Ethylene Glycol and Amino Acid Derivatives of 5-Aminolevulinic Acid as New Photosensitizing Precursors of Protoporphyrin IX in Cells

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Protoporphyrin IX (PpIX) is used as a photosensitizing agent in photodynamic detection and therapy (PDT) of cancer and is synthesized intracellularly from aminolevulinic acid (ALA) precursors. To evaluate means to specifically target ALA derivatives to defined cells, we have synthesized and characterized ethylene glycol esters and amino acid pseudodipeptide derivatives of ALA as potential specific substrates for cellular esterases and aminopeptidases, respectively. The PpIX formation induced by these products was investigated using cultures of human and rat cell lines of carcinoma and endothelial origins. The cytotoxicity of these compounds in the absence of light was also controlled. The results have shown that ethylenglycol esters can induce high levels of PpIX and are useful at concentrations below their cytotoxicity threshold. From the ALA-amino acid derivatives which were evaluated, the highest PpIX production was obtained using ALA derivatives of neutral amino acids, as compared to acidic or basic amino acids.

Introduction

Photodynamic detection and therapy (PDT) involves the detection and destruction of diseased tissue by visible light after loading of the target tissue with a photosensitizer. An approach to PDT of cancer, in preclinical and clinical studies, is based on the endogenous accumulation of protoporphyrin IX (PpIX) following topical or systemic administration of 5-aminolevulinic acid (ALA), used as a precursor photosensitizer.[1] This therapeutic approach is becoming an option in the treatment of diseases, including tumors and pretumoral lesions or vascular proliferative disorders, and has recently been approved in the Food and Drug Administration in the United States for the treatment of actinic keratosis. Cancer treatment is presently attempted by targeting the destruction of the tumor cells or the tumor-associated vascular endothelial cells. Whereas ALA-PDT has proved very successful clinically for superficial applications in skin and hollow organs such as the bladder, problems have arisen in treating deeper lesions following intravenous administration,[2,3,4] resulting in a nonspecific distribution of ALA derivatives in cells of any type[2] and sensitization to light. Thus, to limit unwanted side effects to normal tissue, it would be valuable to design ALA derivatives displaying selectivity for tumor-associated cells or vascular endothelial cells, depending on the particular disease to be treated.

Although tightly regulated PpIX formation exists in nearly all nucleated cell, it preferentially accumulates in tissues with a high cellular turnover such as tumor cells.[5,6] Biological and chemical approaches have been attempted in order to improve the selectivity of ALA delivery or yield of PpIX production from ALA. Using an adenoviral vector, a constitutively active cytoplasmic ALA-synthase, the first regulatory enzyme of the PpIX biosynthetic pathway, was expressed in cultured tumor cells, inducing a large increase in PpIX production and photosensitivity.[7] Another approach was to use more lipophilic ALA derivatives, such as alkyl-esters substrates for cellular esterases,[8−11] as ALA is a hydrophilic molecule with a poor penetration into cellular membranes and interstitial space.[12] However, the use of ALA-esters resulted in a nonspecific distribution of ALA in all cell types, with a certain preference for tumor cells. This approach required 30−150-fold lower concentration of ALA derivatives, depending on the particular ester, than ALA itself for comparable amounts of PpIX produced.[8,10,13]

In an attempt to improve cell selectivity of ALA and its derivatives, we investigated PpIX formation, using direct fluorescence measurement in cells in culture, from ALA precursors, either esters of the polyethylenglycol (PEG) family as substrates for cellular esterases, or basic, acidic, and neutral amino acid derivatives which represent potential substrates for cell-surface and cytoplasmic aminopeptidases and/or ligand for peptide and amino acid transporters. The synthesis, characterization, and evaluation in cell culture of these new ALA derivatives are described. ALA-PEG-esters appeared to be a potentially better precursor than ALA-alkylesters, leading to high levels of production of PpIX in cells and displaying an improved ratio of PpIX production versus cytotoxicity. Of the amino acid derivatives tested, ALA-pseudopeptide derivatives of neutral amino acids induced the highest PpIX production.

Results

Synthesis of ALA Derivative Precursors of PpIX. 1. Synthesis of the Ethylene Glycol Derivatives. The initial feature which was examined for its capacity to obtain derivatives of 5-amino levulinate capable to

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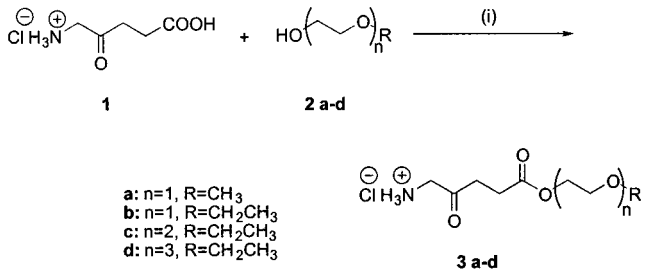
Scheme 1[a]



[a] Reagents and conditions: (i) $SOCl_2$, 50 °C, 5 h.

Table 1. Synthesis of ALA Ethylene Glycol Ester Derivatives[a]

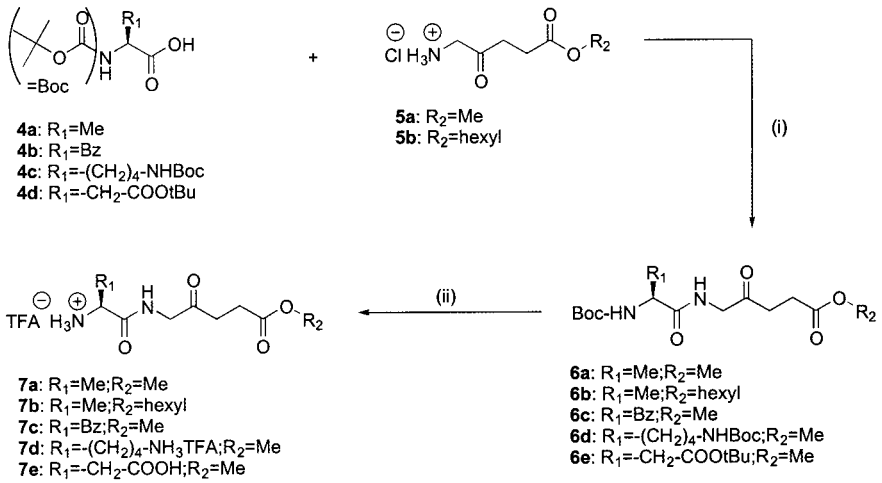
code	yield [%]	name
3a	90	ALA-O-ethylene glycol monomethyl ether
3b	89	ALA-O-ethylene glycol monoethyl ether
3c	87	ALA-O-diethylene glycol monoethyl ether
3d	90	ALA-O-triethylene glycol monoethyl ether

[a] Yield of the syntheses, and numeration and nomenclature of the derivatives used in cell cultures.

induce PpIX production in cells was the use of ethylene glycol derivatives **2a**−**d** as alcohols used for the esterification. Earlier tests with simple alkyl esters had shown that increasing the chain length induced higher production of PpIX.[9,13] A major limitation to this approach was the reduced solubility in water of the esters derived from long chain alcohols. Ethylene glycols have the interesting properties to be at the same time hydro- and lipo-soluble. The esters **3a**−**d** derived 5-amino levulinate (**1**) and the corresponding ethylene glycols **2a**−**d** were obtained in excellent yields starting from the in situ prepared acid chloride and the corresponding alcohols (Scheme 1, Table 1). The major problem was the removal of the corresponding alcohol used in excess. The compounds were fully characterized spectroscopically; however, the elemental analysis indicated that some amount of the corresponding alcohol was still present in our samples. Preliminary tests were undertaken to determine the relative rate of the enzymatic hydrolysis of these esters using α-chymotrypsin and hog liver esterase. The rate of hydrolysis of these esters **3a**−**d** was compared with the rate of hydrolysis of the *n*-hexyl 5-amino levulinate. The rate of the enzymatic hydrolysis of our esters was comparable and in most cases even higher than the rate of the enzymatic hydrolysis of the *n*-hexyl 5-amino levulinate, a compound inducing the production of high levels of PpIX production in vitro and in vivo.

2. Synthesis of the Amino Acid Derivatives. The condensation of 5-aminolevulinate (**1**) to obtain the peptide analogues **6a**−**e** was straightforward[14] (Scheme 2). In our first trials we had used Fmoc-protected amino acids for the condensation reaction. However, it became clear that the deprotection of the Fmoc-group was difficult. The peptide analogues were not stable under basic conditions. So we turned to the Boc-protected amino acids **4a**−**d**. In contrast to the Fmoc-protected peptide analogues, most of the Boc-protected peptides **6a**−**e** were not crystalline, which rendered the purification less convenient. After optimization of the reaction conditions starting from the Boc-peptides **6a**−**e,** the mono-deprotected peptides **7a**−**e** could be obtained in good yield as their trifluoroacetates (Scheme 2, Table 2). To test the importance of the protecting groups of the carboxylate and the amino functions, selective mono-deprotection of the carboxylate group was achieved using pig liver esterase (Scheme 3). Compound **8a** could

Scheme 2[a]



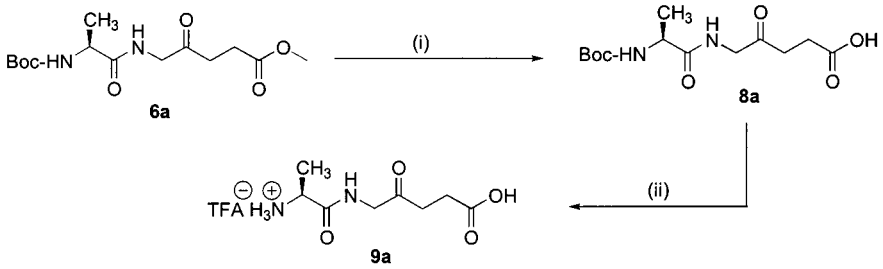
[a] Reagents and conditions: (i) HOBt, EDC, Net_3, room temperature, 12 h; (ii) TFA, room temperature, 1 h.

Table 2. Synthesis of Pseudopeptide Amino Acid ALA Derivatives[a]

code	R_1[b]	R_2[b]	yield [%]	name
6a	methyl-	-O-methyl	72	Boc-Ala-ALA-O-methyl
7a	methyl-	-O-methyl	71	Ala-ALA-O-methyl
7b	methyl-	-O-hexyl	64	Ala-ALA-O-hexyl
7c	benzyl-	-O-methyl	74	Phe-ALA-O-methyl
7d	$-(CH_2)_4-NH_3$·TFA	-O-methyl	69	Lys-ALA-O-methyl
7e	$-CH_2$-COOH	-O-methyl	72	Asp-ALA-O-methyl
8a	methyl-	-H	88	Boc-Ala-ALA
9a	methyl	-H	82	Ala-ALA

[a] Yield of the syntheses, and numeration and nomenclature of the derivatives used in cell cultures. [b] R_1 and R_2 according to Scheme 2.

Scheme 3[a]



[a] Reagents and conditions: (i) PLE, phosphate buffer pH 8.0, room temperature, 5 h; (ii) TFA, room temperature, 1 h.

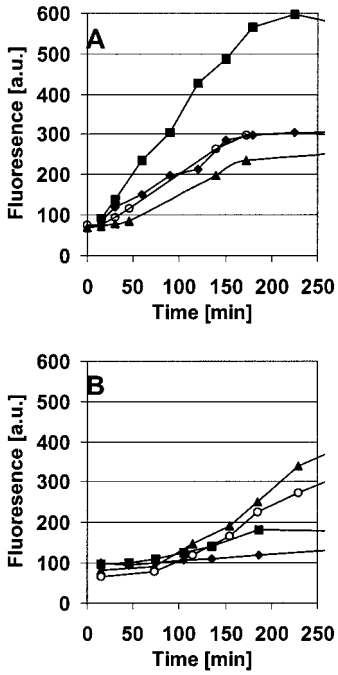


Figure 1. PpIX fluorescence production following exposure of human endothelial HCEC and carcinoma A549 cells to ALA-ethylene glycol esters. (A) Effect of the length of the ether and glycol chain in HCEC endothelial cells: ◆ ALA-O-ethylene glycol monomethyl ether (**3a**) [87 μM]; ■ ALA-O-ethylene glycol monoethyl ether (**3b**) [82 μM]; ▲ ALA-O-diethylene glycol monoethyl ether (**3c**) [100 μM]; ○ ALA-O-triethylene glycol monoethyl ether (**3d**) [90 μM]. (B) Effect of the length of the ether and glycol chain in A549 carcinoma cells: ◆ ALA-O-ethylene glycol monomethyl ether (**3a**) [87 μM]; ■ ALA-O-ethylene glycol monoethyl ether (**3b**) [82 μM]; ▲ ALA-O-diethylene glycol monoethyl ether (**3c**) [79 μM]; ○ ALA-O-triethylene glycol monoethyl ether (**3d**) [85 μM].

be obtained in good yield (Table 2) and high purity. The totally deprotected peptide **9a** was obtained using trifluoroacetic acid. All peptides which contained an amino group had to be stored as their trifluoroacetates at 4 °C.

Evaluation of the Production of PpIX from Synthetic Precursors in Cell Lines. 1. Production of PpIX from the Synthetic ALA Derivatives of Ethylenglycols. The production of PpIX from ALA-esters of the ethylenglycol family **3a**−**d** was evaluated using human cells in culture either from endothelial (HCEC) (Figure 1A) or tumor (A549) origin (Figure 1B). First, the effect of the chain length of the ether moiety was determined. Production of PpIX from the ALA O-methyl ethylene glycol and O-ethyl ethylene glycol esters **3a** and **3 b** were compared in endothelial (Figure 1A) and tumor (Figure 1B) cells. In endothelial cells (Figure 1A), the O-ethyl-derivative **3b** produced higher levels of PpIX than the O-methyl-derivative **3a**, while in tumor cells (Figure 1B), the PpIX level was lower than in endothelial cells for both derivatives. The effect of increasing the chain length of the ethylene glycol moiety was evaluated next. In endothelial cells (Figure 1A), no differences were observed between the mono-, di-, and triethylene glycol ethyl ether **3a, 3c**, and **3d**. In tumor cells (Figure 1B), increasing the ethylene glycol chain length slightly increased the PpIX production. These results suggest that some selectivity for endothelial cells toward tumor cells can be obtained using ALA esters of the ethylene glycol family like **3a**−**d**. The cytotoxicity in the absence of added light of these ethylene glycol derivatives **3a**−**d** was controlled by determining the efficacy index, comparing PpIX fluorescence at 300 min with cell survival (MTT evaluation) after 20 h exposure to increasing concentrations of these compounds. No cytotoxicity was observed for HCEC cells (Figure 2A) or A549 cells (Figure 2B) exposed to low concentrations of ALA-ethylene glycol ethyl ether, when the PpIX fluorescence was already high; while in cells exposed to higher concentrations of the ethylene glycol derivatives, cytotoxicity was observed (Figure 3A−D). The results indicated that endothelial cells are more sensitive than tumor cells to the cytotoxic effect of these derivatives, paralleling the PpIX production.

2. Production of PpIX from the Synthetic ALA Derivatives of Amino Acids. To evaluate a different approach to cell-specific targeting of ALA derivatives, we prepared the amino acid pseudopeptide derivatives of ALA **6a**−**e**, **7a**−**e**, **8a**, and **9a** which are potential substrates for cellular aminopeptidases. Amino acid-ALA derivatives of acidic, basic, or neutral amino acids, as potential substrates for aminopeptidase A (APA), APB, or APN/M, respectively, were synthesized and characterized (listed in Table 2). The expression of these aminopeptidase activities was controlled in the cells lines that were used (Table 3). A particular aminopeptidase activity was expressed at high level by a defined cell line for each of the activities evaluated: APA by EC219 cells, APB by HCEC cells, and APN/M by A549 cells. A549 cells did not express measurable APA activity. By far, at equimolar concentration, the best precursor is the neutral aromatic amino acid-derivative substrate **7c** for APN/M-like aminopeptidases in all cell lines tested and whatever the level of expression of this enzymatic activity by the different cell lines (Figure 4A−C). The results show that basic amino acid derivatives such as **7d** are not precursors for PpIX production whatever the cell line, including HCEC cells which express high levels of APB activity, and the substrate

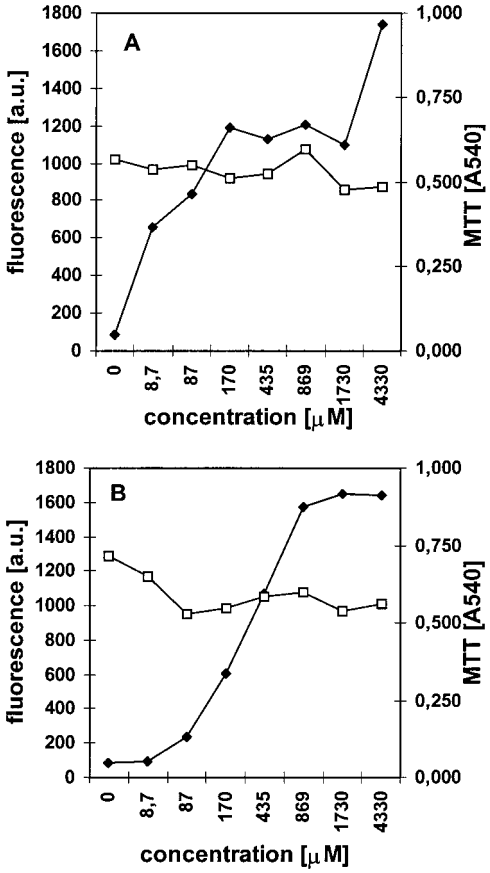


Figure 2. PpIX fluorescence and MTT reduction in HCEC (A) and A549 (B) cells exposed to increasing low concentrations of ALA-O-ethylene glycol monoethyl ether (**3b**). ◆: PpIX fluorescence [a.u.]; □: MTT reduction [A_{540} nm].

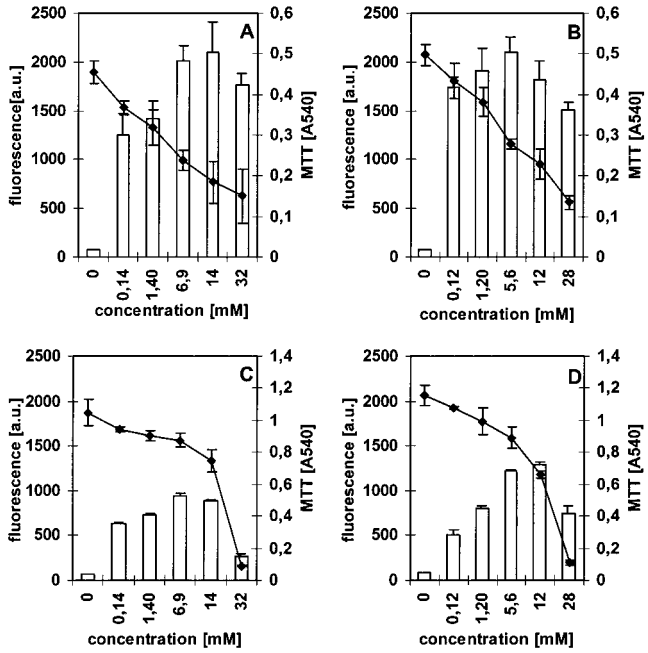


Figure 3. Efficacy of the ALA-O-diethylene glycol and ALA-O-triethylene glycol monoethyl ethers in human endothelial and tumor cells at increasing high concentrations of ALA-ethylene glycol derivatives. PpIX production (fluorescence at 300 min) and cytotoxicity at 20 h (MTT assay, absorbance at 540 nm) of the ALA-O-diethylene glycol monoethyl (**3c**) (A, C) and ALA-O-triethylene glycol monoethyl (**3d**) (B, D) ethers in human endothelial HCEC (A, B) and carcinoma A549 (C, D) cells were determined. Results are means of sextuple wells ± SEM. Bars: PpIX fluorescence [a.u.]; ◆: MTT reduction [A_{540} nm].

concentration used (Figure 3C). Acidic derivatives such as **7e** are poor precursors in the cells expressing APA (Figure 4A,B), EC219, and HCEC cells, while no PpIX production was obtained in APA-negative A549 cells (Figure 4C). However, in EC219 cells expressing high APA activity, PpIX production can be observed using high concentrations, up to 10 mM, of the precursor (results not shown). To test aminopeptidase A accessibility to substrate, we determined in the brain-derived endothelial EC219 cells and in brain-derived endothelial cells of human glioblastoma the in situ activity of APA using Glu-methoxy-naphthylamide substrate which results in a red precipitate of the methoxy-naphthylamine with diazotating reagents following hydrolysis (Figure 5). The results showed that APA is easily accessible in EC219 cells as well as in the vascular system of human glioblastoma. Thus targeting of cellular proteases may be of use in the targeting of photosentizers to defined cells.

The specificity of neutral aliphatic amino acid-ALA derivatives such as **7a** and **7c** for APN/M-like aminopeptidase was further evaluated using the N-substituted Ala-ALA derivative **6a** and **8a**, which would not be substrates for such an enzyme, or the AlaALA-methyl and -hexyl esters **7a** and **7b**, which would make them better cell-permeant with increasing hydrophobicity. The results showed that, as expected, the N-substituted products **6a** (not shown) and **8a** (Figure 6A) are not PpIX precursors, validating the necessity for an APN/M-like activity to release ALA from the amino acid derivative, and that increasing hydrophobicity (Ala-ALA-methyl **7a** versus Ala-ALA-hexyl **7b**) did result in increased PpIX production. Comparison of PpIX production between Ala-ALA **9a** and Ala-ALA hexyl ester derivatives **7b** (Figure 6A) with ALA and ALA-hexyl ester (Figure 6B) demonstrated a comparable behavior with increasing lipophilicity, suggesting a common mechanism for intracellular uptake.

Table 3. Expression of Aminopeptidase (AP) A, B, and N/M Activities in the Endothelial and Carcinoma Cell Lines[a]

cell line substrates	ref	APA[b] Glu-, Asp-X	APB[b] Lys-, Arg-X	APN/M[b] Phe-, Ala-, Leu-X
A549[c]	28	−	+	++
HCEC[d]		+	++	+
EC219[d]	27	++	+	+

[a] Cells were grown to confluence and enzymatic activities were measured. [b] Symbols: −, no measurable activity. +, low enzymatic activity; ++, high enzymatic activity. [c] A549 cells: human lung carcinoma cell line. [d] HCEC and EC219 cells: cerebral endothelial cells of human and rat origin, respectively.

Discussion

The targeted delivery to defined cells of anticancer treatments, including photosensitizers in PDT, is an intense field of research. Precursor photosensitizers, such as ALA, must be internalized by target cells in order to be processed to the photosensitizer PpIX in mitochondria, depending on the presence in target cells not only of the necessary transport systems able to deliver ALA into the cytoplasm, but also of the processing enzymes able to release free ALA from its pro-drug

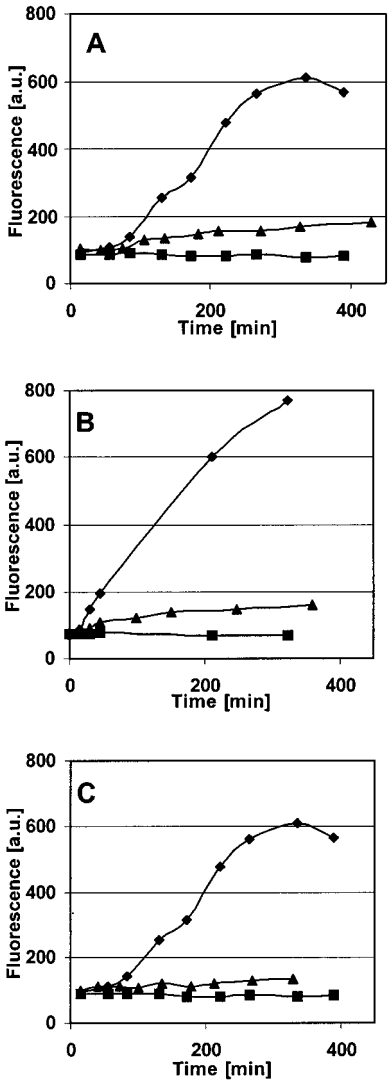


Figure 4. PpIX fluorescence production following exposure of human and rat endothelial cells and human carcinoma A549 cells to ALA-amino acid esters. (A) Acidic, basic, and neutral amino acid derivatives added to human endothelial HCEC cells: ▲ Asp-ALA-O-methyl (**7e**) [1.57 mM]; ■ Lys-ALA-O-methyl (**7d**) [1.17 mM]; ◆ Phe-ALA-O-methyl (**7c**) [1.43 mM]. (B) Acidic, basic, and neutral amino acid derivatives added to rat endothelial EC219 cells: ▲ Asp-ALA-O-methyl (**7e**) [1.88 mM]; ■ Lys-ALA-O-methyl (**7d**) [1.61 mM]; ◆ Phe-ALA-O-methyl (**7c**) [1.68 mM]. (C) Acidic, basic, and neutral amino acid derivatives added to human carcinoma A549 cells: ▲ Asp-ALA-O-methyl (**7e**) [1.57 mM]; ■ Lys-ALA-O-methyl (**7d**) [1.17 mM]; ◆ Phe-ALA-O-methyl (**7c**) [1.43 mM].

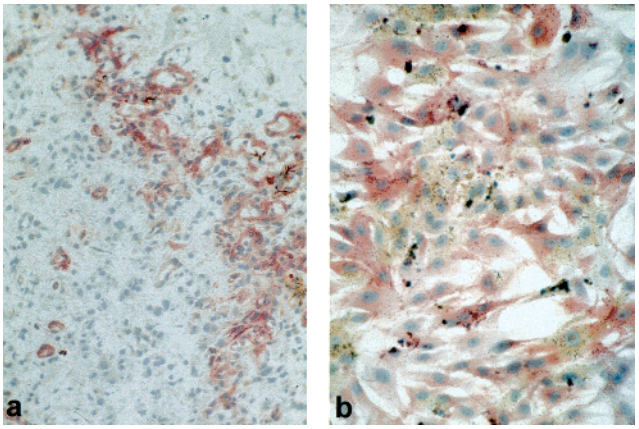


Figure 5. In situ aminopeptidase A activity (histoenzymography) in tumor vasculature of human glioblastoma and intact EC219 cells: (a) human glioblastoma; (b) EC219 cells.

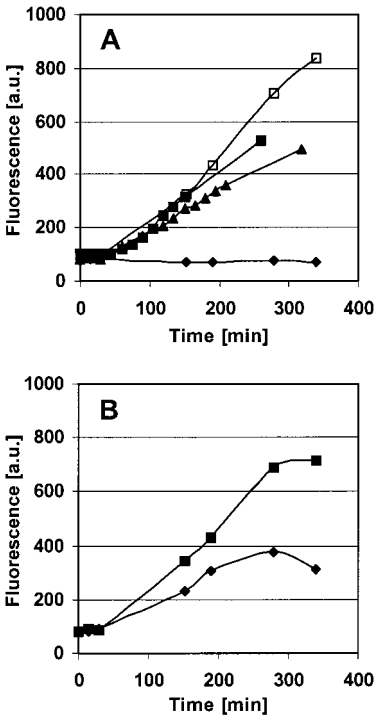


Figure 6. PpIX fluorescence production following exposure of human carcinoma A549 cells to N- and C-blocked Ala-ALA esters. (A) ◆ Boc-Ala-ALA (**8a**) [10.9 mM]; ■ Ala-ALA (**9a**) [11.1 mM]; ▲ Ala-ALA-Me (**7a**) [11.4 mM]; □ Ala-ALA-Hex (**7b**) [1.9 mM]; (B) ◆ ALA [3.9 mM]; ■ ALA-hex [39.8 μM].

and to perform the biosynthetic pathway. These specific metabolic processes may be different in cells of different phenotypes.[15] Our present goal was thus to define means to specifically target ALA delivery either to tumor-associated endothelial cells or to tumor cells. To evaluate the potential of targeting specific cellular esterases, we compared PpIX production obtained following exposure of human endothelial cells or human carcinoma cells to ALA-esters of the ethylene glycol family **3a**−**d** in comparison with information obtained using alkyl esters of ALA.[8,13] In a different approach, based on the previous information that defined acidic, basic, or neutral aminopeptidase activities are differently expressed in tumor vasculature when compared to normal vasculature of the same organ,[16] we prepared amino acid-ALA substrates for aminopeptidases and compared PpIX production in cell lines of endothelial and carcinoma phenotype, expressing high or low levels of the three types of aminopeptidases. ALA esters of the ethylene glycol family **3a**−**d** demonstrated some selectivity for endothelial cells toward tumor cells, and ALA substrates for aminopeptidases, in particular APN/M, demonstrated the potential to obtain PpIX from such prodrugs.

It has been previously shown that ALA-hexyl ester is internalized more rapidly and efficiently that ALA or ALA-methyl ester;[8,9,13,17] however, no differences in the cellular levels of PpIX were observed between endothelial and tumor cells (Juillerat, unpublished results). Using ALA-esters of ethylene glycols **3a**−**d**, we obtained increased PpIX production in endothelial cells

compared to tumor cells, especially for long chain ethers. In addition, the ethylene glycol derivatives **3a**−**d** used in the present experiments displayed a better PpIX fluorescence yield versus cytotoxicity in the absence of light than did the alkyl esters (Juillerat, unpublished results), suggesting that reduced cytotoxic side effects for bystander cells may be obtained using this class of compounds. However, cytotoxicity associated with cell specificity may also be an advantage in cancer treatment. Evidence exists that part of the efficiency of PDT involves vascular damage. PDT may cause tumor regression and cell death (apoptosis or necrosis)[18] either by direct death of tumor cells themselves or also by inducing tumor hypoxia through microvascular shutdown.[19,20] Clinically, the selective targeting of drugs to tumor-associated endothelial cells after systemic injection is also an advantage when compared to targeting the tumor cells themselves.

The use of amino acid derivatives of ALA had not been attempted before, to the best of our knowledge. Substrates for basic aminopeptidases such as **7d** were not precursors for PpIX production, even in cells expressing APB activity, while substrates for acidic aminopeptidases were precursors only in cells expressing APA activity. However, high concentrations of the pro-drug had to be used in order to obtain the intracellular PpIX concentrations which can be relevant for PDT. We did not attempt to determine the kinetic characteristics of hydrolysis of Lys-ALA **7d** or Asp-ALA **7e** by cellular aminopeptidases or the mechanisms of cellular uptake. Thus we cannot with the present experiments determine whether poor kinetic constants, a low cell penetration, or delivery of the prodrug to a cellular compartment different from the cytoplasm where ALA dehydratase is located is responsible for the low PpIX production. Using substrates for APN/M activity, Ala-ALA **7a** and Phe-ALA **7c**, we obtained high yields of PpIX. These enzymes are located both on the cell surface and in the cytoplasm. PpIX production was not observed using the N-terminal Boc-derivative **6a** or **8a**, which are not substrates for such an enzyme, demonstrating the involvement of APN/M activity in the release of ALA. Production of PpIX from Ala-ALA, free acid **9a**, and methyl or hexyl esters **7a**,**b** was comparable to PpIX production obtained from ALA free acid and methyl or hexyl esters, suggesting that the lipophilicity of amino acid pro-drugs facilitates their cell penetration and that both amino acid-ALA and ALA derivatives use a common transport system. However, our approach cannot formally differentiate between the alternative that either (i) Ala-ALA or its esters are first hydrolyzed at the cell surface and then free ALA or its esters are internalized or (ii) the hydrolysis is performed intracellularly following transport of the amino acid-ALA derivative. The mechanism of entry of externally added ALA and its esters into cells have not been determined. ALA uptake by cells in culture has been shown to be inhibited by some amino acids,[21] peptides[22] or modulators of ion-cotransporters,[17,22,23] suggesting the involvement of an amino acid/peptide (co)transport. In mammalian cells transfected with dipeptide transporters, radioactive ALA was translocated by saturable and pH-dependent transport mechanisms using a cation/ALA cotransport.[17,22] Di- and tripeptides and serum, but not amino acids, such as Glu or GABA,[24] competed for transport, indicating that the amino acid transport system involved does not transport acidic and basic amino acid analogues. This information may explain the poor efficiency of acidic- and basic-ALA derivatives as PpIX precursors, suggesting that the hydrolysis of the amino acid-ALA derivative is performed by intracellular aminopeptidases, mainly of the neutral type, following entry of compounds into cells. As APA activity has been mainly described as an ectoenzyme, this may also explain the poor efficiency for this pro-drug in PpIX production.

In conclusion, we demonstrate here the potential of a new approach for the specific targeting of tumor-associated endothelial cells or tumor cells for PDT protocols. The cell-specific delivery of PpIX precursors may be obtained using cell-specific addresses consisting of esters of ethylene glycols or peptide derivatives of ALA for cellular esterases and peptidases, respectively.

Experimental Section

Synthesis. General Synthetic Methods. Melting points were determined on a Gallenkamp MFB-595 apparatus and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer FT-IR 1720 X spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AMX-400 and are reported in ppm relative to TMS and referenced to the solvent indicated. Mass spectra were recorded on the following instruments, using the stated ionization methods: Nermag RC 30−20 mass spectrometer, electron impact ionization mass spectra (EI), and direct chemical ionization mass spectra (DCI) using NH_3 as reactant; Finnigan LCQ mass spectrometer, electrospray ionization (ESI); Bruker FTMS 4.7T BioAPEX II mass spectrometer, high-resolution mass spectra (HRMS) measured using the ESI technique, which allowed to determine the molecular formula of the $[M + H]^+$ or the $[M + Na]^+$ peak. Elemental analyses were obtained from the CHN analyses laboratory of Ciba Specalties in Marly and are within 0.4% of theory unless otherwise noted. Reagents were used as acquired from commercial sources without purification. Anhydrous solvents were obtained by distillation over an adequate drying agent.[25] Solvent was removed by rotary evaporation under reduced pressure, and silica gel chromatography was performed using Merck silica gel 60 with a particle size of 40−63 μm. The purified compounds were analyzed by thin-layer chromatography (silica gel 60 F_{254} 0.2 mm, Merck, solvent CH_2Cl_2/CH_3OH/H_2O 70:30:5, detection with $KMNO_4$). To keep the numbering of the synthesized compounds coherent, the carbon of the ester function or the carbon of the carboxylate function was always attributed the number 1.

General Procedure for the Synthesis of Compounds 3a−**d.** To a solution of 0.5 mL (7 mmol) of thionyl chloride (Fluka) in 3 mL (38 mmol) of methoxyethanol (Fluka, **2a**) was added 500 mg (3 mmol) of 5-aminolevulinic acid hydrochloride (ALA, **1**), and the solution was stirred at 50 °C for 5 h under argon atmosphere. The resulting solution was directly applied to a silica chromatography column (50 g of silica gel (Merck), eluent: dichloromethane/methanol 95/5) to provide the product (**3a**) as yellowish oil.

Compound 3a: 90% yield; ^{1}H NMR (CD_3OD) δ 4.30−4.28 (*m*, 2H, $H_2C(1^2)$), 4.13 (*s*, 2H, $H_2C(5)$), 3.70−3.67 (*m*, 2H, $H_2C(1^3)$), 3.45 (*s*, 3H, $H_3C(1^4)$), 2.95−2.92 (*m*, AA′ of a AA′BB′-system, 2H, $H_2C(3)$), 2.81−2.78 (*m*, BB′ of AA′BB′-system, 2H, $H_2C(2)$); ^{13}C NMR (CD_3OD) δ 203.4 (C(4)), 174.2 (C(1)), 71.7 ($C(1^3)$), 65.1 ($C(1^2)$), 59.4 ($C(1^4)$), 48.5 (C(5)), 35.6 (C(3)), 28.8 (C(2)); MS (EI) *m*/*z* relative intensity 190 (100, $[M - Cl]^+$); HRMS (ESI) calcd for $C_{11}H_{22}NO_5^+$ 190.1073, obs. 190.1071. Anal. calcd for $C_8H_{16}NO_4Cl$: C, 42.58; H, 7.15; N, 6.21. Found: C, 38.97; H, 6.75; N, 5.71.

Compound 3b: 89% yield; ^{1}H NMR (D_2O) δ 4.19−4.17 (*m*, 2H, $H_2C(1^2)$), 4.05 (*s*, 2H, $H_2C(5)$), 3.67−3.64 (*m*, 2H, $H_2C(1^3)$),

3.53 (*q*, $^3J(1,^4\ 1^5) = 7.1$, 2H, $H_2C(1^4)$), 2.87−2.84 (*m*, AA′ of a AA′BB′-system, 2H, $H_2C(3)$), 2.69−2.65 (*m*, BB′ of a AA′BB′-system, 2H, $H_2C(2)$), 1.11 (*t*, $^3J(1,^4\ 1^5) = 7.1$, 3H, $H_3C(1^5)$); ^{13}C NMR (D_2O) δ 206.5 (C(4)), 177.3 (C(1)), 70.3 ($C(1^3)$), 69.3 ($C(1^4)$), 66.8 ($C(1^2)$), 49.6 (C(5)), 36.8 (C(3)), 30.0 (C(2)), 16.6 ($C(1^5)$); MS (EI) *m*/*z* relative intensity 204 (100, $[M - Cl]^+$), 189 (77), 146 (30), 114 (65); HRMS (ESI) calcd for $C_9H_{17}NO_4+$: 204.2448, obs. 204.2444. Anal. calcd for $C_9H_{18}NO_4Cl$: C, 45.10; H, 7.57; N, 5.84. Found: C, 44.45; H, 7.29; N, 5.79.

Compound 3c: 87% yield; mp 25.0−30.0 °C; ^1H NMR (400 MHz, D_2O) δ 4.20−4.18 (*m*, 2H, $H_2C(1^2)$), 4.05 (*s*, 2H, $H_2C(5)$), 3.70−3.68 (*m*, 2H, $H_2C(1^3)$), 3.63−3.61, 3.59−3.56 (*m*, 2H, *m*, 2H, $H_2C(1^4)$, $H_2C(1^5)$), 3.51 (*q*, $^3J(1,^6\ 1^7) = 7.1$, 2H, $H_2C(1^6)$), 2.87−2.83 (*m*, AA′ of AA′BB′-system, 2H, $H_2C(3)$), 2.68−2.65 (*m*, BB′ of AA′BB′-system, 2H, $H_2C(2)$), 1.11 (*t*, $^3J(1,^6\ 1^7) = 7.1$, 3H, $H_3C(1^7)$); ^{13}C NMR (100 MHz, D_2O) δ 206.6 (C(4)), 177.3 (C(1)), 72.2, 71.5 ($C(1^4)$, $C(1^5)$), 71.0 ($C(1^3)$), 69.2 ($C(1^6)$), 66.7 ($C(1^2)$), 49.7 (C(5)), 36.9 (C(3)), 30.0 (C(2)), 16.7 ($C(1^7)$); MS (EI) *m*/*z* relative intensity 248 (46, $[M - Cl]^+$), 117 (25), 99 (68), 86 (45), 72 (100); HRMS (ESI) calcd for $C_{11}H_{22}NO_5+$: 248.1491, obs. 248.1495. Anal. calcd for $C_{11}H_{22}NO_5Cl$: C, 46.56; H, 7.81; N, 4.94. Found: C, 44.93; H, 7.18; N, 4.93.

Compound 3d: 90% yield; ^1H NMR (400 MHz, D_2O) δ 4.31−4.28 (*m*, 2H, $H_2C(1^2)$), 4.15 (*s*, 2H, $H_2C(5)$), 3.79−3.77 (*m*, 2H, $H_2C(1^3)$), 3.75−3.72, 3.73, 3.70−3.67 (*m*, 2H, *′s′*, 4H, m, 2H, $H_2C(1^7)$, $H_2C(1^6)$, $H_2C(1^5)$, $H_2C(1^4)$), 3.64 (*q*, $^3J(1,^8\ 1^9) = 7.0$, 2H, $H_2C(1^8)$), 2.95−2.92 (*m*, AA′ of a AA′BB′-system, 2H, $H_2C(3)$), 2.82−2.79 (*m*, BB′ of a AA′BB′-system, 2H, $H_2C(2)$), 1.28 (*t*, $^3J(1,^9\ 1^8) = 7.1$, 3H, $H_3C(1^9)$); ^{13}C NMR (100 MHz, D_2O) δ 203.5 (C(2)), 174.0 (C(1)), 71.8, 71.7, 71.1 ($C(1^4)$-$C(1^7)$), 70.3 ($C(1^3)$), 67.8 ($C(1^8)$), 65.2 ($C(1^2)$), 48.6 (C(5)), 35.6 (C(3)), 29.0 (C(2)), 15.7 ($C(1^9)$); MS (ESI^+) *m*/*z* relative intensity 315 (25, $[M - HCl + Na]^+$), 293 (100, $[M - Cl]^+$), 201 (27), 196 (56), 179 (99), 149 (37); HRMS (ESI) calcd for $C_{13}H_{26}NO_6^+$: 292.1755, obs. 292.1751.

General Procedures for the Preparation of the Peptide Analogues 7a−e via Coupling of 5-Aminolevulinic Acid Esters with BOC-Protected Amino Acids 4a−d Followed by Acid Induced Deprotection of the BOC-Group. Coupling. A total of 1 mmol of BOC-amino acids (**4a**−**d**) (Novabiochem) dissolved in 8 mL of DMF was activated during 45 min by addition of 0.82 g (6.1 mmol) of *N*-hydroxybenzotriazole (HOBt, Novabiochem) and 1.16 g (6.1 mmol) of *N*-(3-dimethylaminoprpyl)-*N*′-ethylcarbodiimide HCl (EDC, Fluka). A solution of 5.5 mmol of ALA-methyl (**5a**) or ALA-hexyl (**5b**) esters in 6 mL of DMF was added for 10 min, then 0.77 mL (5.5 mmol) of triethylamine (NEt_3, Fluka) was added. After 12 h stirring at room temperature, the DMF was distilled in a Kugelrohr oven (50 °C/0.5 mbar) and the crude material was extracted with 2 × 100 mL of ethyl acetate and washed sequentially with 75 mL of 1 M citric acid, $NaHCO_3$ 10%, and NaCl saturated water. The solution was applied to a silica chromatography column (50 g silica gel (Fluka), eluent: ethyl acetate:hexane, 1:3 to 5:1). The pure products were obtained as solids following evaporation of the solvent.

Compound 6a: 72% yield; ^1H NMR (400 MHz, $CDCl_3$) δ 7.07 (*sbr*, 1H, N*H*-$H_2C(5)$), 5.30 (*sbr*, 1H, N*H*-HC(7)), 4.19−4.16 (*m*, 1H, HC(7)), 4.11 (*d*, $^3J(5, NH) = 5.0$, 2H, $H_2C(5)$), 3.60 (*s*, 3H, $H_3C(1^2)$), 2.71−2.67 (*m*, AA′ of a AA′BB′-system, 2H, $H_2C(3)$), 2.59−2.55 (*m*, BB′ of a AA′BB′-system, 2H, $H_2C(2)$), 1.37 (*s*, 9H, $H_3C(10^a)$, $H_3C(10^b)$, $H_3C(10^c)$), 1.30 (*d*, $^3J(7, 7^2) = 7.1$, 3H, $H_3C(7^2)$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 203.8 (C(4)), 173.0, 172.7 (C(1), C(6)), 155.3 (C(8)), 80 (C(9)), 51.7 ($C(1^2)$), 49.9 (C(7)), 48.9 (C(5)), 34.3 (C(3)), 28.1 ($C(10^a)$, $C(10^b)$, $C(10^c)$), 27.4 (C(2)), 18.4 ($C(7^2)$); MS (EI) $^+$) *m*/*z* relative intensity 317 (29, $[M + H]^+$), 261 (53), 217 (16), 145 (31), 144 (100), 140 (26), 115 (68), 88 (80), 87 (20), 70 (20); HRMS calcd for $C_{14}H_{24}N_2O_6$-Na^+ 339.1526, found 339.1525. Anal. ($C_{14}H_{24}N_2O_6$) C, H, N.

Compound 6b: (65% yield); ^1H NMR (400 MHz, CD_3OD) δ 4.22−4.13 (*m*, 3H, $H_2C(5)$, HC(7)), 4.15 (*t*, $^3J(1^2,\ 1^3) = 6.7$, 2H, $H_2C(1^2)$), 2.87−2.84 (*m*, AA′ of a AA′BB′, 2H, $H_2C(3)$), 2.69−2.66 (*m*, BB′ of a AA′BB′, 2H, $H_2C(2)$), 1.71 (*quint.*, $^3J(1^3,\ 1^4) = {}^3J(1^3,\ 1^2) = 6.7$, 2H, $H_2C(1^3)$), 1.53 (*s*, 9H, $H_3C(10^a)$, $H_3C(10^b)$, $H_3C(10^c)$), 1.47−1.37 (*m*, 6H, $H_2C(1^4)$, $H_2C(1^5)$, $H_2C(1^6)$), 1.42 (*d*, $^3J(7^2,\ 7) = 6.9$, 3H, $H_3C(7^2)$), 1.32 (*t*, $^3J(1,^7\ 1^6) = 6.9$, 3H, $H_3C(1^7)$); ^{13}C NMR (100 MHz, CD_3OD) δ 206.4 (C(4)), 176.5 (C(1)), 174.7 (C(6)), 157.9 (C(8)), 80.9 (C(9)), 66.1 ($C(1^2)$), 51.9 (C(7)), 50.0 (C(5)), 35.5 (C(3)), 32.9 ($C(1^5)$), 29.9 ($C(1^3)$), 29.0 (C(2), $C(10^a)$, $C(10^b)$, $C(10^c)$), 26.9 ($C(1^4)$), 23.9 ($C(1^6)$), 18.7 ($C(7^2)$), 14.8 ($C(1^7)$); MS (DCI) $^+$) *m*/*z* relative intensity 387 (18, $[M]^{+\bullet}$), 331 (40), 313 (25), 288 (40), 287 (51), 270 (55), 269 (100), 201 (13), 144 (25) 114 (12), 100 (29), 99 (20); HRMS (ESI) calcd for $C_{19}H_{34}N_2O_6Na^+$ 409.2309, found 409.2308.

Compound 6c: 75% yield; ^1H NMR (400 MHz, $CDCl_3$) δ 7.30−7.17 (*m*, 5H, HC(7^4), HC($7^{4'}$), HC(7^5), HC($7^{5'}$), HC(7^6)), 6.65 (*sbr*, 1H, N*H*-$H_2C(5)$), 5.00 (*sbr*, 1H, N*H*-HC(7)), 4.41 (*sbr*, 1H, HC(7)), 4.20−4.04 (*m*, 2H, $H_2C(5)$), 3.66 (*s*, 3H, $H_3C(1^2)$), 3.13−2.99 (*m*, 2H, $H_2C(7^2)$), 2.70−2.66 (*m*, AA′ of an AA′BB′ system, 2H, $H_2C(3)$), 2.64−2.59 (*m*, BB′ of an AA′BB′ system, 2H, $H_2C(2)$), 1.38 (*s*, 9H, $H_3C(10^a)$, $H_3C(10^b)$, $H_3C(10^c)$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 203.2 (C(4)), 172.7 (C(1)), 171.4 (C(6)), 155.3 (C(8)), 136.5 ($C(7^3)$), 129.2, 128.6 ($C(7,^4\ 7^{4'}$, $7,^5\ 7^{5'})$), ($C(7,^4\ 7^{4'})$), 126.9 ($C(7^6)$), 80.2 (C(9)), 55.6 (C(7)), 51.9 ($C(1^2)$), 49.0 (C(5)), 38.4 ($C(7^2)$), 34.4 (C(3)), 28.2 ($C(10^a)$, $C(10^b)$, $C(10^c)$), 27.5 (C(2)); MS (APCI) $^+$) *m*/*z* relative intensity 394 (23, $[M + H]^+$), 393 (98, $[M]^{+\bullet}$), 337 (14), 293 (13), 275, 261, 247, 243, 215; HRMS calcd for $C_{20}H_{28}N_2O_6Na^+$ 415.1840, found 415.1838. Anal. ($C_{20}H_{28}N_2O_6$) C, H, N.

Compound 6d: 71% yield; ^1H NMR (400 MHz, CD_3OD) δ 4.23−4.09 (*m*, 3H, $H_2C(5)$, HC(7)), 3.74 (*s*, 3H, $H_3C(1^2)$), 3.15−3.11 (*m*, 2H, $H_2C(7^5)$), 2.88−2.84 (*m*, AA′ of a AA′BB′-system, 2H, $H_2C(3)$), 2.70−2.66 (*m*, BB′ of a AA′BB′-system, 2H, $H_2C(2)$), 1.92−1.84 (*m*, 1H, HC(7^{2a})), 1.76−1.67 (*m*, 1H, HC(7^{2b})), 1.54 (*s*, 18H, $H_3C(7^{8a})$, $H_3C(7^{8b})$, $H_3C(7^{8c})$, $H_3C(10^a)$, $H_3C(10^b)$, $H_3C(10^c)$), 1.64−1.40 (*m*, 4H, $H_2C(7^3)$, $H_2C(7^4)$); ^{13}C NMR (100 MHz, CD_3OD) δ 206.4 (C(4)), 175.9 (C(1)), 175.1 (C(6)), 158.8, 158.2 ($C(7^6)$, C(8)), 80.9, 80.1 ($C(7^7)$, C(9)), 56.3 (C(7)), 52.5 ($C(1^2)$), 49.9 (C(5)), 41.3 ($C(7^5)$), 35.5 (C(3)), 33.2 ($C(7^2)$), 30.9 (*s*, $C(7^4)$), 29.1 ($C(7^{8a})$, $C(7^{8b})$, $C(7^{8c})$ or $C(10^a)$, $C(10^b)$, $C(10^c)$), 29.0 ($C(10^a)$, $C(10^b)$, $C(10^c)$ or $C(7^{8a})$, $C(7^{8b})$, $C(7^{8c})$), 28.7 (C(2)), 24.4 ($C(7^3)$); MS (ESI^+) *m*/*z* relative intensity 475 (23, $[M + H]^+$), 474 (100, $[M]^{+\bullet}$), 418 (8), 374 (11), 318 (3), 300; HRMS (ESI) calcd for $C_{22}H_{39}N_3O_8Na^+$ 496.2629, found 496.2631. Anal. calcd for $C_{22}H_{39}N_2O_8$: C, 55.80; H, 8.30; N, 8.87. Found: C, 55.27; H, 8.40; N, 8.56.

Compound 6e: 73% yield; ^1H NMR (400 MHz, $CDCl_3$) δ 7.16 (*t*, $^3J(5, NH) = 4.8$, 1H, N*H*-$H_2C(5)$), 5.68 (*d*, $^3J(7, NH) = 8.2$, 1H, N*H*-HC(7)), 4.46−4.43 (*m*, 1H, HC(7)), 4.09 (*dxt*, $^3J(5, NH) = 4.6$, $^4J(3, 5) = 2.2$, 2H, $H_2C(5)$), 3.59 (*s*, 3H, $H_3C(1^2)$), 2.79−2.73 (*m*, 1H, HC(7^{2a})), 2.69−2.65 (*m*, AA′ of a AA′BB′-system, 2H, $H_2C(3)$), 2.61−2.54 (*m*, 3H, $H_2C(2)$, HC(7^{2b})), 1.38 (*s*, 9H, $H_3C(7^{5a})$, $H_3C(7^{5b})$, $H_3C(7^{5c})$), 1.36 (*s*, 9H, $H_3C(10^a)$, $H_3C(10^b)$, $H_3C(10^c)$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 203.2 (C(4)), 172.6 (C(1)), 171.0 (C(6)), 170.7 ($C(7^3)$), 155.3 (C(8)), 81.4 ($C(7^4)$), 80.1 (C(9)), 51.7 ($C(1^2)$), 50.6 (C(7)), 49.0 (C(5)), 37.2 ($C(7^2)$), 34.2 (C(3)), 28.1, 27.8 ($C(7^{5a})$, $C(7^{5b})$, $C(7^{5c})$), $C(10^a)$, $C(10^b)$, $C(10^c)$), 27.4 (C(2)); MS (ESI) 439(21, $[M + Na]^+$), 418 (20, $[M + H]^+$), 417 (100, $[M]^{+\bullet}$), 416 (11), 361 (16); HRMS (ESI) calcd for $C_{19}H_{32}N_2O_8Na^+$ 439.2053, found 439.2051.

Procedure for the Deprotection of the N-Terminal Amino Acid Function. A total of 0.3 mmol of protected amino acid-ALA derivatives was dissolved in 3 mL (39 mmol) of TFA under argon atmosphere. After the solution was stirred for 1 h at room temperature, TFA was evaporated under high vacuum. The products were obtained in good purity and characterized without further purification.

Compound 7a: 98% yield; ^1H NMR (400 MHz, DMSO-d_6) δ 8.81 (*t*, $^3J(5, NH) = 5.6$, 1H, N*H*-$H_2C(5)$), 8.26 (*sbr*, 3H, H_3N^+), 4.24 (*dxd*, $^3J(5, NH) = 5.7$, $^2J(5^a,\ 5^b) = 12.7$, 1H, HC(5^a)), 4.16 (*dxd*, $^3J(5, NH) = 5.5$, $^2J(5^a,\ 5^b) = 12.9$, 1H, HC(5^b)), 4.07−4.00 (*m*, 1H, HC(7)), 3.68 (*s*, 3H, $H_3C(1^2)$), 2.86−2.82 (*m*, AA′ of a AA′BB′-system, 2H, $H_2C(3)$), 2.62 (*m*, BB′ of a AA′BB′-system, 2H, $H_2C(2)$), 1.49 (*d*, $^3J(7, 7^2) = 7.0$, 3H, $H_3C(7^2)$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 204.9 (C(4)), 172.9 (C(1)), 170.2 (C(6)), 51.6 ($C(1^2)$), 48.5 (C(7)), 48.4 (C(5)), 34.2 (C(3)), 27.4 (C(2)), 17.5 ($C(7^2)$); MS (ESI) *m*/*z* relative intensity 239 (4, $[M + Na]^+$), 217 (100, $[M + H]^+$), 199 (38), 171, 167, 146 (18); HRMS (ESI) calcd for $[C_9H_{17}N_2O_4]^+$ 217.1183, found 217.1191.

Compound 7b: 99% yield; ^{1}H NMR (400 MHz, D_2O) δ 3.66−3.65 (*AB-system*, $J^2\approx$18.4, 2H, $H_2C(5)$), 3.63 (*q*, $^3J(7, 7^2) = 7.3$, 1H, HC(7)), 3.47 (*t*, $^3J(1^2, 1^3) = 6.7$, 2H, $H_2C(1^2)$), 2.25−2.22 (*m*, AA′ of a AA′BB′, 2H, $H_2C(3)$), 2.04−2.01 (*m*, BB′ of a AA′BB′, 2H, $H_2C(2)$), 1.05−0.96 (*m*, 2H, $H_2C(1^3)$), 1.01 (*d*, $^3J(7^2, 7) = 7.1$, 3H, $H_3C(7^2)$), 0.80−0.73 (*m*, 6H, $H_2C(1^4)$, $H_2C(1^5)$, $H_2C(1^6)$), 0.32 (*t*, $^3J(1,^7 1^6) = 6.8$, 3H, $H_3C(1^7)$); ^{13}C NMR (100 MHz, D_2O) δ 205.5(C(4)), 173.8 (C(1)), 170.4 (C(6)), 64.8 (C(1^2)), 48.6 (C(7)), 48.1 (C(5)), 33.5 (C(3)), 30.7 (C(1^5)), 27.6 (C(1^3)), 26.8 (C(2)), 24.7 (C(1^4)), 21.7 (C(1^6)), 16.0 (C(7^2)), 12.7 (C(1^7)); MS (ESI$^+$) *m/z* relative intensity 309 (12, [M − HTFA + Na]$^+$), 287 (100, [M-TFA]$^+$), 269 (6), 216 (6).

Compound 7c: 99% yield; ^{1}H NMR (400 MHz, D_2O) δ 7.19−7.07 (*m*, 5H, HC(7^4), HC($7^{4'}$), HC(7^5), HC($7^{5'}$), HC(7^6)), 4.10 (*t* $^3J(7, 7^2) = 7.3$, 1H, HC(7)), 3.91−3.90 (*AB-system*, $J^2\approx$18.5, 2H, $H_2C(5)$), 3.44 (*s*, 3H, $H_3C(1^2)$), 3.02 (*dd*, ^2J (7^2_a, 7^2_b) = 14, ^3J (7^2_a, 7) = 7.1, 1H, HC(7^2_a)), 2.96 (*dd*, ^2J (7^2_b, 7^2_a) = 14, ^3J (7^2_b, 7) = 7.1, 1H, HC(7^2_b)), 2.53−2.48 (*m*, AA′ of a AA′BB′, 2H, $H_2C(3)$), 2.39−2.36 (*m*, BB′ of a AA′BB′, 2H, $H_2C(2)$); ^{13}C NMR (100 MHz, D_2O) δ 207.5 (C(4)), 175.7 (C(1)), 169.4 (C(6)), 134.0 (C(7^3)), 129.7, 129.4 ((C($7,^4 7^{4'}, 7,^5 7^{5'}$)), 128.2 (C(7^6)), 54.6 (C(7)), 52.7 (C(1^2)), 48.8 (C(5)), 37.1 (C(7^2)), 34.3 (C(3)), 27.6 (C(2)); MS (ESI$^+$) *m/z* relative intensity 315 (43, [M − TFA]$^+$), 293 (100, [M − HTFA + Na]$^+$), 275 (15), 120 (8).

Compound 7d: 97% yield; ^{1}H NMR (400 MHz, D_2O) δ 4.01 (*d*, $^2J(5_a, 5_b) = 18.8$, 1H, HC(5_a)), 3.91 (*d*, $^2J(5_b, 5_a) = 18.8$, 1H, HC(5_b)), 3.79 (*t*, $^3J(7, 7^2) = 6.6$ 1H, HC(7)), 3.38 (*s*, 3H, $H_3C(1^2)$), 2.72 (*t*, $^3J(7,^5 7^4) = 7.6$, 2H, $H_2C(7^5)$), 2.58−2.55 (*m*, AA′ of a AA′BB′, 2H, $H_2C(3)$), 2.37−2.34 (*m*, BB′ of a AA′BB′, 2H, $H_2C(2)$), 1.68−1.62 (*m*, 2H, $H_2C(7^2)$), 1.44 (*quin.*, $^3J(7,^4 7^5) = (7,^4 7^3) = 6.6$ 2H, $H_2C(7^4)$), 1.26−1.17 (*m*, 2H, $H_2C(7^3)$; ^{13}C NMR (100 MHz, D_2O) δ 209.6 (C(4)), 177.9 (C(1)), 172.3 (C(6)), 55.4 (C(7)), 54.7 (C(1^2)), 51.0 (C(5)), 41.4 (C(7^5)), 36.6 (C(3)), 32.7 (C(7^2)), 29.8 (C(2)), 28.7 (C(7^4)), 23.5 (C(7^3)); MS (ESI$^+$) *m/z* relative intensity 296 (5, [M-2HTFA+Na]$^+$), 274 (100, [M − 2TFA − H]$^+$), 256, (34), 239 (9), 211 (3).

Compound 7e: 99% yield; ^{1}H NMR (400 MHz, D_2O) δ 4.22 (*dd*, $^3J(7, 7^{2a}) = 5.1$, $^3J(7, 7^{2b}) = 7.5$, 1H, HC(7)), 4.03−3.99 (*AB-system*, $J^2\approx$18.3, 2H, $H_2C(5)$), 3.44 (*s*, 3H, $H_3C(1^2)$), 2.89 (*dd*, $^2J(7^{2a}, 7^{2b}) = 18.1$, $^3J(7^{2a}, 7) = 5.1$, 1H, HC(7^2_a)), 2.83 (*dd*, $^2J(7^{2b}, 7^{2a}) = 18.1$, $^3J(7^{2b}, 7) = 5.1$, 1H, HC(7^{2b})), 2.65−2.62 (*m*, AA′ of a AA′BB′, 2H, $H_2C(3)$), 2.44−2.41 (*m*, BB′ of a AA′BB′, 2H, $H_2C(2)$); ^{13}C NMR (100 MHz, D_2O) d 207.6 (C(4)), 175.8 (C(1)), 172.7(C(6)), 169.1 (C(7^3)), 52.6 (C(1^2)), 49.7 (C(7)), 49.0 (C(5)), 35.0 (C(7^2)), 34.4 (C(3)), 27.6 (C(2)); MS (ESI$^+$) *m/z* relative intensity 283 (39, [M − HTFA + Na]$^+$), 261 (100, [M − TFA]$^+$), 243 (65), 225 (9), 183 (13), 146 (5).

Procedure for the Enzymatic Deprotection of the Methyl Ester to Free Acid 8a. A total of 1.58 mmol of Ala-ALA **6a** was dissolved in 50 mL of phosphate buffer pH 8.0, and 0.2 mL of pig liver esterrase (PLE) solution (Sigma, 250 units/mg, 15 mg/mg derivative) was added. The pH was maintained at 8.0 with 0.2 N NaOH for 48 h, then the pH was lowered to 1.5. The solution was extracted with 3 × 100 mL of AcOEt, dried under $MgSO_4$, and purified by column chromatography (55 g silica gel, eluent: dichloromethane/ methanol 5%).

Compound 8a: 88% yield; R_f (AcOEt/hexane/CH_3COOH: 100:20:1) 0.67; ^{1}H NMR (400 MHz, CD_3OD) δ 4.26−4.13 (*m*, 3H, $H_2C(5)$, HC(7)), 2.80−2.75 (*m*, AA′ of a AA′BB′, 2H, $H_2C(3)$), 2.64−2.60 (*m*, BB′ of a AA′BB′, 2H, $H_2C(2)$), 1.53 (*s*, 9H, $H_3C(10^a)$, $H_3C(10^b)$, $H_3C(10^c)$), 1.43 (*d*, $^3J(7, 7^2) = 7.2$, 3H, $H_3C(7^2)$); ^{13}C NMR (100 MHz, CD_3OD) δ 179.7 (C(1)), 176.6 (C(6)), 157.9 (C(8)), 81.0 (C(9)), 51.9 (C(7)), 50.2 (C(5)), 36.2 (C(3)), 31.0 (C(2)), 29.0 (C(10^a), C(10^b), C(10^c)), 18.8 (C(7^2)); MS (APCI) *m/z* relative intensity 304 (17, [M + H]$^+$), 303 (81, [M]$^{+\bullet}$), 247 (100), 229 (12), 203 (10). Anal. ($C_{13}H_{22}N_2O_6$) C, H, N.

Compound 9a. Using the standard deprotection procedure with TFA starting from **8a** (181 mg, 0.6 mmol) the fully deprotected **9a** (151 mg, 82%) was isolated: R_f 0.71; ^{1}H NMR (400 MHz, D_2O) δ 3.02 (*s*, 2H, HC(5)), 3.94 (*q*, $^3J(7, 7^2) = 7.1$, 1H, HC(7)), 2.64 (*t*, $^3J(3, 2) = 6.3$, 2H, HC(3)), 2.44 (*t*, $^3J(2, 3) = 6.3$, 2H, HC(2)), 1.34 (*d*, $^3J(7^2, 7) = 6.3$, 1H, HC(7^2)); ^{13}C NMR (100 MHz, D_2O) δ 207.9 (C(4)), 177.2 (C(1)), 172.8 (C(6)), 49.3 (C(7)), 48.9 (C(5)), 34.4 (C(3)), 27.7 (C(2)), 16.7 (C(7^2)); MS (ESI$^+$) *m/z* relative intensity 225 (53, [M + Na]$^+$), 203 (100, [M + H]$^+$), 185 (63), 145 (17), 132 (22); HRMS (ESI) calcd for $[C_8H_{15}N_2O_4]^+$ 203.1026, found 203.1031.

Cell Lines and Culture Conditions. Human lung carcinoma A549 cell line was from ATCC, human microvascular endothelial cell line HCEC[26] was a kind gift of D.Staminirovic and A. Muruganandam, Ottawa, and the rat microvascular endothelial cell line EC219 has been described.[27] Cells were grown in DMEM medium (Gibco) containing either 4.5 g/L (A459 and HCEC cells) or 1.1 g/L (EC219 cells) glucose, 10% FCS, and penicillin/streptomicin, at 37 °C and 6% CO_2. Cells were subcultured in 48-well dishes (Costar) 48 h prior to experiments in complete culture medium. Aminopeptidase activities were determined in cell extracts essentially as previously described.[27,28] Experiments were repeated at least twice and were performed in sextuple wells. Means of results were used.

PpIX Fluorescence Measurements. For fluorescence measurements, confluent cell layers in 48-well plates were exposed to the indicated concentrations (6 wells per concentration) of the different PPIX precursors in 0.25 mL/well DMEM medium, free of phenol red (Gibco) and of FCS. Increase in fluorescence was measured every 15−60 min in a thermostated fluorescence multiwell plate reader at 37 °C (CytoFluor Series 4000, PerSeptive Biosystems, MA) using excitation filter at $\lambda = 409 \pm 20$ nm and detection filter at $\lambda = 640 \pm 40$ nm.

Determination of Cell Viability. Cell viability was determined after 20 h exposure to ALA derivatives using the evaluation of mitochondrial functions by the MTT assay. Briefly, MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromid, Merck), final concentration 250 mg/mL, was added to each well and incubation was continued at 37 °C for 2 h. The supernatant was removed, the cell layer was dissolved in 0.04 N HCl/2-propanol, and absorbance at 540 nm was quantitated using a 96-well ELISA plate reader (iEMS Reader MF, Labsystems, Bioconcepts, Switzerland).

Evaluation of Product Efficacy (Efficacy Index). The ratio of fluorescence measured at 300 min after initial exposure/ cell viability, as defined by absorbance at 540 nm (MTT reduction) following 20 h exposure to the compound, was used to determine the molecule producing the maximal PpIX fluorescence with the minimal cytotoxicity. Standard deviations (SEM) were calculated.

In Situ Enzymatic Activities (Histoenzymography). EC219 cells grown in 6-well plates and washed from culture medium without further fixation or 5 μm sections of frozen brain tumor samples were exposed at 37 °C to Glu-β-methoxynaphthylamide substrate (Bachem, Switzerland) and Fast Blue B (Sigma, Switzerland) in the same conditions as previously described.[16] They were counterstained with light hematoxylin reagent. Enzymatic activity was visualized as a red precipitate.

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Supporting Information Available: Additional chemical and spectral characteristics of the synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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