular extracts of HT29 and B16 cells treated with the glycoconjugated compounds (data not shown). These results suggest that only limited cellular metabolic degradation of the O-glycoconjugated porphyrins occurs in Y79 cells.

Discussion

The photobiological properties of our glycoconjugated porphyrins are strongly dependent upon the nature of the linker and the nature and anomeric configuration of the glycoside residue.

The increase in the spacer length linking the tetrapyrrolic ring and the sugar moiety resulted in higher cellular uptake in the case of Deg-O-*β*-GalOH (**6**), Deg-S-*β*-GalOH (**13**), and Deg-O-α-ManOH (**10**) derivatives relative to that of nonglycoconjugated compound **2**. In addition, a considerable decrease in drug internalization was observed for Deg-O-α-GalOH (**8**)[2] relative to that for Deg-O-*β*-GalOH (**6**) derivatives. This observation correlates with the idea that modifications in molecular structure result in strong variations of the partition coefficient values. Indeed, it is well established that the overall lipophilicity of the photosensitizer plays an important role in the drug-membrane interaction. The lower cellular uptake of the glycoconjugated derivatives **(8**, **15**, and **16)** can clearly be related to their higher lipophilicity, (log(PC), Table 2**).** In contrast, the most internalized compounds are those with a high amphiphilic character, in particular, TPP(*p*-Deg-O-α-ManOH)$_3$ (**10**) (log(PC) = 0.02).

However, as shown by the effect of glucose, the cellular internalization of glycoconjugated porphyrins may occur via an active mechanism involving a cellular transporter or receptor. It is important to consider the presence of such sugar receptors that can be overexpressed on tumoral cell membranes and be specific for a given cell line. Griegel and co-workers[13] showed

that the plasma membranes of retinoblastoma cells (Y79) bear α-mannose and β-galactose receptors. The melanoma B16 cells and adenocarcinoma colon cells HT29 have been found to overexpress β-galactose and β-glucose receptors, respectively.[9,13] Our data show that the relative drug uptake of the glycoconjugated porphyrins was similar and independent of the cell lines studied. No conclusions can thus be drawn about the involvement of any specific interaction between the carbohydrate moiety and some membrane sugar receptor. However, the inhibition effects observed when the cells were preincubated with glycosylated albumin prior to the incubation with the corresponding glycosylated photosensitizer clearly demonstrates a partial saturation of possible cellular binding sites. To obtain more information on this phenomena, a semiquantitative method was developed on the basis of the use of magnetic nanoparticles coated with glycosylated albumin (manuscript in preparation). In experiments with this system carried out at 4 °C (ip, under conditions where transport across the cell membrane is limited), it was observed that the albumin−glycoside remained located on the cell surface. The number of mannose and galactose receptors determined by colorimetric dosage of iron was found to be similar for B16 and Y79 cells. In contrast, only glucose receptors could be characterized on HT29 cells. These findings are thus consistent with the similar inhibition effects on cellular uptake observed for both B16 and Y79 cell lines as well as the significantly lower inhibition effect observed in the case of HT29 cells. It is also necessary to consider that the targeting of sugar receptors may not be highly selective. Recently, Li et al.[11] showed that the binding affinity between a benzochlorin−galactose conjugate and an isolated galectin was only two times that of a benzochlorin−glucose conjugate. The nonglycoconjugated parent benzochlorin unit was also demonstrated to exhibit some significant galectin-binding nonspecific interaction, which is a general phenomenon for a tetrapyrrolic ring.

The Y79 cells appeared to be highly sensitive to the PDT treatment with glycoconjugated porphyrins.[21] As expected, the most internalized compound, amphiphilic TPP(*p*-Deg-O-α-ManOH)$_3$ (**10**) exhibits good photoactivity ($LD_{50} = 0.35\ \mu M$). Surprisingly, the O-α-galactosylated porphyrin **8**, which is only two times more internalized than the unglycosylated dye (**2**), proved to be highly photoactive ($LD_{50} = 0.05\ \mu M$). Our group[6] and others[12,22] have already observed such a lack of correlation. Several hypotheses such as a localization in different subcellular compartments that plays a major role in the photodynamic efficiency can be considered.[23] The fluorescence imaging experiments clearly shows that compared to unconjugated porphyrin **2** the presence of Deg-O-glycosyl moieties linked to photosensitizers results in modifications of the subcellular distribution. However, it is not possible to establish any significant difference related to the nature of the glycoside. The singlet oxygen producing ability ($^1O_2$) can also be considered. However, the $^1O_2$ quantum yield as determined by the direct measurement of the IR luminescence (1270 nm) of the sensitizer has been found to be quite similar for all of the Deg-O-glycoconjugated porphyrins within experimental errors (around 0.47).[24] The $^1O_2$ formation yield cannot, thus, be considered as a determining parameter in the photoefficiency of these glycoconjugated porphyrins.

It may be noted that the photoefficiency of the glycoconjugated porphyrins has been evaluated at 514 nm. Despite a low penetration depth of 514 nm light in tissues, some effective effects have been observed in patients undergoing PDT treatment at a depth higher than that expected on the basis of tissue optical properties.[25,26] Compared to red (630 nm) light, green light has some potential to cure superficial cancer, preventing deep tissue damages. Owing to the very small size of the retinoblastoma to be treated, damages to healthy retinal tissue could thus be avoided.

In conclusion, in this new series of novel glycoconjugated diethylene-glycol porphyrin derivatives, two of them, TPP(*p*-Deg-O-α-GalOH)$_3$ (**8**) and TPP(*p*-Deg-O-α-ManOH)$_3$ (**10**), exhibit a particularly high in vitro photobiological activity in human retinoblastoma cells. Compared to the values reported in the literature for other tumoral cell lines, very low doses of the drug associated with low light fluences (about 10 times lower) are sufficient to observe an in vitro high photobiological effect in Y79 cells. Moreover, they only undergo a very limited cellular metabolic degradation. It should be noted that enzymatic activities in cells and plasma are, in general, different, and a higher degradation in an in vivo situation cannot be totally excluded. In this context, the replacement of O-β-GalOH by a S-β-GalOH (porphyrin **13)** that has been demonstrated to avoid any enzymatic cleavage could be of interest. On the basis of this in vitro study, work is now in progress to further evaluate the in vivo photodynamic activity of two α-manno/galacto conjugated porphyrins **8** and **10** on nude mice bearing human retinoblastoma xenografts.

Experimental Section

All solvents used were reagent grade. The following reagents have been abbreviated: 1,2-dichloroethane (1,2-DCE), dimethyl formamide (DMF), dimethylesulfoxide (DMSO), fetal calf serum (FCS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), phosphate buffer saline (PBS), and Dulbecco's modified Eagle's medium (DMEM). Dry MeOH was kept over 3 Å sieves, and dichloromethane was distilled from calcium hydride and kept over 4 Å sieves. Dimethylformamide was distilled under slow argon flow and kept over 4 Å sieves. Column chromatography was performed with the indicated solvents using E. Merck silica gel 60 (particle size 0.035−0.070 mm). Macherey-Nagel precoated plates (SIL G-200, 2 mm) were used for preparative thin-layer chromatography. Yields refer to chromatographically and spectroscopically pure compounds. ^1H and ^{13}C NMR spectra were recorded on a Bruker AC-300 spectrometer at ambient temperature using an internal deuterium lock. Chemical shift values are given in ppm relative to tetramethyl silane (TMS). Acidic impurities in $CDCl_3$ were removed by treatment with anhydrous K_2CO_3. Quantitative UV−visible spectra were obtained using a Varian DMS 200 spectrometer (molar extinction coefficient values are given in L/mmol.cm), and fluorescence spectra were obtained using a Cary-Eclipse (Varian) fluorimeter. Microanalyses were performed by the ICSN-CNRS Elemental Analysis Center at Gif-sur-Yvette, France.

Chemistry. 5,10,15-tri{*p*-*O*-2-[(2-ethoxy)-ethanol]phenyl}-20-phenyl porphyrin (TPP(*p*-Deg-OH)$_3$) (2). A mixture of 5,10,15-tri(*para*-hydroxyphenyl)-20-phenyl porphyrin (TPP(*p*-OH)$_3$) (65 mg, 0.1 mmol), 2-(2-chloro-ethoxy)-ethanol (106 μL, 1 mmol), KI (20 mg, 0.12 mmol), and K_2CO_3 (0.400 g, 2.9 mmol) in dry DMF (10 mL) was stirred at 100 °C for 4 h under argon. The mixture was concentrated under vacuum, then taken-up in $CH_2Cl_2-H_2O$, and washed with H_2O (3×). The organic layer was dried over Na_2SO_4 and concentrated. Compound **2** (82 mg, 90%) was obtained as a red powder after silica gel column chromatography (CH_2Cl_2/MeOH; 100:1) and crystallization from CH_2Cl_2−heptane. UV−vis spectrum in (CH_2Cl_2): λ_{max}, nm (ε): 420 (436), 517 (16.4), 553 (10), 592 (5.1), 648.5 (5.2). ^1H NMR ($CDCl_3$) δ 8.84 (m, 8H), 8.2 (dd, 2H, J = 7.7 and 2 Hz), 8.09 (t, 6H, J = 8.6 Hz), 7.74 (m, 2H), 7.25 (t, 3H, J = 8.6 Hz), 7.24 (t, 3H, J = 8.6 Hz), 4.36 (m, 6H), 4.01 (m, 6H), 3.86 (t, 6H, J = 4.4 Hz), 3.78 (t, 6H, J = 4.4 Hz). ^{13}C NMR ($CDCl_3$) δ 158.47, 142, 135.56, 134.81, 131, 127.66, 126.57, 119.9, 119.8, 119.7, 112.8, 72.69, 69.76, 67.63, 61.89. Anal. ($C_{56}H_{54}N_4O_9$, 2 H_2O) C, H, N.

2-[2-(2-Bromo-ethoxy)-ethoxy]-*O*-2′,3′,4′,6′-tetraacetyl-D-galactose (α-3 and β-3). BF_3 etherate (7.5 mL, 61 mmol) was added

slowly (15 min) to a solution of 1,2,3,4,6-pentaacetyl β-D-galactose (5.85 g, 15 mmol) and 2-(2-bromoethoxy)-ethanol[15] (3.73 g, 18 mmol) in dry CH_2Cl_2 (30 mL) at 0 °C under argon. The solution was stirred for 1 h at 0 °C and overnight at room temperature. The mixture was then poured in ice water and extracted with CH_2Cl_2. The organic phases were combined, washed with water and aqueous $NaHCO_3$ and water, dried over Na_2SO_4, filtered, and concentrated under vacuum. The two products were obtained as colorless oils after silica gel column chromatography (EtOAc/heptane; 2:3). The less polar component corresponded to the α anomer **a-3** (2.11 g, 28%) and the more polar component to ***β*-3** (1.47 g, 19%). Intermediate fractions containing a mixture of the two products were also collected (**α-3**/***β*-3**; 10.5%). **α-3**: ^{1}H NMR ($CDCl_3$) δ 5.44 (s, 1H), 5.38 (dd, 1H, J = 8.5 and 2.8 Hz), 5.15 (m, 2H), 4.31 (t, 1H, J = 6.6 Hz), 4.08 (d, 2H, J = 6.5 Hz), 3.79 (t, 4H, J = 5.6 Hz), 3.67 (m, 2H), 3.44 (t, 2H, J = 5.7 Hz), 2.13 (s, 3H), 2.06 (s, 3H), 2.03 (s, 3H), 1.97 (s, 3H). Anal. ($C_{18}H_{27}BrO_{11}$, 2 H_2O) C, H. ***β*-3**: ^{1}H NMR ($CDCl_3$) δ 5.37 (d, 1H, J = 2.8 Hz), 5.19 (dd, 1H, J = 8 and 10.5 Hz), 4.99 (dd, 1H, J = 3.4 and 10.5 Hz), 4.59 (d, 1H, J = 8 Hz), 4.13 (m, 2H), 3.92 (m, H), 3.77 (m, 4 H), 3.62 (m, 2H), 3.39 (m, 2H), 2.17 (s, 3H), 2.05 (s, 3H), 2.03 (s, 3H), 1.97 (s, 3H). Anal. ($C_{18}H_{27}BrO_{11}$, EtOAc) C, H.

2-[2-(2-Bromo-ethoxy)-ethoxy]-*O*-2′,3′,4′,6′-tetraacetyl-α-D-mannose (4). Prepared as in **3** from 1,2,3,4,6-pentaaacetyl-α-D-mannose. Compound **4** (5.63 g; 75%) was obtained as a colorless oil after silica gel column chromatography (CH_2Cl_2/Et_2O; 10:1). ^{1}H NMR ($CDCl_3$) δ 5.36−5.26 (m, 3H), 4.88 (d, 1H, J = 1.5 Hz), 4.28 (dd, 1H, J = 12.8 and 5.7 Hz), 4.11 (m, 1H, J = 12.6 and 2.5 Hz), 4.11 (m, 1H), 3.84 (m, 1H), 3.82 (t, 2H, J = 6.1 Hz), 3.68 (m, 3H), 3.46 (t, 2H, J = 6.1 Hz), 2.14 (s, 3H), 2.10 (s, 3H), 2.02 (s, 3H), 1.98 (s, 3H). Anal. ($C_{18}H_{27}BrO_{11}$) C, H.

5,10,15-tri{*p*-*O*-[2-(2-*O*-(2′,3′,4′,6′-tetraacetyl-*β*-D-galactosyloxy)-ethoxy)-ethoxy] phenyl}-20-phenyl porphyrin (TPP(*p*-Deg-*O*-*β*-GalOAc)$_3$) (5). A mixture of TPP(*p*-OH)$_3$ (45 mg, 67.8 μmol), compound ***β*-3** (0.450 g, 0.9 mmol), and K_2CO_3 (0.355 g, 2.5 mmol) in dry DMF (30 mL) was stirred under argon at 60 °C overnight. The mixture was concentrated under vacuum, then taken-up in CH_2Cl_2/H_2O, and washed with H_2O (3×). The organic layer was dried over Na_2SO_4 and concentrated. The product was partially purified by three recrystallizations from CH_2Cl_2/heptane and then further purified by silica gel column chromatography (CH_2Cl_2/acetone; 10:1) and crystallization from CH_2Cl_2/heptane. Porphyrin **5** (50 mg; 38%) was obtained as a red powder. UV−vis spectrum in CH_2Cl_2: λ_{max}, nm (ϵ): 421 (429), 517.5 (18.5), 554 (12.8), 593 (8.2), 649.5 (8.5). ^{1}H NMR ($CDCl_3$) δ 8.85 (s, 2H), 8.84 (s, 2H), 8.83 (s, 2H), 8.82 (s, 2H), 8.21 (dd, 2H, J = 5.8 and 1.8 Hz), 8.12 (dd, 6H, J = 8.5 Hz), 7.75 (m, 1H, J = 6.5 Hz), 7.75 (m, 2H), 7.28 (d, 6H, J = 8.5 Hz), 5.43 (d, 3H, J = 3.3 Hz), 5.29 (t, 6H, J = 9.4 Hz), 5.09 (dd, 3H, J = 10.4 and 3.4 Hz), 4.67 (d, 3H, J = 7.9 Hz), 4.40 (t, 6H, J = 4 Hz), 4.20 (m, 6H), 4.08 (m, 6H), 4.03 (t, 6H, J = 4.5 Hz), 3.97 (m, 6H), 3.87 (m, 6H), 3.87 (m, 3H), 2.17 (s, 9H), 2.14 (s, 9H), 2.06 (s, 9H), 2.0 (s, 9H), − 2.77 (s, 2H). ^{13}C NMR ($CDCl_3$) δ 170.38, 170.24, 170.14, 169.54, 158.53, 142.19, 135.55, 134.81, 134.5, 131, 127.64, 126.63, 119.85, 119.79, 119.7, 112.78, 101.44, 70.92, 70.71, 70.61, 70.03, 69.23, 68.87, 67.7, 67.08, 61.3, 20.84, 20.66, 20.58. Anal. ($C_{98}H_{108}N_4O_{36}$, H_2O) C, H, N.

5,10,15-tri{*p*-*O*-[2-(2-*O*-*β*-D-galactosyloxy)-ethoxy)-ethoxy]-phenyl}-20-phenyl porphyrin (TPP(*p*-Deg-*O*-*β*-GalOH)$_3$) (6). To a solution of **5** (40 mg, 2.1 μmol) in dry MeOH (10 mL) and dry CH_2Cl_2 (10 mL) was added a solution of NaOMe in MeOH (100 μL; 1 M), and the mixture was slowly stirred for 1.5 h at room temperature. IWT TMD-8 ion-exchange resin (1 g) was then added, and gentle stirring was continued for 30 min. The reaction mixture was filtered, and the recovered resin was washed with MeOH and pyridine. The combined filtrate and washings were then evaporated to dryness and crystallized from MeOH/1,2-DCE. Product **6** (29 mg, 98%) was obtained as a red powder. MALDI-TOF MS calcd for $C_{74}H_{84}N_4O_{24}$ (MH^+), 1413.55; found, 1413.60 (additional small peaks 1163.48, 1251.58). UV−vis spectrum in MeOH/pyridine (24:1): λ_{max}, nm (ϵ): 417 (392.1), 515.5 (17.3), 551.5 (12.4), 592 (7.7), 648.5 (8). ^{1}H NMR (pyridine d_5) δ 9.17 (s, 4H), 9.13 (d, 2H, J = 5.1 Hz), 9.06 (d, 2H, J = 4.6 Hz), 8.37 (t, 2H, J = 3.2 Hz), 8.26 (d, 6H, J = 8.2 Hz), 7.8 (m, 3H), 7.42 (d, 6H, J = 7.8 Hz), 4.9 (d, 3H, J = 7.9 Hz), 4.6 (m, 3H), 4.56 (t, 3H, J = 8.5 Hz), 4.51 (m, 3H), 4.49 (m, 3H), 4.43 (t, 6H), 4.40 (m, 3H), 4.21 (dd, 3H, J = 8.3 and 3 Hz), 4.11 (m, 3H), 4.08 (m, 3H), 4.05 (m, 6H), 3.93 (t, 6H), −2.28 (s, 2H). ^{13}C NMR (pyridine d_5) δ 159.43, 142.62, 136, 135.04, 131.8, 128.3, 127.39, 120.83, 120.69, 120.49, 113.59, 105.57, 77.14, 75.43, 72.61, 71.21, 70.35, 70.1, 69.11, 68.25, 62.53. Anal. ($C_{74}H_{84}N_4O_{24}$, 6 H_2O) C, H, N.

5,10,15-tri{*p*-*O*-[2-(2-*O*-(2′,3′,4′,6′-tetraacetyl-α-D-galactosyloxy)-ethoxy)-ethoxy] phenyl}-20-phenyl porphyrin (TPP(*p*-Deg-*O*-α-GalOAc)$_3$) (7). Prepared as in **5**. Compound **7** (66 mg, 51%) was obtained as a red powder after silica gel column chromatography (CH_2Cl_2/acetone; 8:1) and crystallization from CH_2Cl_2/heptane. UV−vis spectrum in CH_2Cl_2: λ_{max}, nm (ϵ): 421 (439.2), 517.5 (18.2), 554 (12.5), 593 (7.8), 649.5 (8.8). ^{1}H NMR ($CDCl_3$) δ 8.87 (s, 6H), 8.83 (d, 2H, J = 4.7 Hz), 8.21 (d, 2H, J = 6.2 Hz), 8.12 (d, 6H, J = 8.2 Hz), 7.74 (m, 1H, J = 6.5 Hz), 7.74 (m, 2H), 7.31 (d, 6H, J = 8.2 Hz), 5.52 (m, 3H), 5.47 (dd, 3H, J = 10.7 and 3.3 Hz), 5.28 (d, 3H, J = 3.5 Hz), 5.19 (dd, 3H, J = 14.2 and 3.5 Hz), 4,67 (d, 10% of β-isomer **5**) 4.42 (t, 6H, J = 4 Hz), 4.41 (m, 3H), 4.17 (d, 3H, J = 1.6 Hz), 4.15 (d, 3H, J = 2.4 Hz), 4.04 (m, 6H), 3.95 (m, 3H), 3.87 (m, 6H), 3.81 (m, 3H), 2.16 (s, 9H), 2.15 (s, 9H), 2.08 (s, 9H), 2 (s, 9H), −2.77 (s, 2H). ^{13}C NMR ($CDCl_3$) δ 170.48, 170.31, 170.09, 158.57, 142.25, 135.62, 134.87, 134.56, 131, 127.66, 126.68, 119.89, 119.85, 119.75, 112.82, 96.38, 70.46, 70.01, 68.2, 67.8, 67.66, 66.3, 61.73, 20.91, 20.78, 20.72, 20.7. Anal. ($C_{98}H_{108}N_4O_{36}$, 2 H_2O) C, H, N.

5,10,15-tri{*p*-*O*-[2-(2-*O*-α-D-galactosyloxy)-ethoxy)-ethoxy]-phenyl}-20-phenyl porphyrin (TPP(*p*-Deg-*O*-α-GalOH)$_3$) (8) (Containing 10% of *β*-isomer 6). Prepared as in **6**. Product **8** (29 mg, 98%) was obtained as a red powder after crystallization from MeOH/1,2-DCE. MALDI-TOF MS calcd for $C_{74}H_{84}N_4O_{24}$ (MH^+) 1413.55; found, 1413.49 (additional small peaks 1163.48, 1236.51, 1251.58). UV−vis spectrum in MeOH/pyridine (24:1): λ_{max}, nm (ϵ): 417 (411.8), 515.5 (17.7), 552 (12.4), 592 (7.6), 648.5 (7.9). ^{1}H NMR (pyridine d_5) δ 9.16 (s, 4H), 9.13 (d, 2H, J = 4.8 Hz), 9.06 (d, 2H, J = 4.7 Hz), 8.37 (m, 2H), 8.25 (d, 6H, J = 7.6 Hz), 7.8 (m, 3H), 7.44 (d, 6H, J = 8.3 Hz), 5.5 (d, 3H, J = 3.6 Hz), 4.68 (m, 3H), 4.64 (m, 3H), 4.61 (m, 3H), 4.5 (m, 6H), 4.43 (t, 6H), 4.25 (m, 3H), 4.11 (m, 6H), 3.98 (m, 3H), 3.97 (m, 3H), 3.96 (t, 6H), −2.29 (s, 2H). ^{13}C NMR (pyridine d_5) δ 159.43, 142.65, 136.1, 135.03, 134.79, 132, 128.3, 127.37, 120.83, 120.68, 120.48, 113.58, 101.19, 72.91, 71.76, 71.12, 70.96, 70.70, 70.1, 68.25, 67.85, 62.79. Anal. ($C_{74}H_{84}N_4O_{24}$, 8 H_2O) C, H, N.

5,10,15-tri{*p*-*O*-[2-(2-*O*-(2′,3′,4′,6′-tetraacetyl-α-D-mannosyloxy)-ethoxy)-ethoxy] phenyl}-20-phenyl porphyrin (TPP(*p*-Deg-*O*-α-ManOAc)$_3$) (9). Prepared as in **5**. Compound **9** (151 mg, 23%) was obtained as a red powder after column chromatography on silica gel (CH_2Cl_2/acetone; 10:1) and crystallization from CH_2Cl_2/heptane. UV−vis spectrum in CH_2Cl_2: λ_{max}, nm (ϵ): 419 (429.5), 516 (16.8), 553 (10.5), 592 (5.5), 647.5 (5.3). ^{1}H NMR ($CDCl_3$) δ 8.86 (s, 4H), 8.63 (dd, 4H), 8.22 (dd, 2H, J = 7.6 and 1.6 Hz), 8.10 (d, 6H, J = 8.5 Hz), 7.75 (m, 3H), 7.31 (d, 6H, J = 8.6 Hz), 5.47 (dd, 3H, J = 10.1 and 3.4 Hz), 5.35 (m, 6H), 4.99 (s, 3H), 4.42 (m, 9H), 4.36 (dd, 3H, J = 4.8 and 12 Hz), 4.16 (m, 3H), 4.06 (m, 6H), 3.95 (m, 6H), 3.88 (m, 6H), 3.60 (m, 6H), 2.18 (s, 9H), 2.14 (s, 9H), 2.04 (s, 9H), 2.00 (s, 9H), −2.76 (s, 2H). ^{13}C NMR ($CDCl_3$) δ 170.7, 170.1, 170, 169.8, 158.7, 142.3, 135.7, 134.9, 134.65, 131, 127.8, 126.8, 112.9, 70.4, 70.1, 69.7, 69.2, 68.6, 67.8, 67.6, 66.3, 62.5, 21.2, 20.85, 20.78, 20.75. Anal. ($C_{98}H_{108}N_4O_{36}$) C, H, N.

5,10,15-tri{*p*-*O*-[2-(2-*O*-α-D-mannosyloxy)-ethoxy)-ethoxy]-phenyl}-20-phenyl porphyrin (TPP(*p*-Deg-*O*-α-ManOH)$_3$) (10). Prepared as in **6**. Compound **10** (103 mg, 100%) was obtained as a red powder after crystallization from MeOH/1,2-DCE. MALDI-TOF MS calcd $C_{74}H_{84}N_4O_{24}$ (MH^+) 1413.55; found, 1413.61. UV−vis spectrum in MeOH/pyridine (24:1): λ_{max}, nm (ϵ): 417.5 (359.2), 516 (15.3), 551.5 (10.5), 592 (6.3), 648 (6). ^{1}H NMR (pyridine d_5) δ 9.16 (s, 4H), 9.13 (d, 2H, J = 4.8 Hz), 9.05 (d, 2H, J = 5.2 Hz),

8.36 (m, 2H), 8.25 (d, 6H, $J = 8.5$ Hz), 7.79 (m, 3H), 7.43 (d, 6H, $J = 8.6$ Hz), 5.51 (s, 3H), 4.65 (m, 6H), 4.63 (m, 3H), 4.46 (m, 3H), 4.41 (m, 9H), 4.22 (m, 3H), 4.04 (m, 6H), 3.91 (m, 6H), 3.89 (m, 3H), −2.31 (s, 2H). ^{13}C NMR (pyridine d_5) δ 159.41, 142.66, 136.15, 136, 135.03, 132, 128.26, 127.36, 120.7, 113.57, 102, 75.55, 73.16, 72.21, 70.9, 70.1, 69.3, 68.2, 67.05, 63.34. Anal. ($C_{74}H_{84}N_4O_{24}$, $2H_2O$) C, H, N.

5,10,15-tri{*p*-*O*-[2-(2-bromo-ethoxy)-ethoxy]phenyl}-20-phenyl porphyrin (TPP(*p*-Deg-Br)$_3$) (11). A mixture of TPP(*p*-OH)$_3$ (100 mg, 0.15 mmol), 2-dibromoethyl ether (0.524 g, 2.26 mmol), and K_2CO_3 (0.621 g, 4.5 mmol) in dry DMF (20 mL) was stirred at 50 °C for 4 h. The mixture was concentrated under vacuum, then taken-up in CH_2Cl_2/H_2O, and washed with H_2O (3×). The organic layer was dried over Na_2SO_4 and concentrated. Compound **11** (87 mg; 51%) was obtained as a red powder after silica gel column chromatography (CH_2Cl_2) and crystallization from CH_2Cl_2/heptane. UV−vis spectrum in CH_2Cl_2: λ_{max}, nm (ϵ): 421 (542.5), 517.5 (22.7), 554 (15), 593 (9.3), 649 (9). ^{1}H NMR ($CDCl_3$) δ 8.85 (m, 8H), 8.21 (dd, 2H, $J = 9.1$ and 2 Hz), 8.10 (d, 6H, $J = 8.4$ Hz), 7.75 (m, 3H), 7.26 (d, 6H, $J = 8.8$ Hz), 4.38 (m, 6H, $J = 5.8$ Hz), 4.03 (m, 6H), 4.0 (t, 6H, $J = 6.3$ Hz), 3.59 (t, 6H, $J = 6.3$ Hz), −2.75 (s, 2H). ^{13}C NMR ($CDCl_3$) δ 158.2, 142.1, 135.5, 135, 134.7, 130.8, 127.5, 126.9, 119.5, 112.8, 71.4, 69.9, 67.5, 30. Anal. ($C_{56}H_{51}N_4O_6Br_3$) C, H, N.

5,10,15-tri{*p*-*O*-[2-(2-*S*-(2′,3′,4′,6′-tetraacetyl-β-D-thiogalactosyloxy)-ethoxy)-ethoxy}phenyl-20-phenyl porphyrin (TPP(*p*-Deg-*S*-β-GalOAc)$_3$) (12). To a mixture of **11** (87 mg, 78 μmol) and K_2CO_3 (100 mg, 0.72 mmol) in dry acetone (25 mL), freshly prepared 1-thio-2,3,4,6-tetraacetyl-β-D-galactose from 1-pseudo-thio-ureyl-2,3,4,6-tetraacetyl-β-D-galactose (170 mg, 0.38 mmol) was added. The mixture was stirred overnight at room temperature, then concentrated under vacuum and diluted in CH_2Cl_2, washed with H_2O, dried over Na_2SO_4, and concentrated. Pure product **12** was obtained as a red powder after silica gel column chromatography (CH_2Cl_2/Et_2O; 1:1) and crystallization from CH_2Cl_2/heptane (112 mg, yield 73%). UV−vis spectrum in CH_2Cl_2: λ_{max}, nm (ϵ): 421 (403.8), 517.5 (16.2), 554 (10.6), 593 (6.1), 649 (6). ^{1}H NMR ($CDCl_3$) δ 8.86 (m, 8H), 8.21 (dd, 2H, $J = 5.9$ and 1.5 Hz), 8.12 (d, 6H, $J = 8.4$ Hz), 7.75 (m, 3H), 7.30 (d, 6H, $J = 8.5$ Hz), 5.47 (d, 3H, $J = 2.8$ Hz), 5.29 (t, 3H, $J = 10$ Hz), 5.10 (dd, 3H, $J = 10$ and 3.4 Hz), 4.67 (d, 3H, $J = 9.9$ Hz), 4.41 (m, 6H), 4.23 (dd, 3H, $J = 11.4$ and 6.5 Hz), 4.16 (dd, 3H, $J = 11.4$ and 6.5 Hz), 4.03 (m, 6H), 3.99 (m, 3H), 3.91 (m, 6H), 3.12 (m, 6H), 2.94 (m, 6H), 2.19 (s, 9H), 2.11 (s, 9H), 2.07 (s, 9H), 1.99 (s, 9H), −2.75 (s, 2H). ^{13}C NMR ($CDCl_3$) δ 170.41, 170.24, 170.06, 169.66, 158.56, 142.25, 135.61, 134.92, 134.56, 131, 127.69, 126.69, 119.91, 119.84, 119.74, 112.86, 84.23, 74.56, 71.92, 71.49, 69.7, 67.71, 67.39−67.35, 61.52, 29.65, 20.91, 20.75, 20.63. Anal. ($C_{98}H_{108}N_4O_{33}S_3$, 2 H_2O) C, H, N.

5,10,15-tri{*p*-*O*-[2-(2-*S*-β-D-thiogalactosyloxy)-ethoxy)-ethoxy]-phenyl}-20-phenyl porphyrin (TPP(*p*-Deg-*S*-β-GalOH)$_3$) (13). Prepared as in **6**. Titled product **13** (29 mg, yield 98%) was obtained as a red powder after crystallization from MeOH/1,2-DCE. MALDI-TOF MS calcd for $C_{74}H_{84}N_4O_{21}S_3$ (MH^+) 1461.48; found, 1461.44. UV−vis spectrum in MeOH/pyridine (24:1) λ_{max}, nm (ϵ): 424.5 (370.3), 519 (17.1), 556 (12.7), 595.5 (7.7), 652 (7.9). ^{1}H NMR (pyridine d_5) δ 9.17 (s, 4H), 9.13 (d, 2H, $J = 4.7$ Hz), 9.07 (d, 2H, $J = 4.5$ Hz), 8.37 (m, 2H), 8.25 (d, 6H, $J = 7.5$ Hz), 7.80 (m, 3H), 7.41 (d, 6H, $J = 7.5$ Hz), 5.06 (d, 3H, $J = 9.4$ Hz), 4.65 (m, 3H), 4.57 (t, 3H, $J = 9$ Hz), 4.49 (m, 6H), 4.38 (s broad, 6H), 4.21 (m, 3H), 4.15 (m, 3H), 4.05 (dd, 6H, $J = 11.3$ and 6.5 Hz), 3.99 (broad, 6H), 3.38 (m, 3H), 3.2 (m, 3H), −2.27 (s, 2H). ^{13}C NMR (pyridine d_5) δ 159.4, 142.66, 136, 135.05, 132, 128.33, 127.4, 120.84, 120.7, 120.53, 113.6, 87.95, 81.18, 76.75, 72.1, 71.77, 70.57, 69.75, 68.19, 62.67, 30. Anal. ($C_{74}H_{84}N_4O_{21}S_3$, $3ClCH_2CH_2Cl$) C, H, N, S.

5,10,15-tri{*p*-*O*-[2-(2-*S*-(2′,3′,4′,6′-tetraacetyl-α-D-thiomannosyloxy)-ethoxy)-ethoxy] phenyl}-20-phenyl porphyrin (TPP(*p*-Deg-*S*-α-ManOAc)$_3$) (14). Prepared as in **12**. Pure product **14** (132 mg, yield 86%) was obtained as a red powder after silica gel column chromatography (CH_2Cl_2/Et_2O; 1:1) and crystallization from CH_2Cl_2/heptane. UV−vis spectrum in CH_2Cl_2: λ_{max}, nm (ϵ): 421 (380), 517.5 (14.4), 552 (9.6), 593 (4.6), 649.5 (4.6). ^{1}H NMR ($CDCl_3$) δ 8.84 (m, 8H), 8.22 (dd, 6H, $J = 7$ and 2 Hz), 8.11 (d, 2H, $J = 8.5$ Hz), 7.75 (m, 3H), 7.29 (d, 2H, $J = 8.5$ Hz), 5.47 (d, 3H, $J = 1.3$ Hz), 5.44 (dd, 3H, $J = 2.5$ and 1.3 Hz), 5.35 (m, 6H), 4.47 (m, 3H), 4.40 (m, 6H), 4.37 (dd, 3H, $J = 12.2$ and 2.1 Hz), 4.16 (dd, 3H, $J = 12.4$ and 5.2 Hz), 4.02 (m, 6H), 3.90 (m, 6H), 3.03 (m, 3H), 2.89 (m, 3H), 2.18 (s, 9H), 2.14 (s, 9H), 2.05 (s, 9H), 2.0 (s, 9H), −2.76 (s, 2H). ^{13}C NMR ($CDCl_3$) δ 170.6, 169.9, 169.7, 169.6, 158.5, 142.2, 135.6, 134.8, 134.5, 131, 127.6, 126.6, 119.8, 119.7, 112.8, 82.9, 71, 70.8, 69.7, 69, 69.1, 67.6, 62.4, 30.7, 20.9, 20.8, 20.7, 20.6. Anal. ($C_{98}H_{108}N_4O_{33}S_3$) C, H, N, S.

5,10,15-tri{*p*-*O*-[2-(2-*S*-α-D-thiomannosyloxy)-ethoxy)-ethoxy]-phenyl}-20-phenyl porphyrin (TPP(*p*-Deg-*S*-α-ManOH)$_3$) (15). Prepared as in **6**. Porphyrin **15** (31 mg, yield 100%) was obtained as a red powder after crystallization from MeOH/1,2-DCE. MALDI-TOF MS calcd $C_{74}H_{84}N_4O_{21}S_3$ (MH^+) 1461.48; found, 1461.60. UV−vis spectrum in MeOH/pyridine (24:1): λ_{max}, nm (ϵ): 417 (275.7), 515 (11.6), 552.5 (7.7), 592.5 (4), 647.5 (3.9). ^{1}H NMR (pyridine d_5) δ 9.17 (s, 4H), 9.13 (d, 2H, $J = 4.8$ Hz), 9.06 (d, 2H, $J = 4.8$ Hz), 8.38 (dd, 2H, $J = 7.1$ and 2.2 Hz), 8.26 (dd, 2H, $J = 8.7$ and 2.3 Hz), 7.80 (m, 3H), 7.44 (dd, 6H, $J = 8.7$ and 2.3 Hz), 6.09 (d, 3H, $J = 0.9$ Hz), 4.75 (m, 3H), 4.72 (m, 3H), 4.70 (m, 3H), 4.65 (m, 3H), 4.63 (m, 3H), 4.46 (m, 3H), 4.39 (m, 6H), 3.99 (m, 6H), 3.94 (m, 6H), 3.26 (m, 6H), 3.04 (m, 6H), −2.28 (s, 2H). ^{13}C NMR (pyridine d_5) δ 159.3, 142.6, 136, 135, 134.7, 133, 128, 127.3, 120.7, 120.6, 120.4, 113.5, 86.8, 75.9, 73.6, 73.5, 71.3, 69.6, 69.4, 68, 63, 30.8. Anal. ($C_{74}H_{84}N_4O_{21}S_3$, H_2O) C, H, N, S.

General Procedure for In Vitro Experiments. Cell Culture Conditions. Human retinoblastoma Y79 cells were obtained from the American Type Culture Collection (HTB-18) and grown in suspension in COON's modified culture medium[27] (VWR) supplemented with 20% of fetal calf serum (FCS), glucose, glutamine, and antibiotics. For experiments, the cells were resuspended at 10^6 cells/mL and incubated with the photosensitizer. Stock solutions of the photosensitizer (5 mg/mL) were prepared in dimethyl sulfoxide (DMSO), and dilutions were performed in a culture medium containing FCS (2%). Incubations were performed in the dark. The cell culture conditions of adherent human colorectal adenocarcinoma cells (HT29) and murine melanocyte cells (B16) have been previously described[7]. All reagents were obtained from Bio-Media (France).

Cell Extraction. Treated Y79 cells were centrifuged and washed three times with cold PBS. After sonication, methanol (2 mL) was added to the pellet, and the resulting suspension was left in the dark for 30 min with sustained shaking and then centrifuged (1700*g*, 10 min). The photosensitizer concentration of the supernatant was determined by absorption spectroscopy. The photosensitizer was recovered in greater than 90% yield. The concentration was related to the number of cells determined by counting.

Evaluation of the Affinity of Glycosylated Porphyrins/Glycosylated Albumin. Glycosylated albumin was prepared according to Mc Broom et al.[18] The albumin concentration was determined by colorimetry (phenol-sulfuric acid) and the amount of proteins by the determination of the total nitrogen amount. The ratios of galactose/albumin and mannose/albumin were 0.1 mM/g and 0.230 mM/g, respectively (±10−15%). Solutions of glycoconjugated porphyrin (2 μM) and the corresponding glycosylated albumin (2.6 mg/mL) in COON's medium were left to incubate (1 h, 37 °C). The protein was precipitated with trifluoroacetic acid and centrifuged. Methanol (2 mL) was then added to the pellet and shacken for 30 min in the dark. The ratio of the porphyrin/albumin association (methanol phase) to free porphyrins (Coon's aqueous phase) concentrations was determined by absorption spectroscopy.

Phototoxicity Assay. The phototoxicity of the dye was determined in Y79 cells following a detailed procedure described in ref 7.[7] Briefly, the cells were placed into 24-well plates at 2×10^5 cells/mL in COON's medium containing 2% FCS. The cells were incubated with the dye, centrifuged, washed, and resuspended in fresh complete medium. Irradiations were performed at 514 nm (1 J/cm^2) with an Ar^+ laser. Cell viability was measured 24 h later by

the determining mitochondrial activity using a colorimetric MTT assay according to Mossmann.[28] Optical densities of the microplates were determined at 570 and 640 nm using a Labsystem instrument. In the case of adherent cells, HT29 and B16, the cells were seeded into 96-well plates at 10^5 cells/mL (200 μL/well), and incubations and irradiations were performed in DMEM containing dyes in the presence of 2% FCS at 514 nm (5 J/cm^2).

Fluorescence Imaging Spectroscopy. Subcellular localization was studied by using a Nikon epifluorescence microscope (Optiphot-2) as described in ref 7.[7]

Flow Cytometry. Cellular uptake was determined from flow cytometry measurements using a FACScan instrument (Becton-Dickinson, Heidelberg, Germany), gating the forward (FSC) and side (SSC) scatters to exclude debris. A CellQuest program was used. The cells were grown in suspension and incubated 1−6 h in the presence of 2−6 μM dye in the dark (37°C). The cells were centrifuged, washed, and resuspended in the medium (200 μL, 10^6 cells). The excitation wavelength was 488 nm (Argon laser), and the fluorescence of treated cells was collected with a FL3-H detector equipped with a 600 nm long band-pass filter. For each determination, the cellular distribution was also provided. Data were analyzed in triplicate, and the results for 10^4 cells were acquired with gating on cells exhibiting normal FSC and SSC characteristics to exclude debris. Untreated cells were used as the control.

Mass Spectrometry Analysis. Positive ion MALDI-TOF mass spectra of chemical samples and cellular extracts of treated cells were recorded using a PerSeptive Biosystems Voyager Elite (Framingham, USA) time-of-flight mass spectrometer equipped with a 337 nm nitrogen laser (VSL 337ND). It was operated in the reflectron delayed extraction mode (acceleration voltage: 20 kV; percent grid voltage: 60%; extraction delay: 125 ns), and the laser fluence was adjusted with a variable-beam attenuator. The ions were detected by a dual channel plate detector. For sample preparation, the matrix, α-cyano-4-hydroxycinnamic acid (HCCA), was dissolved at a concentration of 0.1 M in MeOH. Ten microliters of the matrix solution was added to the evaporated supernatant of the cellular extract and 1 μL deposited onto a polished gold sample stage and allowed to dry in air.

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Supporting Information Available: Elemental analysis information, ^{1}H, ^{13}C NMR attributions, and ^{1}H, ^{13}C NMR, and MALDI-TOF spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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