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A study of the stability of tri(glucosyloxyphenyl)chlorin, a sensitizer for photodynamic therapy, in human colon tumoural cells: a liquid chromatography and MALDI-TOF mass spectrometry analysis

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Abstract—Asymmetrical glycoconjugated tetrapyrrolic macrocycles are under study as efficient sensitizers for photodynamic therapy (PDT). In this context, tri(*meta*-*O*-β-glucopyranosyloxyphenyl)chlorin [TPC(*m*-*O*-Glu)$_3$] **2a/3a** was found to be four times more photoactive in vitro than Foscan®. In a further study of this interesting glycoconjugate, its metabolism by cellular glycosidases in HT29 cells has to be explored. Cellular extracts of HT29 cells incubated with TPC(*m*-*O*-Glu)$_3$ (24 h, 6 μM) were analyzed by MALDI-TOF mass spectrometry and high performance liquid chromatography (HPLC). In MALDI-TOF mass spectra, the presence of compounds distinct from TPC(*m*-*O*-Glu)$_3$ (*m*/*z* 1151) were observed at *m*/*z* 989, 827 and 665 corresponding to the loss of one, two or three glucose units (162 u) and were be ascribed to TPC(*m*-OH)(*m*-*O*-Glu)$_2$ **2/3b**,**b′**,**b″**, TPC(*m*-OH)$_2$(*m*-*O*-Glu) **2/3c**,**c′**,**c″** and TPC(*m*-OH)$_3$ isomers **2d/3d**, respectively. The porphyrins resulting from chlorin oxidation TPP(*m*-*O*-Glu)$_3$ **4a**, TPP(*m*-OH)(*m*-*O*-Glu)$_2$ **4b**,**b″**, TPP(*m*-OH)$_2$(*m*-*O*-Glu) **4c**,**c″** and TPP(*m*-OH)$_3$ **4d** were also observed. The HPLC profile ($\lambda_{anal.} = 420$ nm) showed eight peaks consistent with mass spectra. The kinetics of deglucosylation was studied from HPLC profiles between 1 and 48 h incubation. The concentration of triglucoconjugated and diglucoconjugated molecules was maximum around 3 and 8 h incubation, respectively, whereas, totally deglucosylated species appeared only after incubation for more than 10 h. The fully deglycosylated porphyrin TPP(*m*-OH)$_3$ is the final metabolite, being observed at a concentration 15 times higher than that of the remaining TPC(*m*-*O*-Glu)$_3$ **2a/3a**. Compared to the photobiological activity of the parent molecule [TPC(*m*-*O*-Glu)$_3$], a three times higher TPP(*m*-OH)$_3$ concentration was necessary to observe a similar in vitro photoactivity.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; HPLC, high performance liquid chromatography; HT29, human colorectal adenocarcinoma cells; MALDI-TOF, matrix assisted laser desorption ionization-time of flight; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; PBS, phosphate buffer saline; PDT, photodynamic therapy; *m*-THPC, 5,10,15,20-tetrakis(*meta*-hydroxyphenyl)chlorin; TPC(*m*-*O*-Glu)$_3$, 5,10,15-tri(*meta*-*O*-β-D-glucopyranosyloxyphenyl)-20-phenyl-2,3-chlorin and 5,10,15-tri(*meta*-*O*-β-D-glucopyranosyloxyphenyl)-20-phenyl-7,8-chlorin; TPC(*m*-OH)(*m*-*O*-Glu)$_2$, 5,10-di(*meta*-*O*-β-D-glucopyranosyloxyphenyl)-15-(*meta*-hydroxyphenyl)-20-phenyl-2,3-chlorin, 10,15-di(*meta*-*O*-β-D-glucopyranosyloxyphenyl)-5-(*meta*-hydroxyphenyl)-20-phenyl-2,3-chlorin, 5,10-di(*meta*-*O*-β-D-glucopyranosyloxyphenyl)-15-(*meta*-hydroxyphenyl)-20-phenyl-7,8-chlorin, 5,15-di(*meta*-*O*-β-D-glucopyranosyloxyphenyl)-10-(*meta*-hydroxyphenyl)-20-phenyl-2,3-chlorin, 5,15-di(*meta*-*O*-β-D-glucopyranosyloxyphenyl)-10-(*meta*-hydroxyphenyl)-20-phenyl-7,8-chlorin and 5,10-di(*meta*-*O*-β-D-glucopyranosyloxyphenyl)-15-(*meta*-hydroxyphenyl)-20-phenyl-17,18-chlorin; TPP(*m*-OH)(*m*-*O*-Glu)$_2$, 5,10-di(*meta*-*O*-β-D-glucopyranosyloxyphenyl)-15-(*meta*-hydroxyphenyl)-20-phenyl porphyrin and 5,15-di(*meta*-*O*-β-D-glucopyranosyloxyphenyl)-10-(*meta*-hydroxyphenyl)-20-phenyl porphyrin; TPC(*m*-OH)$_2$(*m*-*O*-Glu), 5-(*meta*-*O*-β-D-glucopyranosyloxyphenyl)-10,15-di(*meta*-hydroxyphenyl)-20-phenyl-2,3-chlorin 10-(*meta*-*O*-β-D-glucopyranosyloxyphenyl)-5,15-di(*meta*-hydroxyphenyl)-20-phenyl-2,3-chlorin, 5-(*meta*-*O*-β-D-glucopyranosyloxyphenyl)-10,15-di(*meta*-hydroxyphenyl)-20-phenyl-7,8-chlorin and 10-(*meta*-*O*-β-D-glucopyranosyloxyphenyl)-5,15-di(*meta*-hydroxyphenyl)-20-phenyl-7,8-chlorin; TPP(*m*-OH)$_2$(*m*-*O*-Glu), 5-(*meta*-*O*-β-D-glucopyranosyloxyphenyl)-10,15-di(*meta*-hydroxyphenyl)-20-phenyl porphyrin and 10-(*meta*-*O*-β-D-glucopyranosyloxyphenyl)-5,15-di(*meta*-hydroxyphenyl)-20-phenyl porphyrin; TPC(*m*-OH)$_3$, 5,10,15-tri(*meta*-hydroxyphenyl)-20-phenyl-2,3-chlorin and 5,10,15-tri(*meta*-hydroxyphenyl)-20-phenyl-7,8-chlorin; TPP(*m*-OH)$_3$, 5,10,15-tri(*meta*-hydroxyphenyl)-20-phenyl porphyrin.

Keywords: Photodynamic therapy; Glucoconjugated chlorins; Metabolization; Deglucosylation.
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1. Introduction

Photodynamic therapy (PDT) is an emerging treatment modality of solid tumours based on the administration of a light-activated drug (photosensitizer) followed by the laser illumination of pathological areas. Upon sensitizer photoactivation, the generation of reactive oxygen species leads to irreversible destruction of the treated tissues.[1,2]

Several important milestones in this field include the regulatory approval given in 2000 for Visudyne® (BPD-MA) for the treatment of age-related macular degeneration (AMD), and for Foscan® (*m*THPC, 2002) as an agent for palliative treatment of head and neck cancer. However, for these, and other promising molecules under study, the risk of long-term cutaneous photosensitivity remains a significant adverse effect. Continued research is thus necessary to develop new sensitizers with high photoactivity and increased therapeutic index.

It has been demonstrated that the overall lipophilicity of the sensitizer plays an important role in PDT efficacy[2,3] and in this context, glycoconjugation is undergoing evaluation as an effective mean to modulate the amphiphilicity of tetrapyrrolic compounds. Of note is the work by Pandey and co-workers[4] who have recently prepared a number of galactosylated chlorins and shown that the position of the sugar motif has a strong influence on photobiological activity.[5] In our laboratories, efforts have been focused on the preparation and in vitro evaluation of the phototoxicity of a broad series of neutral tri and tetraglycoconjugated tetrapyrrolic macrocycles.[6,7] It was found that, in general, the triglycoconjugated sensitizers exhibit higher photobiological activity than either the parent tetrapyrroles or the symmetrical tetraglycoconjugated derivatives.[6] To look at the origin of this effect in more detail, a recent study was made of the cellular internalization and photodynamic activity of new tri and tetraglucosylated conjugate of Foscan®, tri(*meta*-*O*-β-glucopyranosyloxyphenyl)chlorin [TPC(*m*-*O*-Glu)$_3$] and tetra(*meta*-*O*-β-glucopyranosyloxyphenyl)chlorin [TPC(*m*-*O*-Glu)$_4$].[8] The triglucoconjugated TPC(*m*-*O*-Glu)$_3$ is four times more photoactive in vitro than Foscan® itself and could be of potential interest in the PDT treatment of tumours.

At present, little is known concerning the metabolization of glycosylated porphyrins or chlorins either in vivo or in cell based assays. In vivo metabolism is a particularly important issue when considering the use of glycoconjugated compounds in PDT treatment, since cleavage of the glycoside bond by glycosidases will result in modifications of amphiphilic properties, biodistribution, blood clearance and drug–cell interactions. Metabolism of these agents may in turn alter their photobiological activity.

The level of glycosidase activity and expression has been found to be organ dependent[9] and to be higher in certain tumoural tissues compared to normal tissues.[10,11] Glucosidases have also been identified in human plasma and in erythrocytes membranes,[12] the β-glucosidase activity in human plasma being about 20-fold lower than that of α-glucosidases. Glycosidases promoted metabolism of glycoconjugated porphyrins/chlorins can thus occur at a number of levels after administration in vivo of these agents. However, a certain specificity and stability can be achieved. Monsigny et al.,[13] showed that glycosyl moieties of glycoconjugated drugs could be recognized by membrane lectins, the drug being released in different organelles (endoplasmic reticulum and Golgi apparatus) that contain specific glycosidases. The resulting deglycosylation processes were shown to depend on the nature of the link between the drug and the glycosyl moieties.

Concerning the porphyrin/chlorin component in such tetrapyrrolic sensitizers, there was little data pertaining to their metabolism.[14,15] As Photofrin®, the first commercialized sensitizer, is a mixture of porphyrin derivatives, HPLC analysis of tissue extracts is difficult to interpret. The presence of a metabolite of Foscan® (*m*THPC) was detected by Whelpton et al. in the rat liver.[16] In contrast, Cai et al. have studied the metabolism of Foscan® and found no evidence either in vitro and in vivo (absence of light) that this agent is metabolized.[17] Visudyne®, a monoacid benzoporphyrin derivative, was reported to be hydrolyzed to its diacid derivative in vivo. However, the extent of formation of this metabolite was less than 10% of the administered dose.[18] Similarly, no metabolites were detected in a study of mono-L-aspartyl chlorin e6 metabolism in a mouse mammary tumour model[19] or for palladium bacteriopheophorbide (Tookad®), a new sensitizer in clinical trial for the PDT treatment of prostate cancer, and gallium-porphyrin (ATX-70).[20,21]

In the present study, we have explored the possible cellular in vitro metabolization of TPC(*m*-*O*-Glu)$_3$ **2a**/**3a** (Fig. 1), a compound that exhibits interesting photobiological characteristics for PDT treatment,[8] using MALDI-TOF mass spectrometry and high performance liquid chromatography (HPLC). We provide data to demonstrate that the three sugar motifs in the chlorin **2a**/**3a** undergo sequential hydrolysis, and that these processes are interconnected to the oxidative metabolism of the chlorin system to the corresponding porphyrins.

2. Results and discussion

2.1. Synthesis

The isomeric glucoconjugated chlorins TPC(*m*-*O*-Glu)$_3$ **2a** and **3a** (a 50/50 mixture of two inseparable isomers)[22] were obtained by diimide reduction and deacetylation of the pentaacetate derivative **1** of triglucosylated porphyrin [TPP(*m*-*O*-Glu)$_3$] **4a** (Scheme 1).[8] In a similar way, the unsubstituted chlorins TPC(*m*-OH)$_3$ **2d**/**3d** were obtained in two steps from TPP(*m*-OMe)$_3$ **5**. Porphyrin **4d** [TPP(*m*-OH)$_3$] was obtained by direct treatment of **5**

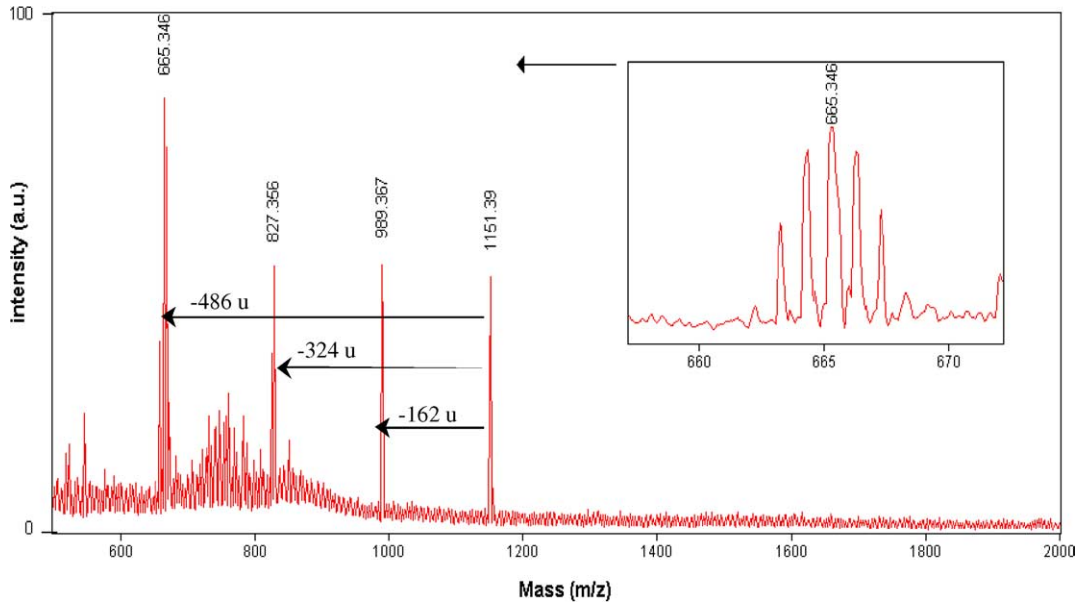
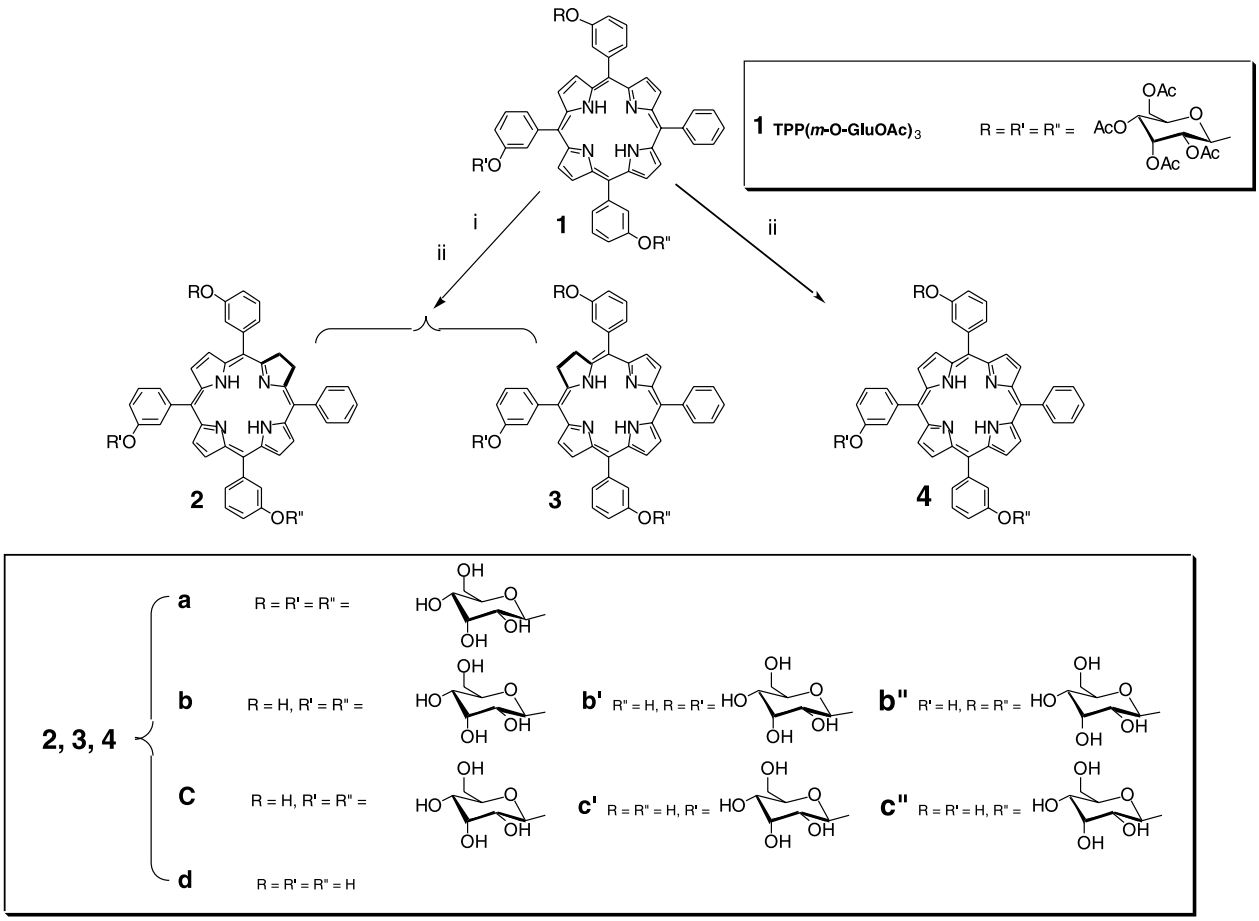


Figure 1. Typical MALDI-TOF mass spectrum of a cellular extract of HT29 cells treated with **2a/3a** (6 μM, 24 h incubation). The isotopic distribution of each main signal indicates the presence of two species separated by 2 u (as an example: see insert in the case of peak at m/z 665).



Scheme 1. Synthesis and structure of glycoconjugated chlorins and porphyrins. Reagents and conditions: (i) (a) toluene-4-sulfonohydrazide, anhydrous K_2CO_3, dry pyridine, 100 °C; (b) *ortho*-chloranil, ethyl acetate; (ii) MeONa/MeOH, 20 °C.

with BBr_3. As determined by NMR, the mixture of triglycosylated chlorins **2a/3a** contains a small quantity (<10%) of the triglucosylated porphyrin **4a** as a contaminant.[22]

The possible transformation of the glucoconjugated chlorins **2a/3a** by cellular enzymes has been investigated using HT29 human adenocarcinoma colon cells. Extraction was performed in the dark on treated cells with **2a/3a** and cellular extracts were then analyzed by MALDI-TOF mass spectrometry and HPLC.

2.2. Mass spectrometry analysis of cellular extracts

A typical MALDI-TOF mass spectrum of a cellular extract of HT29 treated with TPC(*m*-*O*-Glu)$_3$ **2a/3a** (6 μM, 24 h incubation) recorded prior to any chromatographic separation is shown in Figure 1. In addition to the protonated molecule for the two isomeric triglucosylated chlorins **2a/3a** (m/z 1151), intense signals at m/z 989, 827 and 665 were observed. These signals corresponded to the loss of one, two or three glucose units (162 u) from these chlorins, and were assigned to TPC(*m*-OH)(*m*-*O*-Glu)$_2$ (six isomers; **2/3b**,**b′**,**b″**), TPC(*m*-OH)$_2$(*m*-*O*-Glu) (six isomers; **2/3c**,**c′**,**c″**) and TPC(*m*-OH)$_3$ (two isomers; **2d/3d**).

The isotopic distribution observed for these signals (see for example insert Fig. 1) was different from that expected for a single species, and clearly indicated the presence of the corresponding porphyrin derivatives, TPP(*m*-*O*-Glu)$_3$ **4a** (m/z 1149), TPP(*m*-OH)(*m*-*O*-Glu)$_2$ **4bb″** (m/z 987), TPP(*m*-OH)$_2$(*m*-*O*-Glu) **4c**,**c″** (m/z 825), and TPP(*m*-OH)$_3$ **4d** (m/z 663).

As a control, **2a/3a** was kept in culture medium (6 μM) in the dark for 24 h at 37 °C, extracted, and then dried under vacuum. In the mass spectrum of the extracted control sample the protonated molecule at m/z 1151 was observed. Compound **4a** was also detected corresponding to the porphyrin contaminant[8] with an abundance (approx. 7%), which was lower than that measured in all cases for the cellular extracts.

It can thus been assumed that the loss of sugars only results from an enzymatic reaction between cellular β-glucosidase and the sensitizer, the tetrapyrrolic ring aromatization implicating an other enzymatic pathway. Activity of intact cell β-glucosidase has been evaluated to $6.9(\pm1)\times10^{-8}$ nmol/min/cell in HT29. As a comparison, mannosidase activity was two times lower [$3.5(\pm0.13)\times10^{-8}$ nmol/min/cell]. In as far as enzymatic activities are specific of the cell line and sugar moieties, deglycosylation processes may be expected to be highly dependent on the chemical structure of glycoconjugated compounds.

It is interesting to note that glucosidic bond metabolization processes do not depend upon the saturated or unsaturated character of the tetrapyrrolic ring. Besides the molecular ion at m/z 1149, the mass spectrum of a cellular extract of HT29 treated with **4a** (6 μM, 24 h incubation), also displayed three peaks at m/z 987, 825 and 663, corresponding to the loss of one, two and three sugar units. An identical degradation pattern was found for cells treated with the analogous *para*-substituted compound, TPP(*p*-*O*-Glu)$_3$ indicating the cellular metabolization is not affected by the *meta*- or *para*-position of sugar units (spectra not shown). However, one cannot exclude the metabolic pathway could be affected by the chemical structure of the sensitizer.

The metabolic degradation of glucoconjugated compounds have to be considered whatever the cell line used depending on the glucosidase activity. As an example, similar degradation pattern of the TPC(*m*-*O*-Glu)$_3$ **2a/3a** has also been observed with the loss of one, two and three glucose units in cellular extracts of treated cells (6 μM, 24 h incubation) in the case of murine melanoma cells (B16).

2.3. HPLC analysis

Chromatographic analysis of cellular extracts of treated cells was conducted in order to elucidate the different steps of the deglucosylation process. The HPLC profile [absorption detection ($\lambda = 420$ nm)] of a cellular extract of HT29 cells (Fig. 2) incubated with **2a/3a** (24 h incubation) was consistent with the mass spectra displayed with eight peaks or group of peaks. Chromatograms obtained with a dual absorption ($\lambda = 420$ nm) or fluorescence detection ($\lambda_{exc} = 420$ nm, $\lambda_{em} = 650$ nm) were similar in both cases indicating the eight absorbing species are fluorescent (data not shown).

MALDI-TOF mass spectra of HPLC fractions allowed to directly ascribed fraction 1 to **2a/3a** (m/z 1151), fraction 2 to TPC(*m*-OH)(*m*-*O*-Glu)$_2$ (six isomers) (m/z 989) and fraction 3 to **4a** (m/z 1149) (see Table 1). Peaks 7 and 8 could be assigned by using model compounds. MALDI-TOF mass spectra and HPLC characteristics of **2d/3d** (m/z 665, 29 min retention time) and **4d** (m/z 663, 37 min retention time) clearly indicated that peaks 7 and 8 corresponded to the totally deglucosylated chlorin and porphyrin, respectively (see Table 1).

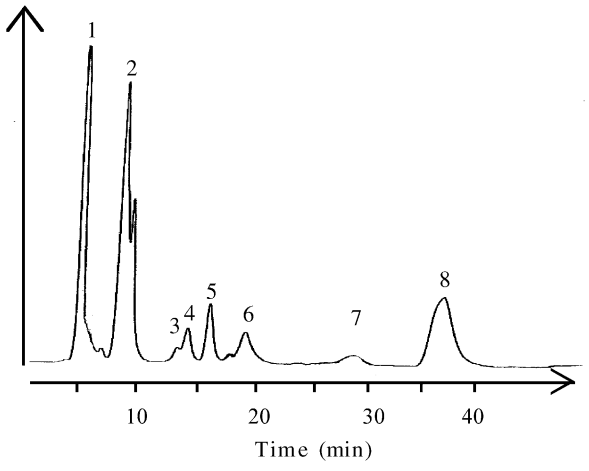


Figure 2. Representative HPLC profile (absorption detection $\lambda = 420$ nm) of a cellular extract of HT29 cells treated with **2a/3a** (6 μM, 24 h incubation). Fraction numbers 1 to 8 correspond to the f_n values reported in Table 1.

Table 1. Fraction number (f_R), corresponding retention times (t_R) and m/z values obtained from HPLC and MALDI-TOF mass spectrometry analysis of cellular extracts of HT29 treated with TPC(*m*-*O*-Glu)$_3$ **2a/3a** (6 μM, 24 h incubation)

f_n	t_R (min)	m/z	Compound
1	5.1–5.3	1151	**2a/3a**: TPC(*m*-*O*-Glu)$_3$
2	7.8–8.2	989	**2/3b,b′,b″**: TPC(*m*-OH)(*m*-*O*-Glu)$_2$
3	13	1149	**4a**: TPP(*m*-*O*-Glu)$_3$
4	15.2	987	**4b,b″**: TPP(*m*-OH)(*m*-*O*-Glu)$_2$
5	16	827	**2/3c,c′,c″**: TPC(*m*-OH)$_2$(*m*-*O*-Glu)
6	19	825	**4c,c″**: TPP(*m*-OH)$_2$(*m*-*O*-Glu)
7	29.5	665	**2d/3d**: TPC(*m*-OH)$_3$
8	37.8	663	**4d**: TPP(*m*-OH)$_3$

Assignment of peaks 4, 5 and 6 could not be done from MALDI-TOF data. However, on the basis of respective retention times and polarity considerations, peak 4 was assigned to diglucoconjugated porphyrin isomers, and peaks 5 and 6 to monoglucosylated chlorin and porphyrin isomers, respectively (Table 1). Occurrence of deglucosylation processes results in the existence of isomeric forms that could explain the structure observed for peak 2 and the poor resolution of some other peaks.

The HPLC analysis wavelength of 420 nm corresponded to optimized conditions to observe both chlorin and porphyrin derivatives. Nevertheless, HPLC profile does not reflect the concentration of each species since maximum extinction coefficients are different and highly dependent on the solvent environment. The concentration of metabolites isolated from the cellular extracts was determined from calibration curves performed in the case of peaks 1, 2, 3, 7 and 8 using the corresponding model compounds. Pure mono and diglucoconjugated derivatives were not available.

2.4. Kinetics of TPC(*m*-*O*-gluOH)$_3$ deglucosylation

Figure 3A and B show the time course of A/A_{max} ratio where A is the peak area obtained for a given incubation time t and A_{max} the peak area corresponding to the maximum value observed for each product isolated from the cellular extracts.

The concentration of **2a/3a** first sharply increased reaching a maximum value [$2.8(\pm0.03)\times10^{-6}$ μmol/μg of proteins] at 3 h incubation. That concentration decreased with increasing incubation time—lowered by 60% at 8 h—and remained quite constant between 8 and 14 h incubation. This suggests a possible competition between cellular uptake and metabolization processes the latter becoming predominant for long incubation times. The concentration of **2a/3a** determined at 24 h was $0.55(\pm0.07)\times10^{-6}$ μmol/μg of proteins and only $0.24(\pm0.05)\times10^{-6}$ μmol/μg of proteins at 48 h incubation.

Kinetics of formation of the triglucoconjugated porphyrin **4a** (peak 2) (Fig. 3B) also shows a maximum concentration at 3 h incubation that subsequently decreased for longer times. The ratio of **4a** concentration

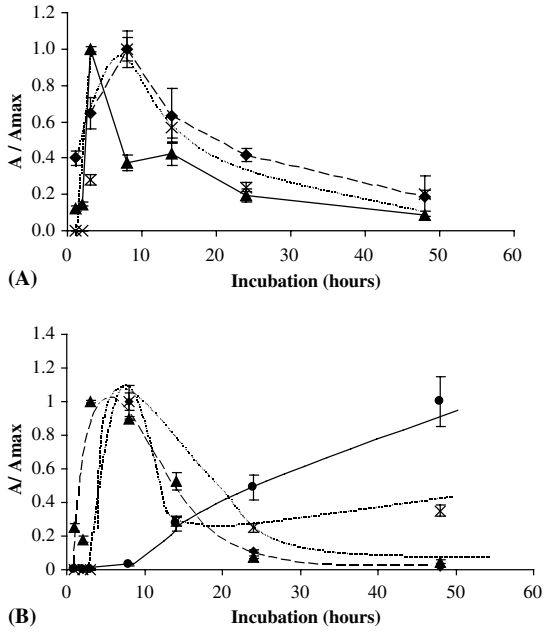


Figure 3. Time course of metabolites determined from HPLC analysis of cellular extract of HT29 cells treated with **2a/3a** (6 μM, incubation times going from 1 to 48 h). (A) Chlorin derivatives: ▲ **2a/3a**, ◆ **2/3b,b′,b″**, × **2/3c,c′,c″**. (B) Porphyrin derivatives: ▲ **4a**, ◆ **4b,b″**, × **4c,c″**, ● **4d**. The maximum concentration was $2.8\pm0.03\times10^{-6}$ μmol/μg of proteins in the case of **2a/3a** and $3.6\pm0.03\times10^{-6}$ μmol/μg of proteins in the case of **4d**. Errors bars, SE ($n=3$).

Table 2. Ratio of TPP(*m*-*O*-Glu)$_3$/TPC(*m*-*O*-Glu)$_3$ **(4a/2a–3a)** and TPP(*m*-OH)$_3$/TPC(*m*-*O*-Glu)$_3$ **(4d/2a–3a)** concentration as determined from HPLC profiles

	0 h	3 h	8 h	14 h	24 h	48 h
4a/2a–3a	0.07	0.12	0.33	0.18	0.06	0.07
4d/2a–3a	0	0	0.12	1.2	4.36	15

to that of **2a/3a** (**4a/2a–3a**) increased during the first 8 h from 7% to 33% then falling down to 6% at 24 h. As mentioned before this value corresponds to the initial amount of contaminant porphyrin. The time course of the relative concentration of triglucoconjugated porphyrin to chlorin (Table 2) clearly shows that **2a/3a** chlorin oxidation does result from a cellular process.

Both species corresponding to peaks 4, 5 and 6 (Fig. 3A and B) exhibited a maximum concentration around 8 h decreasing at longer times.

The two compounds resulting from the loss of three glucose units, appeared only after incubation for more than 10 h. The determination of the time course of **2d/3d** abundance was somewhat hazardous since peak 7 was poorly resolved with a low intensity. As shown in Figure 3B, **4d** concentration slowly increased with incubation time. Concentrations of $2.4(\pm0.8)\times10^{-6}$ μmol/μg of proteins and $3.6(\pm0.8)\times10^{-6}$ μmol/μg of proteins were measured at 24 and 48 h, respectively. These concentrations were much higher than that of the parent molecule **2a/3a** (Table 2).

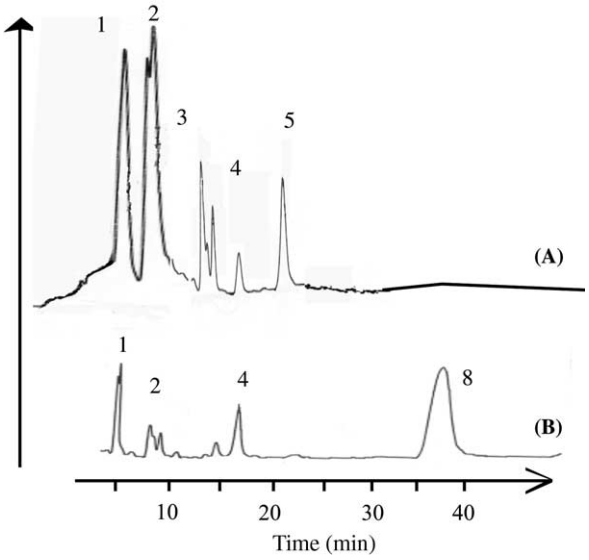
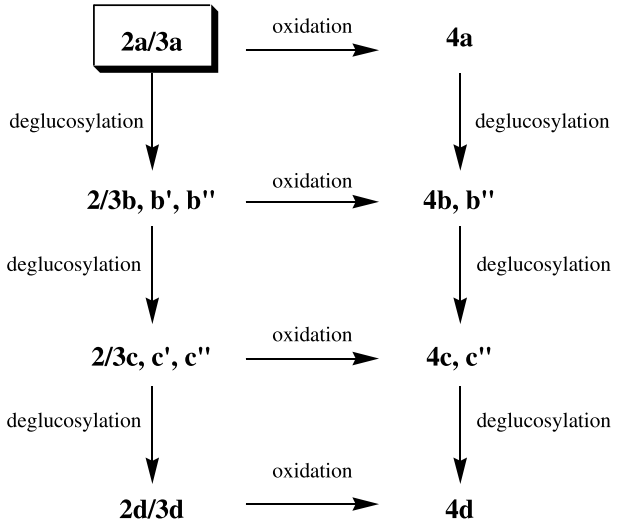


Figure 4. HPLC profile (absorption detection, λ 420 nm) of a cellular extract of HT29 treated with **2a/3a** (6 μM): (A) 3 h incubation, (B) cells washed at 3 h incubation and kept in free drug medium up to 24 h. Fraction numbers 1 to 8 correspond to the f_n values reported in Table 1.

It is interesting to note that the ratio of **4a** to **2a/3a** concentration increased with time (Table 2). In addition the comparison of the HPLC profile of a cellular extract obtained at 3 h incubation (Fig. 4A) and that of an extract obtained with cells first incubated for 3 h, then washed and kept in the dark in free drug medium up to 24 h (Fig. 4B) showed that **4d** becomes the most abundant species in the cellular extract. These findings clearly show that the deglucosylated porphyrin **4d** observed only resulted from a metabolism process and can thus be assumed to be the final metabolite of the triglucosylated chlorin **2a/3a**. A possible metabolic pathway is illustrated in Scheme 2.



Scheme 2. Possible metabolic pathway of glycoconjugated chlorin **2a/3a**.

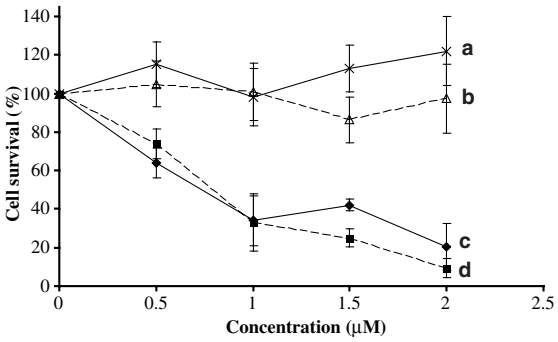


Figure 5. Concentration dependence of toxicity in HT29 (3 h incubation) expressed as cell survival fraction in the dark and exposed to 514 nm light with a light fluence of 5 J/cm^2: **2a/3a** (a) and (c), **4d** (b) and (d). Experiments have been triplicated. Errors bars, SE.

2.5. Phototoxicity of the final metabolite

In as far as the **4d** concentration becomes much higher than the maximal concentration of the initial compound, the question arises about the consequences of metabolization processes on the sensitizer photoactivity for long incubation times. The phototoxicity of **4d** in HT29 cells has been determined and compared to that of **2a/3a**. Cells were incubated for 3 h in order to minimize metabolization of the parent molecule. In both cases the cytotoxicity was found negligible for a dose going from 0.5 to 2 μM (Fig. 5). The phototoxicity (514 nm, 5 J/cm^2) of **2a/3a** and **4d** was found quite similar (χ^2 test, NS, $\alpha = 2.5\%$) with a LD_{50} value around 0.7 μM (dose corresponding to a survival fraction of 50%). However, the phototoxicity must be related to the respective amount of internalized drug. As determined by cellular extraction, **4d** concentration was found to be $5.9(\pm 0.8)\times 10^{-6}$ μmol/μg of proteins whereas it was lower [$2.5(\pm 0.8)\times 10^{-6}$ μmol/μg of proteins] for **2a/3a**. A 2–3 higher concentration of deglucosylated porphyrin is thus necessary to observe a similar activity to that of the initial glucoconjugated chlorin.

3. Conclusion

In conclusion, metabolism of the triglucoconjugated chlorin, TPC(*m*-*O*-Glu)$_3$, **2a/3a** results in the formation of partially or totally deglucosylated compounds, chlorins by action of cellular glucosidases and porphyrins by oxidation of chlorin macrocycles. The final metabolite resulting from the loss of three glucose units and oxidation of macrocycle exhibits a biological activity lower than that of the initial compound. It may thus be assumed that cellular metabolization of TPC(*m*-*O*-Glu)$_3$ affects the global photoactivity of this compound. In vivo metabolization studies are currently under investigation.

4. Experimental

Acetonitrile (HPLC grade), methanol (HPLC grade), DMSO (analytical-reagent grade), trifluoroacetic acid (>99% pure) were purchased from VWR (France).

4.1. Chemistry

All chemicals used were of reagent grade and were purchased from Aldrich or Fluka. Merck silica gel 60 (0.040–0.060 mm) was used for column chromatography. Methylene chloride stabilized with ethanol (6 mL/L) used for synthesis of 5,10,15-tri[3-*O*-(2′,3′,4′,6′-tetraacetyl-β-D-glucopyranosyl)phenyl]-20-phenyl porphyrin, was kept on $CaCO_3$ overnight, filtered then distilled on $CaCO_3$ and stored on molecular sieve 4 Å. Pyrrole was distilled on CaH_2. BF_3-etherate was distilled. Macherey–Nagel precoated plates (SIL G-200, 2 mm) were used for preparative thin layer chromatography. Elemental analysis were carried out by the 'Service Central de Microanalyse du CNRS'. ^{1}H NMR spectra were obtained in the indicated deuteriated solvents with Brucker AM-300 instruments. Acidic impurities of chloroform-d_3 were removed with anhydrous K_2CO_3. Chemical shift values were given in ppm relative to TMS. Coupling constants were given in Hz. Optical spectra were recorded using a Varian DMS 200 spectrometer.

4.1.1. 3-*O*-(2′,3′,4′,6′-Tetraacetyl-β-D-glucopyranosyloxy)-benzaldehyde. A solution of 3-hydroxybenzaldehyde (5.12 g, 42×10^{-3} mol) in methylene chloride (50 mL) was vigorously stirred with an aqueous solution of sodium hydroxide (5%, 70 mL) and tetrabutylammonium bromide (2.26 g, 7×10^{-3} mol) at room temperature. To this mixture, a solution of 2,3,4,6-tetraacetyl-α-D-glucopyranosyl bromide (11.510 g, 28×10^{-3} mol) in methylene chloride (20 mL) was added. The mixture was vigorously stirred at room temperature for three days. After separation, the organic layer was washed by aqueous sodium hydroxide solution (5%, 2×20 mL) and water. The organic phase was dried over sodium sulfate, filtered and concentrated under vacuum. The crude yellow oil was purified by chromatography on silica gel eluted by a mixture of ethyl acetate/heptane (1/1, v/v) affording the pure titled product after crystallization from a mixture of ethanol/water (2.710 g, yield 21%).

Anal. Calcd for $C_{21}H_{24}O_{11}$, theoretical: C, 55.75; H, 5.35. Found: C, 55.65; H, 5.57.

^{1}H NMR ($CDCl_3$): 9.96 (s, 1H, CHO), 7.57 (d, 1H, H phenyl, $J = 7.3$ Hz), 7.50 (d, 1H, H phenyl), 7.44 (d, 1H, H phenyl, $J = 7.5$ Hz), 7.25 (m, 1H, H phenyl), 5.29 (d, 1H, HC_1 'ose', $J = 7.8$ Hz), 5.30 (m, 1H, H 'ose'), 5.15 (m, 2H, H 'ose'), 4.21 (m, 2H, HC_6 'ose'), 3.91 (m, 1H, HC_5 'ose'), 2.08 (s, 3H, acetyl), 2.04 (s, 6H, acetyl), 2.02 (s, 3H, acetyl).

4.1.2. 5,10,15-Tri[3-*O*-(2′,3′,4′,6′-tetraacetyl-β-D-glucopyranosyloxy)phenyl]-20-phenyl porphyrin 1. Freshly distilled pyrrole (0.410 g, 6.1×10^{-3} mol) in methylene chloride (70 mL), 3-*O*-(2′,3′,4′,6′-tetraacetyl-β-D-glucopyranosyloxy)benzaldehyde(2.26 g, 5×10^{-3} mol) and freshly distilled benzaldehyde (0.177 g, 1.7×10^{-3}) in methylene chloride (70 mL) were added to methylene chloride (750 mL) purged by argon during 30 min. The mixture was stirred under argon for a further 10 min. Then a BF_3-etherate solution in methylene chloride (0.3 mL, 0.5 M) was added and the mixture was stirred under argon then after 2 h BF_3-etherate solution (0.3 mL, 0.5 M) was added again. The mixture was stirred at room temperature overnight. *o*-Chloranil (1.2 g, 4.87×10^{-3} mol) was added and the solution was refluxed for 2 h and Et_3N (1.5 mL) was added. Silica gel (10 g) was added to the dark solution and the solvent was evaporated. The absorbed crude products were placed on the top of a silica gel chromatographic column. The porphyrin mixture was eluted with methylene chloride/acetone (10/1, v/v). The fifth red fraction was collected and purified again by preparative thin layer silica gel chromatography eluted with methylene chloride/acetone (10/1, v/v). The titled pure triglycosylated porphyrin was crystallized from methylene chloride/heptane (405 mg, yield 15%).

Anal. Calcd for $C_{86}H_{84}N_4O_{30}\cdot9H_2O$, theoretical: C, 56.89; H, 5.66; N, 3.09. Found: C, 56.71; H, 4.52; N, 2.68. UV–vis spectrum: λ_{max}, nm (ε, L/mmol cm): (CH_2Cl_2) 417 (410.1), 513.5 (18.6), 548 (8.1), 588 (6.9), 644 (4.7). ^{1}H NMR ($CDCl_3$): 8.88 (m, 8H, pyrrole), 8.21 (d, 2H, *ortho*-phenyl), 7.93 (m, 3H, *para*-phenoxy), 7.88 (br d, 3H, *ortho*′-phenoxy), 7.78 (d, 3H, *para*- and *meta*-phenyl), 7.68 (m, 3H, *meta*-phenoxy), 7.44 (d, 3H, *ortho*-phenoxy, $J = 7.4$ Hz), 5.37 (m, 6H, HC_1 and HC_2 or HC_3 'ose'), 5.31 (m, 3H, HC_3 or HC_2 'ose'), 5.17 (m, 3H, HC_4 'ose'), 4.18 (m, 3H, HC_{6a} 'ose'), 4.04 (m, 3H, HC_{6b} 'ose'), 3.80 (m, 3H, HC_5 'ose'), 2,09 (s, 9H, acetyl), 2.03 (s, 9H, acetyl), 1.98 (s, 9H, acetyl), 1.39 (s, 9H, acetyl), −2.80 (s, 2H, NH). ^{13}C NMR ($CDCl_3$): 170.3 (C=O acetyl), 170.2 (C=O acetyl), 169.3 (C=O acetyl), 155.3 (*meta*-phenoxy), 143.5 (*meso*-phenoxy), 141.8 (*meso*-phenyl), 134.5 (*ortho*-phenyl), 131 (CH pyrrole), 129.8 (*para*-phenoxy), 127.8 (*meta*-phenoxy), 126.8 (*meta*-phenyl), 123 (*para*-phenyl), 123–122.5 (br, *ortho*′-phenoxy), 120.6 (*meso*-C-phenyl), 119.3–119.1 (*meso*-C-phenoxy), 116.6 (*ortho*-phenoxy), 99.3 (C_1 'ose'), 72.7 ($C_{2/3}$ 'ose'), 72.2 (C_5 'ose'), 71.2 ($C_{3/2}$ 'ose'), 68.2 (C_4 'ose'), 61.9 (C_6 'ose'), 20.7 (CH_3 acetyl), 20.6 (CH_3 acetyl), 20.5 (CH_3 acetyl), 19.9 (CH_3 acetyl).

4.1.3. 5,10,15-Tri(3-methoxyphenyl)-20-phenyl porphyrin 5. Freshly distilled pyrrole (2.75 mL, 4×10^{-2} mol), benzaldehyde (1.01 mL, 10^{-2} mol) and *meta*-anisaldehyde (3.35 mL, 3×10^{-2} mol) are dissolved in propionic acid (250 mL). The solution was refluxed for 1 h then quickly cooled. The crude black solution was concentrated under vacuum then methanol (100 mL) was added. The blue precipitate was filtered and washed with cold methanol. The mixture of porphyrins was separated by silica gel chromatography column eluted with a mixture of methylene chloride/heptane (5/1, v/v). The third red band was collected and purified again by silica gel chromatography column eluted by toluene. The pure blue crystals of 5,10,15-tri(3-methoxyphenyl)-20-phenyl porphyrin was obtained after crystallization from methylene chloride/methanol mixture (456 mg, yield 6.5%).

Anal. Calcd for $C_{47}H_{36}N_4O_3$, theoretical: C, 80.09; H, 5.15; N, 7.95. Found: C, 79.43; H, 5.17; N, 7.88. UV–vis spectrum: λ_{max}, nm (ε, L/mmol cm): (CH_2Cl_2) 418.5 (502.9), 515 (21.7), 549 (8.9), 590 (7.3), 646.5 (5.9). ^{1}H NMR ($CDCl_3$): 8.88 (s, 6H, pyrrole), 8.83 (d, 2H, pyrrole, $J = 4.2$ Hz), 8.21 (d, 2H, *ortho*-phenyl, $J = 6.1$ Hz), 7.80 (m, 3H, *meta*- and *para*-phenyl), 7.80 (m, 6H, *ortho*-phenoxy), 7.64 (t, 3H, *meta*-phenoxy, $J = 7.7$ Hz), 7.32 (d, 3H, *para*-phenoxy, $J = 8$ Hz), 3.98 (s, 9H, OCH_3), −2.79 (s, 2H, NH). ^{13}C NMR ($CDCl_3$): 157.7 (*meta*-phenoxy), 143.2 (*meso*-phenoxy), 134.3 (*ortho*-phenyl), 130.8 (C-pyrrole), 127.3 (*meta*′-phenoxy), 120 (*meso*-C), 113.2 (*para*-phenoxy), 55.3 (OCH_3).

4.1.4. 5,10,15-Tri(3-hydroxyphenyl)-20-phenyl porphyrin 4d. 5,10,15-Tri(3-methoxyphenyl)-20-phenyl porphyrin **5** (100 mg, 0.14×10^{-3} mol) was dissolved in dry methylene chloride (25 mL) at −20 °C under argon. BBr_3 (0.604 mL, 6.3×10^{-3} mol) was slowly added. The green solution was stirred at −20 °C for 3 h then at room temperature overnight. The green mixture was diluted in cold water and neutralized by a saturated aqueous solution of $NaHCO_3$. The solution was extracted by ethyl acetate. The organic phase was washed with water (twice), dried with sodium sulfate, filtered then evaporated. The pure porphyrin was obtained after crystallization from ethyl acetate/heptane (81 mg, yield 84%).

Anal. Calcd for $C_{44}H_{30}N_4O_3$, theoretical: C, 79.74; H, 4.56; N, 8.45. Found: C, 79.32; H, 4.75; N, 8.25. UV–vis spectrum: λ_{max}, nm (ε, L/mmol cm): (acetone) 415.5 (368.7), 512.5 (17.8), 546.5 (7.8), 589.5 (6.1), 645.5 (4.7). ^{1}H NMR (acetone-d_6): 8.97 (s, 4H, pyrrole), 8.96 (d, 2H, pyrrole, $J = 8.1$ Hz), 8.84 (d, 2H, $J = 4.7$ Hz), 8.20 (dd, 2H, $J = 2$ and 7.4 Hz), 7.72 (m, 9H, *ortho*-phenoxy, *meta*-phenyl and *para*-phenyl), 7.57 (m, 3H, *meta*-phenoxy, $J = 8$ Hz), 7.31 (d, 3H, *para*-phenoxy, $J = 8$ Hz), −2.72 (s, 2H, NH). ^{13}C NMR (acetone-d_6): 143.7 (*meso*-phenoxy), 134.6 (*ortho*-phenyl), 131.5 (C-pyrrole), 128.2 (*para*-phenyl), 128.1 (*meta*′-phenoxy), 127.1 (*ortho*-phenoxy), 126.8 (*meta*-phenyl), 122.4 (*ortho*-phenoxy), 120.5 (*meso*-C), 115.4 (*para*-phenoxy).

4.1.5. 5,10,15-Tri(3-methoxyphenyl)-20-phenyl 2,3-chlorin and 5,10,15-tri(3-methoxyphenyl)-20-phenyl 7,8-chlorin. 5,10,15-Tri(3-methoxyphenyl)-20-phenyl porphyrin **5** (200 mg, 0.284 mmol) and anhydrous K_2CO_3 (265 mg) were added to dry pyridine (13 mL) under argon. Toluene-4-sulfonohydrazide (86 mg, 0.46 mmol) was then added and the mixture was heated under argon at 100–105 °C for 24 h. Further quantities of toluene-4-sulfonohydrazide (86 mg in 0.5 mL of dry pyridine) were added after 2, 4, 6 and 8 h. After cooling, the crude mixture was treated with ethyl acetate (90 mL), water (45 mL) and heated, at 100 °C, for 1 h. After cooling, the organic phase was separated and washed with HCl (2 M, 100 mL), water (75 mL) and saturated water solution of $NaHCO_3$. The presence of chlorin and bacteriochlorin was controlled by UV–visible spectroscopy (bands at 651 and 738 nm, respectively). *ortho*-Chloranil (196 mg) was slowly added to the stirred organic solution at 25 °C until the absorption peak at 735 nm (bacteriochlorin) disappeared. The solution was washed with aqueous solution of $NaHSO_3$ (5%, 100 mL), water (100 mL) and dried over anhydrous sodium sulfate. The filtered solution was concentrated under vacuum. The residue was purified by column chromatography on silica gel eluted by a mixture of methylene chloride/heptane (5/1, v/v). The pure product (red band) was crystallized from a mixture of methylene chloride/heptane (121 mg, yield 60%).

Microanalysis calcd for $C_{47}H_{38}N_4O_3$: C, 79.86; H, 5.42; N, 7.93. Found: C, 79.75; H, 5.26; N, 7.62. UV–visible spectrum in CH_2Cl_2 λ_{max} (ε, L/mmol cm): 401 (178.9), 419.5 (233.9), 518.5 (18.4), 545 (12.9), 598 (8.5), 651.5 (37.7). ^{1}H NMR ($CDCl_3$): 8.61*, 8.56** (d, 2H, pyrrole, $J = 4.9$ Hz), 8.46*, 8.40** (m*, d**, 2H, pyrrole, $J^* = 4.50$ Hz), 8.23*, 8.17** (d, 2H, pyrrole, $J = 4.85$ Hz), 8.10 (dd, 1H, *ortho*-phenyl, $J = 2$ and 7.6 Hz), 7.87 (dd, 1H, *para*-phenyl, $J = 2.4$ and 7 Hz), 7.69 (t, 6H, *para* and *meta*-phenyl and *ortho*-phenoxy, $J = 7$ Hz), 7.58 (q, 3H, phenoxy, $J = 7.5$ Hz), 7.44 (m, 3H, *para*-phenoxy), 7.26 (dd, 2H, *meta*-phenoxy, $J = 2.6$ and 8.20 Hz), 7.20 (dd, 1H, *meta*-phenoxy, $J = 2.5$ and 8.3 Hz), 4.19*, 4.17** (m, 2H, CH_2 pyrrole), 3.94*, 3.93** (s, 9H, OMe), −1.47 (s, 2H, NH).

4.1.6. 5,10,15-Tri(3-hydroxyphenyl)-20-phenyl 2,3-chlorin and 5,10,15-tri(3-hydroxyphenyl)-20-phenyl 7,8-chlorin 2a/3a. 5,10,15-Tri(3-methoxyphenyl)-20-phenyl 2,3-chlorin and 5,10,15-tri(3-methoxyphenyl)-20-phenyl 7,8-chlorin (112 mg, 0.16×10^{-3} mol) was dissolved in dry methylene chloride (30 mL) at −20 °C under argon. BBr_3 (0.685 mL, 7.1×10^{-3} mol) was slowly added. The green solution was stirred at −20 °C for 3 h then at room temperature overnight. The green mixture was diluted in cold water and neutralized by a saturated aqueous solution of $NaHCO_3$. The solution was extracted by ethyl acetate. The organic phase was washed with water (twice), dried with sodium sulfate, filtered then evaporated. The pure chlorin was obtained after crystallization from ethyl acetate/heptane (100 mg, yield 95%).

Microanalysis calcd for $C_{44}H_{32}N_4O_3 \cdot H_2O$: C, 77.40; H, 5.02; N, 8.21. Found: C, 77.23; H, 4.91; N, 8.04. UV–visible spectrum in acetone λ_{max} (ε, L/mmol cm): 404 (107.1), 416 (121), 516 (10.2), 542 (7), 597 (4.3), 650.5 (24.4). ^{1}H NMR (acetone-d_6): 8.55*, 8.47** (d, 2H, pyrrole, $J = 4.5$ Hz), 8.53 (s, 3H, OH), 8.33*, 8.23** (d, 2H, pyrrole, $J = 4.5$ Hz), 8.17*, 8.07** (d, 2H, pyrrole, $J = 4.6$ Hz), 7.98 (m, 1H, *ortho*-phenyl), 7.79 (m, 1H, *para*-phenyl), 7.59 (m, 3H, *para* and *meta*-phenyl), 7.44 (m, 6H, *ortho*-phenoxy), 7.26 (m, 3H, *para*-phenoxy), 7.09 (dd, 3H, *meta*-phenoxy, $J = 8$ and 20 Hz), 4.10*, 4.07** (m, 2H, CH_2 pyrrole), −1.60 (s, 2H, NH).

4.2. Cell culture and sensitizer incubation

Human colorectal adenocarcinoma cells (HT29) were allowed to grow to confluence in Dulbecco's modified medium (DMEM) supplemented with 10% FCS, gluta-

mine and antibiotics. All reagents were obtained from Bio-Media (France). Cells were subcultured by dispersal with 0.25% trypsin and seeded (10^6 cells/mL). Incubations were performed in the dark. Stock solutions of **2a/3a** (5 mg/mL) were prepared in DMSO and dilutions were performed in culture medium in the presence of 2% of fetal calf serum (FCS).

4.3. Cell extraction

Treated cells were washed three times with cold PBS and then removed using a cell scraper. After sonication, EtOAc (3 mL) was added to 600 μL of cell suspension in PBS. The resulting solution was left in the dark for 30 min with sustained shaking and then centrifuged at 1700*g* for 10 min. The supernatant was removed and evaporated to dryness for further analysis. The sensitizer was recovered in greater than 90% yield. As determined by mass spectrometry, this procedure does not promote any deglucosylation of our glycoconjugated chlorins (ratio of **2a/3a** to **4a** remains the same). The corresponding protein concentration was determined by Lowry's method[23] using a protein assay (Kit P56 56, Sigma Aldrich—France).

4.4. Mass spectrometry analysis

Positive ion MALDI-TOF mass spectra 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. Ions were detected by a dual channel plate detector. The mass spectrum presented in Figure 1 represents an average over 256 consecutive laser shots (3 Hz repetition rate). For sample preparation, the matrix, α-cyano-4-hydroxycinnamic acid (HCCA), was dissolved at a concentration of 0.1 M in methanol. Ten microliters of matrix solution were added to the evaporated supernatant of cellular extract and 1 μL deposited onto a polished gold sample stage and allowed to dry in air.

4.5. HPLC separation

HPLC separations were achieved using a Spheri 5RP18 column (220 mm×4.6 mm; 5 μm particle diameter). The mobile phase was 0.1% aqueous trifluoroacetic acid (TFA)/acetonitrile (30/70, v/v) pumped at a flow rate of 0.8 mL/min (LC-10AS pump, Shimadzu, Japan). Dried extracts were diluted in 250 μL of mobile phase from which 100 μL aliquots were injected. A Shimadzu SPD-6AV UV–visible spectrophotometric detector set at 420 nm was used along with a fluorescence detector (FR-551) set at 420 and 650 nm, for the excitation and emission wavelengths, respectively. The HPLC device was equipped with a CR5A chromatopac integrator (Shimadzu, Japan).

4.6. Phototoxicity assay

The phototoxicity of TPC(*m*-*O*-Glu)$_3$ **2a/3a** was determined in HT29 cells following the detailed procedure in Ref. 8. Briefly, cells were seeded into 96-well plates at 10^5 cells/mL and allowed to grow in an incubator (5% CO_2, 37 °C, humidified atmosphere, Jouan, France). On the day of experiment, fresh DMEM with 2% FCS containing the photosensitizer at a final concentration between 0.5 and 2 μM were added in each well. Cells were incubated for 3 h and then washed before fresh complete medium was added. Irradiations were performed at 514 nm (5 J/cm^2) with an Ar^+ laser (Spectra Physics-2020) under sterile conditions. Cell viability was measured 24 h later by determination of mitochondrial activity using the MTT assay according to Mossmann.[24] Optical densities of microplates were determined at 570 and 640 nm using a Labsystem®.

4.7. Intact cell enzymatic assay

Glucosidase activity in HT29 cells was determined according to Sawkar et al.[25] Cells were allowed to grow for 24 h into 24-well plates (2×10^5 cells/well). Cellular medium was replaced by 150 μL of NaOAc buffer (pH 4) and the enzymatic reaction was started by the addition of 100 μL of 4-methylumbelliferyl glucoside (5 mM). One hour later, the reaction was stopped and the cells were lysed by the addition of 2 mL of glycine buffer (pH 10). The concentration of released 4-methylumbelliferone (4-MU) was measured by fluorescence spectroscopy ($\lambda_{exc.}$ 365 nm, $\lambda_{em.}$ 445 nm). Enzyme concentration was determined using a calibration curve established for 4-MU in glycine buffer.

Note: *2,3-chlorin or 7,8-chlorin isomer, **7,8-chlorin or 2,3-chlorin isomer. The ratio of isomers is 1/1.

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