Improved Peptide Prodrugs of 5-ALA for PDT: Rationalization of Cellular Accumulation and Protoporphyrin IX Production by Direct Determination of Cellular Prodrug Uptake and Prodrug Metabolization

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Twenty-seven dipeptide derivatives of general structure Ac-Xaa-ALA-OR were synthesized as potential prodrugs for 5-aminolaevulinic acid-based photodynamic therapy (ALA-PDT). Xaa is an α-amino acid, chosen to provide a prodrug with appropriately tailored lipophilicity and water solubility. Although no simple correlation is observed between downstream production of protoporphyrin IX (PpIX) in PAM212 keratinocytes and HPLC-derived descriptors of compound lipophilicity, quantification of prodrug uptake reveals that most of the dipeptides are actually more efficiently accumulated than ALA in PAM212 and also A549 and Caco-2 cell lines. Subsequent ALA release is the limiting factor, which emphasizes the importance of decoupling prodrug uptake and intracellular metabolization when assessing the efficacy of ALA derivatives for PDT. In agreement with PpIX fluorescence studies, at a concentration of 0.1 mM, L-Phe derivatives **4m** and **4o**, and L-Leu, L-Met, and L-Glu derivatives **4f**, **4k**, and **4u**, exhibit significantly enhanced photoxicity in PAM212 cells compared to ALA.

Introduction

5-Aminolaevulinic acid-based photodynamic therapy (ALA-PDT[a]) is gaining increasing acceptance in medicine as an effective technique for the treatment of a variety of neoplastic lesions and premalignant disorders.[1] In mammalian cells, ALA is metabolized to protoporphyrin IX (PpIX), the precursor of heme and a potent photosensitizer (Figure 1).[2−4] Under physiological conditions, high intracellular concentrations of heme cause the feedback inhibition of ALA biosynthesis in mitochondria, but this may be bypassed by external administration of ALA, and together with the relatively slow downstream transformation of PpIX into heme by ferrochelatase, this results in the accumulation of the natural photosensitizer. Clinically, when sufficient intracellular levels of PpIX are attained, the targeted tissue is irradiated with visible light to activate the sensitizer and trigger a chain of events that ultimately result in cell death. At a molecular level, this involves the interaction of the excited photosensitizer with molecular oxygen, leading to the generation of electrophilic species (singlet oxygen and/or radicals) that cause oxidative damage to cellular constituents such as phospholipidic membranes, nucleic acids, and proteins.[5]

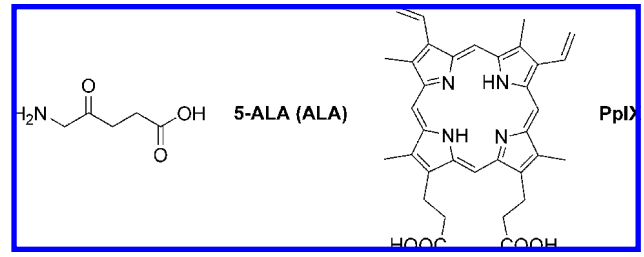

Figure 1. 5-Aminolaevulinic acid (ALA) and protoporphyrin IX (PpIX).

ALA-PDT currently finds wide use in dermatology for the treatment of actinic keratosis, squamous cell carcinoma, and Bowen's disease,[6,7] as well as cutaneous microbial infections such as acne, onychomycosis, and verrucae.[8−10] Promising results have also emerged from studies of photorejuvenation of the skin after sun-induced damage.[6] In gastroenterology and urology, PpIX from administered ALA may be used not only to directly treat conditions such as Barret's esophagus, inflammatory bowel disease, and bladder cancer by PDT[11,12] but also as a diagnostic tool for the visualization of precancerous changes in the mucosae by fluorescence spectroscopy.[13,14] Generally, PpIX generated by exogenous ALA administration presents several advantages over other types of photosensitizers that have been applied in PDT, such as synthetic porphyrin, chlorin, or phthalocyanine derivatives. For example, the risk of overtreatment with ALA-PDT is limited due to the fast clearance of ALA from the body, while saturation of PpIX production at high ALA doses also prevents excessive PpIX production in tissues.[15] The relatively rapid photobleaching of PpIX also means that the phototoxic effects of ALA-PDT are nonpersistent.[16]

The main drawbacks associated with ALA-PDT and ALA fluorescence diagnosis (ALA-FD) result from the hydrophilic nature of ALA itself. At physiological pH, ALA is a zwitterion, which severely impairs its ability to cross cell membranes and results in poor penetration and nonhomogeneous distribution

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[a] Abbreviations: ALA, 5-aminolaevulinic acid; PDT, photodynamic therapy; PpIX, protoporphyrin IX; FD, fluorescence diagnosis; Su, succinimido; DIPEA, diisopropylethylamine; TIPS, triisopropylsilane; PyBrOP, bromo-tris-pyrrolidino phosphoniumhexafluorophosphate; DMAP, 4-dimethylaminopyridine; PBS, phosphate-buffered saline; PAM212, murine keratinocyte cell line; A549, human Caucasian lung carcinoma cell line; Caco-2, human colonic adenocarcinoma cell line; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; R^2, linear correlation; LOD, limit of detection; LOQ, limit of quantification; SD, standard deviation, RSD, relative standard deviation.

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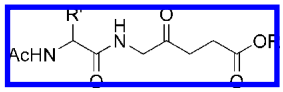


Figure 2. Generic structure of dipeptide ALA prodrugs.

in the target tissue.[17] To overcome this obstacle, forms of delivery involving the use of penetration enhancers have been devised,[18] and much effort has been put into the development of ALA prodrugs with more favorable lipid solubility[19,20] based upon initial observations that administration of ALA esters (methyl, ethyl, propyl) caused a quicker, more homogeneous, and more intense porphyrin fluorescence in mice skin than ALA.[21−24] Numerous prodrugs of this type have been synthesized with linear, branched, or cyclic alkyl ester groups, as well as examples with aromatic, heteroaromatic, perfluorinated hydrocarbon, or ethylene glycol-type moieties.[19] Other ester-based strategies for improving uptake include the incorporation of ALA into dendrimeric structures,[25−28] and the synthesis of glycoside esters of ALA, designed to potentially target tumoral cells that overexpress nutrient transporters.[29] Most recently, acyloxyalkyl esters have also been proposed as multifunctional ALA prodrugs that may induce cell death by both PDT-dependent and independent mechanisms.[30]

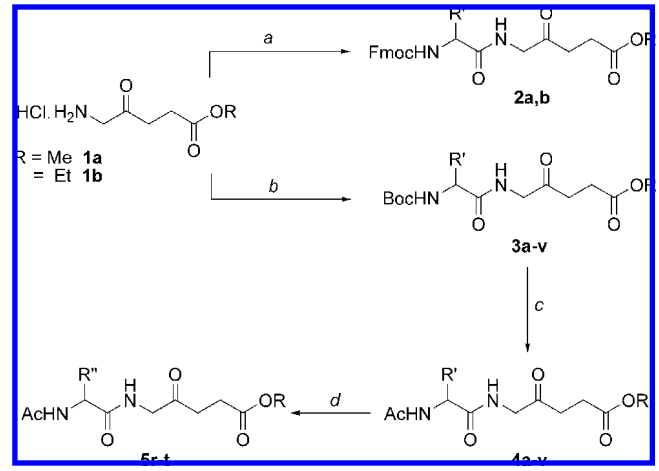
An attractive way to obtain ALA prodrugs that have both improved physicochemical properties and can selectively release ALA in specific cell lines is to incorporate ALA into a short peptide derivative.[31,32] Following this approach, upon cellular uptake, ALA release is mediated by the action of cytoplasmic esterases and/or proteases, and it may be possible to design ALA prodrugs that target disease-dependent levels of a given enzymatic activity. In this general context, we have identified prodrugs of the general structure shown in Figure 2 as potential candidate derivatives that provide enhanced intracellular ALA delivery. Such molecules are stable at physiological pH, unlike ALA, its esters, and dipeptide derivatives with a free amino terminus,[33−35] but they may be fine-tuned in terms of their overall lipophilicity to favor passive uptake while still retaining water solubility, by variation of R′ (the side chain of the amino acid coupled to ALA) and the ester moiety R.

Results and Discussion

Synthesis. The desired *N*-acetylated dipeptides were obtained according to the method previously described, with some important modifications, as outlined in Scheme 1.

We have previously reported that the coupling of *N*-urethane-protected amino acids to ALA can be successfully accomplished without the use of excess reagents provided that a low overall concentration of ALA relative to the acylating species is maintained throughout the course of the reaction.[36] Thus, when ALA, as the hydrochloride salt, is slowly neutralized in the presence of an equimolar quantity of the appropriate activated amino acid derivative, the formation of the required coupled product is favored over competing intermolecular Schiff base formation.[37,38] In this original procedure, the key acylation reaction was followed by conversion of the resulting dipeptide to the methyl ester by treatment with diazomethane.[39] While this provided efficient access to the versatile derivatives **3**, it was only compatible with relatively small scale syntheses. To simplify the procedure, methyl aminolaevulinate hydrochloride **1a** was used directly in the coupling reaction, and under carefully controlled conditions with a fixed concentration of reagents (0.07 M for **1a** and the amino acid succinimido ester derivative, 0.14 M for DIPEA; see Scheme 1), the desired *N*-urethane-protected dipeptides were obtained in excellent yields on gram scales

Scheme 1[a]



[a] Reagents and conditions: (a) Fmoc-Xaa-OSu, DIPEA, THF, 0 °C to rt; (b) Boc-Xaa-OSu, DIPEA, THF, 0 °C to rt; (c) HCl, 1,4-dioxane, then Ac_2O, DIPEA, CH_2Cl_2, 0 °C to rt (**4a**−**v**); (d) side-chain deprotection step (**5r**−**t**; see Table 1 for details).

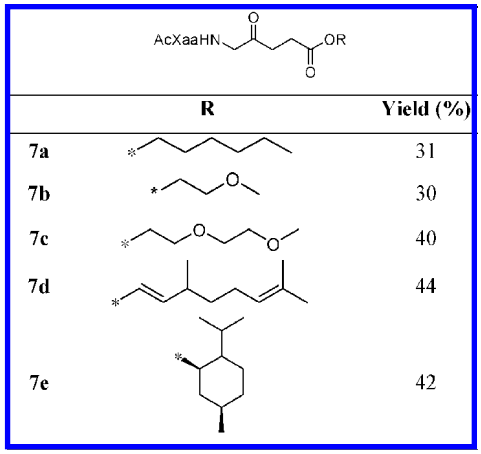
Table 1. Preparation of *N*-Urethane-Protected and *N*-Acetyl Dipeptide Derivatives

	PG−NH		AcHN	
Xaa		**Yield (%)**		**Yield (%)**
Gly	**3a**	72	**4a**	52
L-Ala	**3b**	80	**4b**	84
D-Ala	**3c**	93	**4c**	74
L-Val	**3d**	77	**4d**	85
D-Val	**3e**	53	**4e**	75
L-Leu	**3f**	90	**4f**	94
D-Leu	**3g**	90	**4g**	93
L-Ile	**3h**	92	**4h**	65
L-Pro	**3i**	94	**4i**	80
D-Pro	**3j**	80	**4j**	85
L-Met	**3k**	95	**4k**	70
L-Ser	**3l**	96	**4l**	72
L-Phe	**3m**	92	**4m**	92
D-Phe	**3n**	87	**4n**	86
L-Phe	**3o**[a]	84	**4o**	80
L-Trp	**3p**	97	**4p**	92
D-Trp	**3q**	95	**4q**	86
L-Tyr(Bn)	**3r**	98	**4r**[b]	85
L-His(Trt)	**3s**	82	**4s**[c]	70
L-Lys(Z)	**3t**	93	**4t**[d]	75
L-Glu(OBu^t)	**3u**	94	**4u**	51
L-Asp(OBu^t)	**3v**	98	**4v**	64
L-Ser(Bu^t)	**2a**	91		
L-Lys(Boc)	**2b**	93		

[a] Coupling reaction was performed with ethyl-5-aminolaevulinate hydrochloride (**1b**). [b] Conditions for final side chain deprotection: H_2, Pd/C, CH_3OH, rt, (**5r**, 89%). [c] Conditions for final side chain deprotection: TFA, CH_2Cl_2, TIS, rt, (**5s**, 85%). [d] Conditions for final side chain deprotection: H_2, Pd/C, CH_3OH, HCl, rt, (**5t**, 96%).

(Table 1). Use of an increased addition time of 7 h was found to give optimum results for coupling with various Boc- (entries **3a**−**u**) and Fmoc- (entries **2a** and **2b**) amino acids, in both L- and D-forms, with a range of side chain protection and with significant improvements in overall yield relative to our original procedure. The procedure could also be readily adapted to other simple esters of ALA (see entry **3o**). Compound **1a** and the ethyl ester, **1b**, were obtained on a multigram scale by a novel esterification procedure by treatment of ALA with trimethyl-orthoformate or triethylorthoformate in the corresponding

Table 2. Preparation of L-Phenylalanyl Ester Prodrugs



alcohol in the presence of a catalytic amount of an acidic ion-exchange resin at room temperature. This was found to give a substantial improvement in both yield and ease of product isolation compared to the widely used thionyl chloride esterification method.[40]

The desired *N*-acetylated dipeptides **4** and **5** were obtained from **3** following cleavage of *N*-terminal Boc protection with 4 M HCl in dioxane and subsequent acetylation with acetic anhydride (2 equiv) in the presence of DIPEA (2.2 equiv). As shown in Table 1, this two-step transformation generally proceeded with high efficiency (54−94%). Final side chain deprotection for His derivative **4s** was accomplished by acidolysis of the trityl group with TFA/CH_2Cl_2/TIPS. For Tyr(Bn) and Lys(Z) derivatives **5r** and **5t**, final deprotection was effected by hydrogenolysis (10% Pd−C). In the case of the Lys derivative, it proved essential to perform the hydrogenolysis reaction in the presence of HCl in order to trap the ε-amino group as the hydrochloride salt and prevent intra- or intermolecular Schiff base formation with ALA keto functions.[41] Interestingly, it was preferable to prepare Ser derivative **4l** without the use of side chain protection because Boc removal was found to result in partial cleavage of Ser(Bn), thus leading to a mixture of the desired *N*-acetylated dipeptide and *N*,*O*-diacetylated material. This problem was circumvented by coupling of Boc-L-Ser-OSu to **1a**, subsequent Boc group removal, and performing the acetylation reaction with just 1 equiv of acetic anhydride.

As reported previously,[36] ALA-containing dipeptides such as **4m** could be readily converted into a variety of other ester derivatives via the intermediate acid. Thus, saponification of **4m** with LiOH in aqueous CH_3OH, gave **6**, which then underwent PyBroP-mediated esterification[42] with hexan-1-ol, 2-methoxyethanol, 2-(2-methoxy)-ethoxyethanol, geraniol, and menthol to give the novel derivatives, **7a**−**e**, in satisfactory yields (Table 2).

Evaluation of Lipophilicity of the Dipeptides. We have previously speculated that increasing the lipophilicity of prodrugs of the general structure described should result in enhanced intracellular delivery of ALA and PpIX production, assuming predominant uptake by passive diffusion. To investigate whether a correlation could be established between the lipophilicity of the dipeptides **4a**−**v** and **5r**−**t** and the corresponding efficiency of PpIX production, it was therefore necessary to establish the relative lipophilicity of all the compounds under study. The classic descriptor for the lipophilicity of a drug is the octanol/water partition coefficient (log *P*), however experimental log *P* determinations by the "shake-flask" method may be both lengthy and laborious for a large compound series. Not only are relatively large amounts of analytically pure material required, but quantification of compounds that are sparingly soluble in one of the phases may not be feasible without a suitably sensitive analytical technique. To address these difficulties, several chromatographic descriptors for lipophilicity have been suggested based upon the principle that the behavior of a chemical entity in an RP-HPLC system is mainly the result of partition equilibrium between a lipophilic solid phase and a predominantly aqueous mobile phase. Such chromatographic hydrophobicity indices are amenable to rapid high-throughput determination, under either gradient or isocratic elution RP-HPLC conditions, with isocratic elution-based techniques showing good correlation to the traditional log *P* values. In this context, the *isocratic chromatographic hydrophobicity index*, $\varphi_{0,}$ developed by Valkó et al., is a very useful descriptor that characterizes compound hydrophobicity and has been shown to correlate well with log *P* for a wide range of drug molecules.[43−45] φ_o is derived from the relationship between the percentage of organic solvent in the mobile phase, φ, and the isocratic retention factor, log *k*, expressed by the eqs 1−3:

$$\log k = \log(t_R - t_0)/t_0 \quad (1)$$

(where t_R = retention time and t_0 = dead time).

$$\log k = S\varphi + \log k_w \quad (2)$$

$$\varphi_0 = -\log k_w/S \quad (3)$$

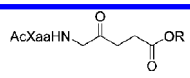
(where log k_w represents the retention factor on extrapolation to pure aqueous buffer). φ_0 thus corresponds to the value of φ needed to obtain log $k = 0$, i.e., a retention time that is double the dead time ($t_R = 2t_0$). To determine φ_0, the log *k* values for a given compound are thus measured using mobile phases with different solvent composition φ, and the former are plotted as a function of φ according to eq 2. φ_0 is then obtained from the slope and intercept of the straight line according to eq 3 (φ_0 = −slope/intercept). Values of φ_0 depend on pH and the type of organic solvent employed. Parallel determinations with more than one solvent are generally recommended to confirm the validity of any trend within a compound series.

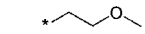
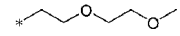
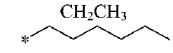
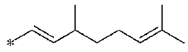
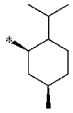
Applying this method, we calculated the φ_0 for the compounds of Table 1 under isocratic conditions with either CH_3CN or CH_3OH as the organic component and 50 mM aq ammonium formate (pH = 6.8). The correlation between log *k* and φ was found to be linear for both solvents, provided that values of φ were chosen such that $-1.5 < \log k < 0.6$. Under these conditions, the linear range was greater for determinations with CH_3OH.

The experimentally determined φ_0 values obtained with both solvents are reported in Table 3, alongside the calculated log *P* (clogP). The compounds are listed in order of increasing values of φ_0. Gratifyingly, for the prodrugs under study, an acceptable linear correlation between the experimental and calculated hydrophobicity descriptors was observed ($R = 0.966$ and $R = 0.865$ for CH_3CN and CH_3OH, respectively), further validating the use of φ_0 as an alternative to log *P*.

Enhancement of PpIX Fluorescence with ALA Peptide Prodrugs. The production of PpIX after incubation with either ALA or L-amino acid-containing prodrugs was evaluated in PAM212 keratinocytes as a function of the dose and incubation

Table 3. φ_0 (CH_3CN and CH_3OH) and clogP Values for L-Amino Acid-Containing ALA Dipeptides[a]



	Xaa	R	φ_0 (CH_3CN)	φ_0 (CH_3OH)	clogP
5t	Lys	CH_3	8.48	13.61	-1.27
4a	Gly	CH_3	10.31	18.77	-2.82
4b (4c)	Ala	CH_3	14.18 (17.30)	17.08 (17.30)	-2.33
4l	Ser	CH_3	14.61	36.43	-3.19
5s	His	CH_3	15.59	28.89	-3.11
4i (4j)	Pro	CH_3	18.69 (18.70)	27.47 (27.34)	-2.12
4v	Asp	CH_3	19.62	24.27	-3.11
4u	Glu	CH_3	23.14	35.01	-2.83
4d (4e)	Val	CH_3	25.64 (24.74)	41.60 (40.76)	-1.45
4k	Met	CH_3	28.89	41.40	-2.00
5r	Tyr	CH_3	34.14	40.73	-1.05
4m (4n)	Phe	CH_3	34.71 (37.71)	52.96 (52.62)	-0.66
4f (4g)	Leu	CH_3	36.67 (33.89)	50.92 (50.76)	-1.10
4h	Ile	CH_3	37.14	46.91	-1.03
4p (4q)	Trp	CH_3	37.35 (37.32)	22.08 (22.08)	-1.12
7b	Phe		38.26	79.03	0.56
7c	Phe		38.88	58.35	0.42
4o	Phe	CH_2CH_3	44.01	57.58	0.98
7a	Phe		56.64	97.31	3.09
7d	Phe		82.33	105.36	1.63
7e	Phe		53.22	110.53	4.45

[a] The values for the corresponding D-amino acids are shown in parentheses.

time. The results of these experiments are summarized in Figure 3. Panel (A) shows that at 0.1 mM drug concentration after 4 h of incubation, six dipeptides (**4b**, **4d**, **4f**, **4m**, **4k**, and **4u**) displayed an enhanced PpIX fluorescence compared to equimolar ALA, while dipeptides **5r** and **4a** showed comparable efficacy to ALA. When the incubation time was extended to 6 h, the difference between the efficacy of the aforementioned dipeptides and ALA increased, especially for derivatives **4f**, **4m**, **4k**, and **4u**. At 0.01 mM (Figure 3B), both after 4 and 6 h of incubation, no difference was observed between the PpIX production induced by **4a**, **4d**, **5r**, and ALA, whereas dipeptides **4b**, **4f**, **4m**, and **4k** gave a marked enhancement. It is worth underlining that at 0.01 mM, the PpIX fluorescence induced by ALA alone is not significantly different from the background fluorescence registered in unexposed cells.

In our previous study, compound **4m** was found to be more efficient than ALA in enhancing PpIX production in PAM212 keratinocytes, especially at low concentrations (0.01 mM and 0.1 mM).[35] Besides giving further evidence of the efficacy of compound **4m**, the present experiments show that other dipeptides, such as **4f**, **4k**, and, to a lesser extent **4b** and **4u**, are similarly able to induce PpIX production in the cells when administered at a concentration at which ALA is ineffective.

Comparison of the results of Figure 3 with the experimentally determined hydrophobicity descriptor for each compound indicates that some of the dipeptides that are most efficient in inducing PpIX production do indeed display correspondingly higher values of φ_0 (L-Leu **4f**, $\varphi_0 = 36.67$; L-Phe **4m**, $\varphi_0 = 34.71$; L-Met **4k**, $\varphi_0 = 28.89$; Table 3). However, high levels of PpIX are also obtained after exposing the cells to compounds that are notably more hydrophilic as demonstrated by their φ_0 values, such as L-Ala **4b** ($\varphi_0 = 14.18$) and L-Glu **4u** ($\varphi_0 = 23.14$). In contrast, L-Trp derivative **4p** ($\varphi_0 = 37.35$) gives no apparent increase in PpIX production at either concentration relative to ALA.

No PpIX production was detected in a parallel series when PAM212 cells were exposed to the dipeptides **4c**, **4e**, **4g**, **4j**, **4n**, and **4q**, which contain D-amino acids (data not shown). These results confirm that the release of ALA from the prodrugs is enzyme-mediated, hence PpIX production cannot be elicited using these compounds in cells that do not possess D-peptidase activity.

The induction of PpIX production after incubation with compounds **7a**−**e** and **4o** was also evaluated as above. As shown in Figure 4, the results indicate that at 1 mM the dipeptides and ALA induce PpIX production with similar efficiency, but at lower doses, a marked enhancement of PpIX fluorescence compared to that seen with ALA is observed after exposure to compounds **4o** and **7a**−**e**. Still no direct correlation is evident between the efficacy of the dipeptides studied in terms of enhancing PpIX production and a simple increase in their lipophilicity. As such, this alone is not a sufficient explanation for the improved properties of some of the derivatives relative to ALA.

Quantitative Determination of Intracellular Prodrug Accumulation. Assessments of improvements in the cellular uptake of a given ALA prodrug or formulation for PDT are frequently based upon a direct measurement of PpIX fluorescence. While this provides a convenient readout of apparent efficacy whenever different modalities of treatment need to be compared (i.e., different prodrugs, different incubation times, etc.), it should be borne in mind that the increase in fluorescence measured in pharmacokinetics experiments in fact represents the end result of a sequence of processes, namely the penetration of the prodrug into the cells, the metabolization of the prodrug and subsequent release of ALA, and ultimately the conversion of ALA into PpIX. As pointed out by other authors,[24,46] it is particularly important to decouple the stages of prodrug uptake and intracellular metabolization in order to properly rationalize structure−function relationships. In the light of the apparent lack of correlation between the lipophilicity of our prodrug derivatives and improvements in PpIX production, we decided, in parallel, to unequivocally determine their uptake in PAM212 cells. This would allow us to determine whether the lack of induction of PpIX production displayed by several of our dipeptides could be ascribed to poor cellular penetration or instead to lack of release of ALA due to inefficient metabolization of the prodrugs.

To compare the extent of intracellular accumulation of the prodrugs studied, we chose to use a HPLC-fluorescence method recently developed in our laboratories.[47] The method, based on a procedure originally reported by Tomokuni[48] and later optimized by Oishi,[49] relies on the reaction of ALA with two molecules of acetylacetone and one molecule of formaldehyde to give 2,6-diacetyl-1,5-dimethyl-7-(2-carboxyethyl)-3-*H*-pyrrolizine, a stable fluorescent derivative that can be isolated and quantified by HPLC (inset Figure 5). We have successfully applied this method for the detection and quantification of free ALA directly in cell lysates over a broad range of concentrations, and we also showed that it could be successfully adapted to the quantification of ALA esters following hydrolysis with dilute aq HCl.

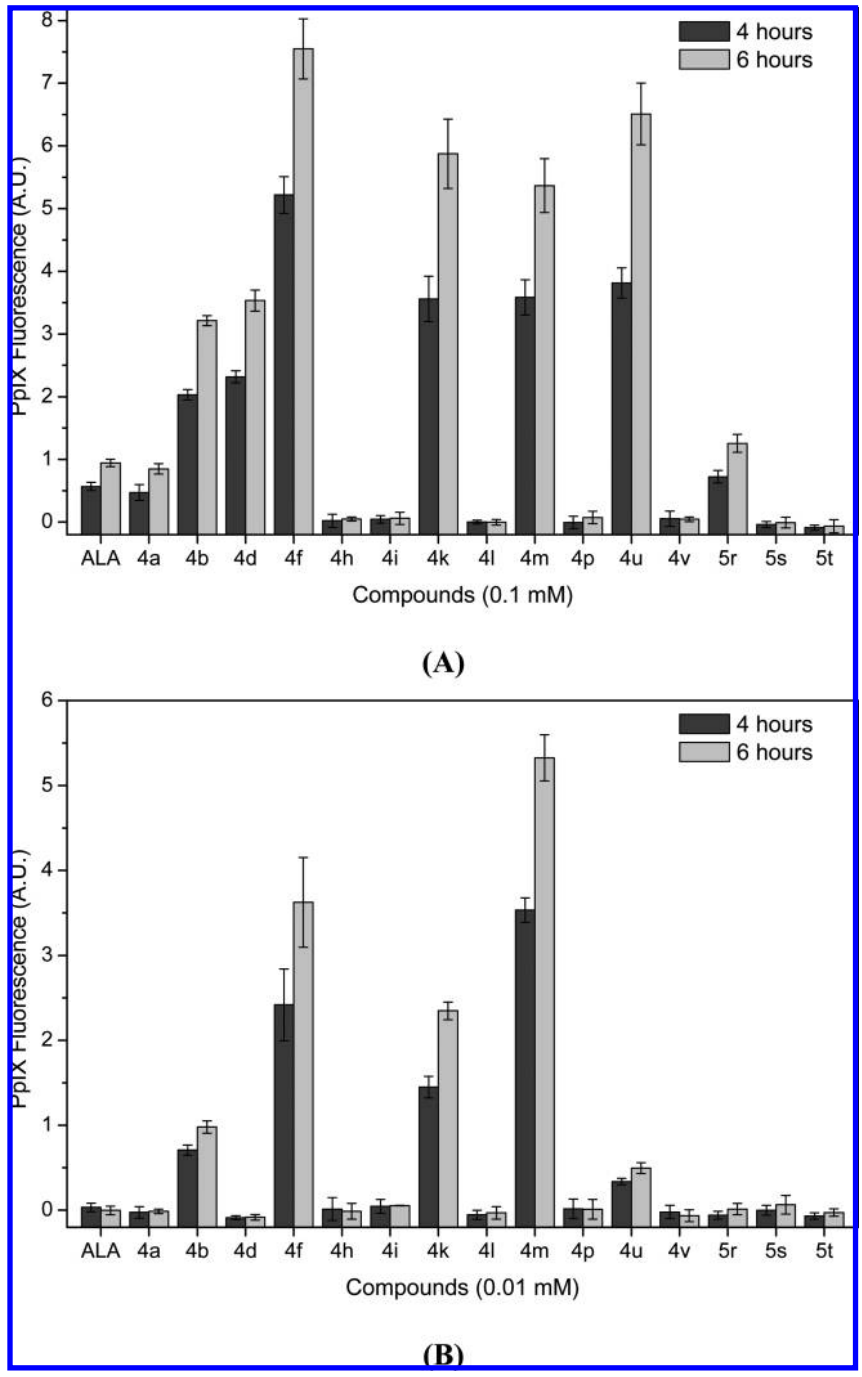


Figure 3. PpIX fluorescence registered after exposure of PAM212 cells to 0.1 mM (A) and 0.01 mM (B) ALA and dipeptides. The results obtained after 4 h (dark-gray) and 6 h (light-gray) of incubation are shown.

For the dipeptides evaluated previously, complete hydrolysis to liberate ALA could be achieved upon refluxing in either 6 M HCl for 2 h, or 4 M HCl for 3 h, as judged by analytical HPLC (data not shown). We had established during the optimization of our analytical method above that the ALA derivatization reaction does not proceed effectively in strongly acidic media (pH < 3), hence neutralization of the hydrolyzed samples prior to derivatization was required. After several attempts, it was found that this could be best achieved by adding solid $NaHCO_3$ to the hydrolysis mixtures. This allows neutralization without risking bringing the pH to alkaline, which could lead to ALA degradation, and also as a solid base does not cause dilution of the sample. The optimized conditions for the quantitative determination of ALA after hydrolysis of the dipeptide prodrugs thus consisted of 3 h reflux in 4 M HCl, neutralization with solid $NaHCO_3$, followed by the derivatization test and HPLC analysis according to our procedure. This method was validated over the range 0.6−65 μM for linearity, accuracy, and precision (see Supporting Information).

Once the method was validated, the stage was set to measure the accumulation of ALA and a selected group of dipeptides in PAM212 keratinocytes. Based upon the measured φ_0 values, we chose examples of lipophilic dipeptides that should be expected to accumulate effectively if uptake by passive diffusion is significant.[46] Alongside these, more hydrophilic species were examined whose membrane permeability should be correspondingly poor. The following compounds were therefore included:

(1) **4m**, **4f**, and **4k** (containing the amino acids Phe, Leu, and Met) as examples of lipophilic dipeptides which, as expected, efficiently induce PpIX production.

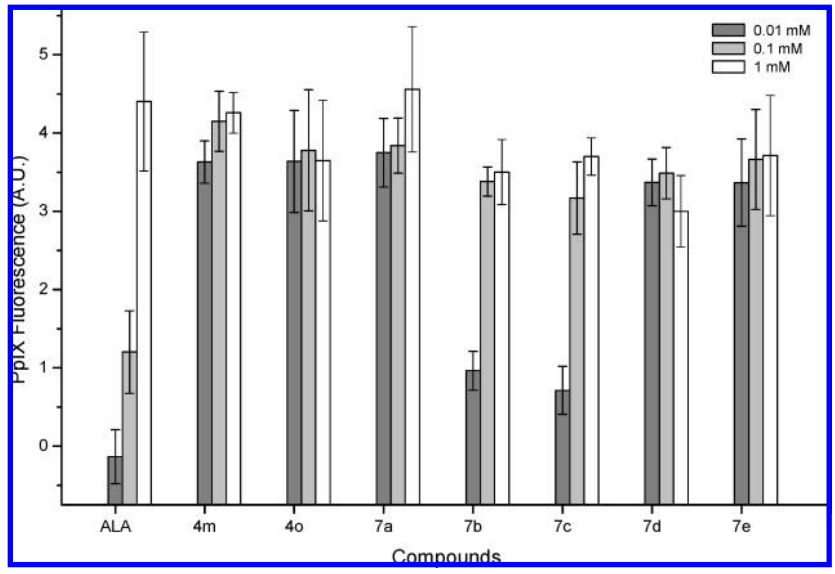


Figure 4. PpIX fluorescence registered after exposing the cells to 0.01 mM (dark-gray), 0.1 mM (gray), and 1 mM (white) ALA and compounds **7a**−**e**, **4m**, and **4o** after 4 h of incubation.

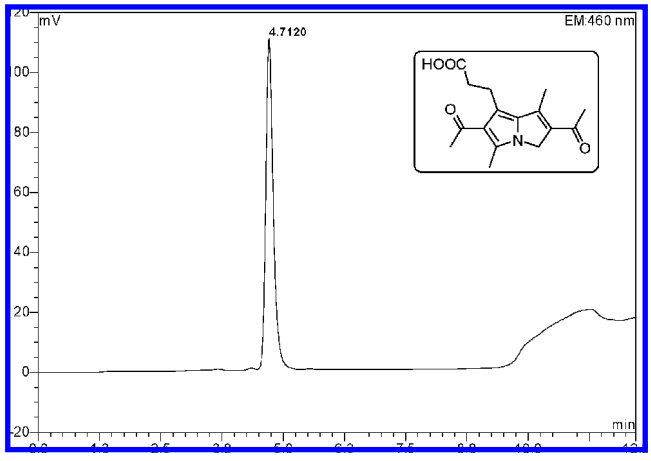


Figure 5. Analytical HPLC following derivatization of 10 μM aq ALA. Conditions (see Experimental Section): Phenomenex C18 column; mobile phase: 0.1% aq acetic acid (solvent A), 0.1% acetic acid in CH_3OH (solvent B); fluorescence detection: $\lambda_{exc} = 370$ nm, $\lambda_{em} = 460$ nm. (Inset: 2,6-diacetyl-1,5-dimethyl-7-(2-carboxyethyl)-3-*H*-pyrrolizine).

(2) **5t** and **4v** (Lys, Asp), which being charged species at physiological pH are likely to show poor accumulation and hence not to promote PpIX production.

(3) **4h** and **4p** (Ile, Trp) as examples of dipeptides that on the basis of their value of φ_0 would be expected to induce PpIX production but fail to do so.

(4) **4l** and **4b** (Ser, Ala), which display intermediate values of φ_0 and do not induce PpIX production.

(5) **4u** (Glu), hydrophilic but nevertheless efficient in inducing PpIX production.

Besides ALA itself as the reference drug, we also evaluated ALA-Hex, the *n*-hexyl ester of ALA, as a standard for an ALA prodrug, which, although chemically different from the compounds of our study, has been extensively characterized with respect to efficacy of PpIX production and mechanisms of cellular uptake.[49]

PAM212 cells were incubated for 4 h at 37 °C, with either 0.1 or 1 mM of drug. The cells were then lysed, and for each drug, PpIX production was quantified by fluorescence spectroscopy and total ALA content determined following hydrolysis and derivatization according to the methods described above. Figures 6 and 7 summarize the data obtained in terms of ng/mg or μg/mg of protein, respectively, for ALA and PpIX.

The results displayed in Figure 6 now reveal that the majority of the dipeptides do in fact penetrate PAM212 cells more efficiently than ALA itself. This is particularly true for the species with high φ_0, such as **4m** (Phe) and the corresponding hexyl-esters **7a**, **4f** (Leu), **4h** (Ile), **4k** (Met), and **4p** (Trp). Strikingly, the more hydrophilic dipeptides **5t**, **4l**, **4b**, and **4v** (Lys, Ser, Ala, Asp, respectively) although unable to elicit elevated levels of PpIX, appear to be able to penetrate the cells just as efficiently as ALA. The values of intracellular ALA measured in PAM212 keratinocytes after incubation with 0.1 mM drug for 4 h show that compound **4m**, the corresponding Phe hexyl esters **7a**, **4f**, **4h**, and **4k** are all 2−5 times more effectively accumulated than equimolar ALA. The same difference is found when the cells are exposed to a higher dose of drugs (1 mM), where in addition a larger number of dipeptides seem to penetrate more efficiently than ALA.

Alongside the general finding that most of the dipeptides accumulate more effectively than ALA, the results obtained under these conditions also appear consistent with some contribution to uptake from diffusion mechanisms. Comparison of total accumulated ALA and measured hydrophobicity do indeed show a generally good correlation, although for some molecules other uptake mechanisms must also come into play. For example, compound **4n** gives a lower value of intracellular ALA compared to its enantiomer **4m**. On the basis of φ_0, the two enantiomeric prodrugs would be expected to show comparable values of intracellular ALA if a nonspecific phenomenon such as passive diffusion was the predominant uptake mechanism involved. This is consistent with our previous observation of the involvement of active transport in the uptake of **4m** in PAM212 cells.[35] Similarly, the relatively high efficacy of accumulation of Asp derivative **4v** in spite of its low lipophilicity also suggests the role of some active transport mechanism. Notwithstanding the precise mechanism of uptake in these and other cases, it

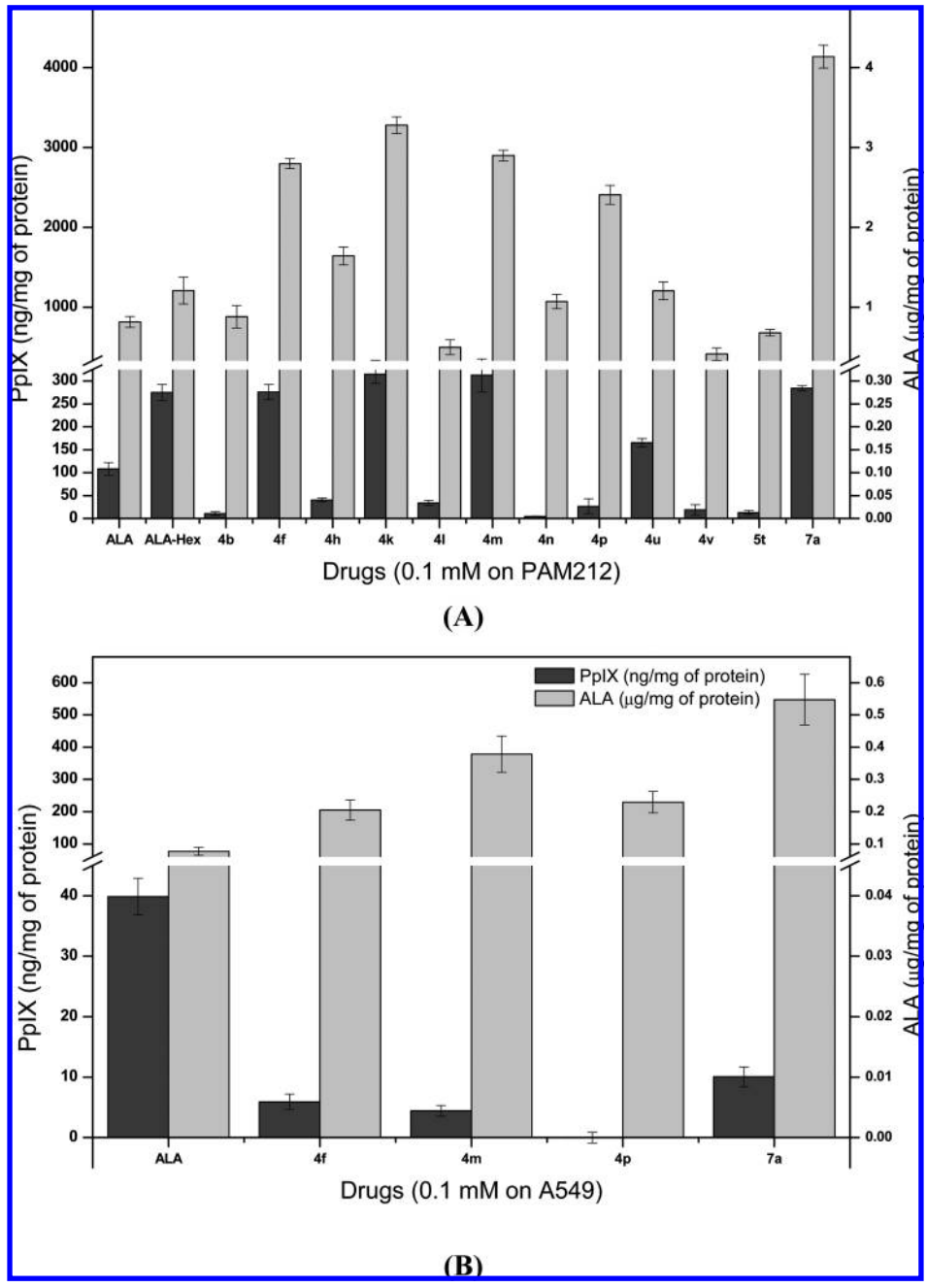


Figure 6. PpIX production and intracellular ALA concentration after incubation of PAM12 keratinocytes (A) and A549 cells (B) with 0.1 mM concentrations of selected prodrugs.

should be noted that for D-Phe derivative **4n**, a simple assessment of PpIX fluorescence might have led to the conclusion that no drug was internalized at all.

Because all the dipeptides examined are at least as efficient as ALA in gaining access to PAM212 cells, it is reasonable to assume that the poor performance of certain derivatives in terms of PpIX production reflects a slower metabolization and a corresponding lower affinity for the esterases and/or peptidases involved in the release of ALA from the prodrug. To confirm this hypothesis and further define the general utility of the above prodrugs, we examined a selection of derivatives in two other cell lines. Berger et al. have shown that in A549 human lung carcinoma cells, PpIX synthesis can be efficiently induced by ALA prodrugs such as L-Phe-ALA-Me and L-Ala-ALA-Me, which are potential substrates for APN/M aminopeptidases, which are expressed in this cell line.[34] In preliminary studies (data not shown) it emerged that our dipeptide prodrugs are not able to induce the production of PpIX in A459 cells. This result was not completely unexpected because our *N*-acetylated prodrugs are rather potential substrates for acylpeptide hydrolase, which is sparingly expressed in the A549 line.[50] When we exposed A549 cells to compounds **4f**, **4m**, **4p**, and **6a** and subsequently quantified ALA according to the HPLC-fluorescence method described above, the uptake observed was, although reduced compared to PAM212, still significantly higher for the dipeptides than ALA (see Supporting Information and Figure 6B). These data again prove conclusively that the absence of PpIX production may be attributed to poor metabolization rather than simply inefficient uptake

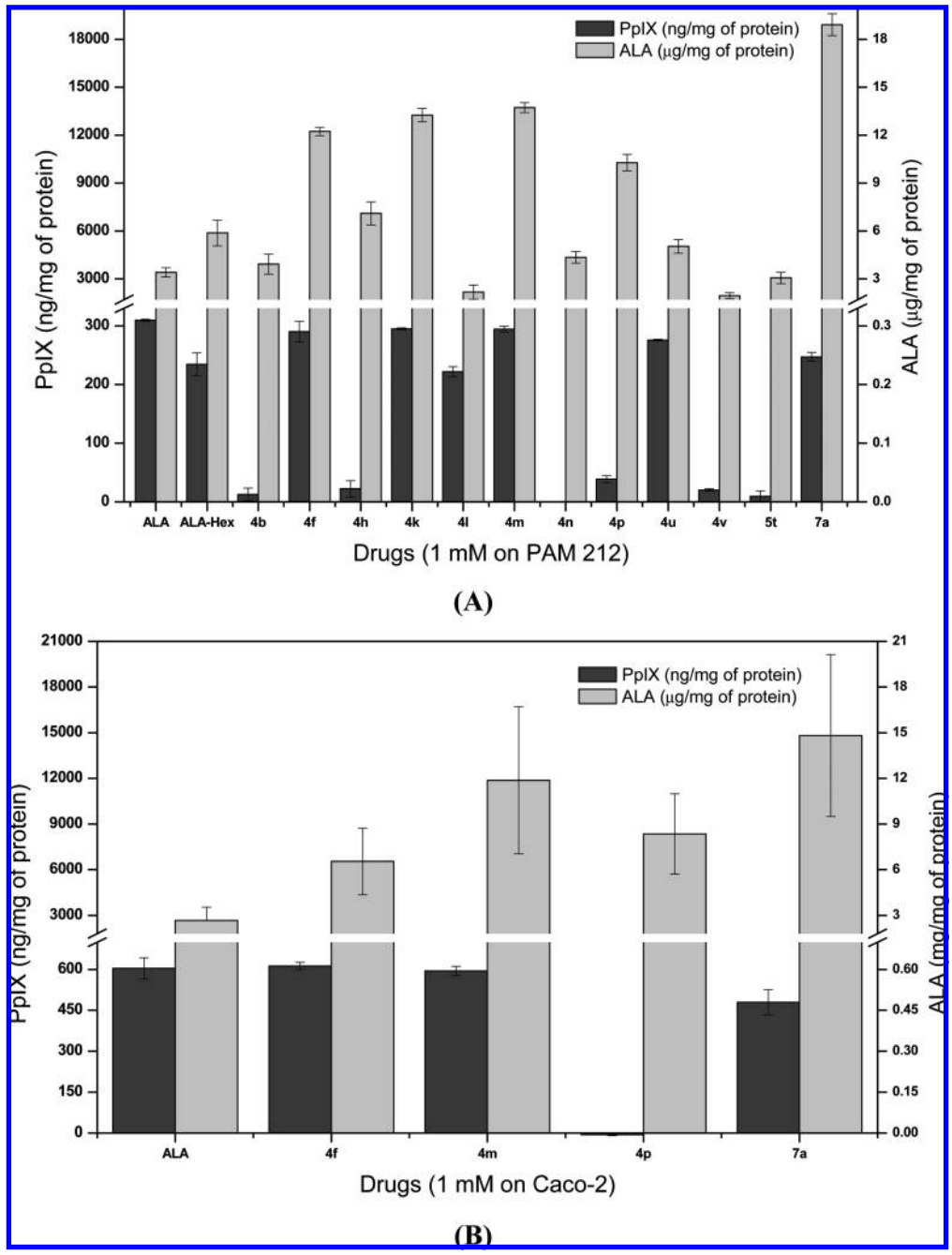


Figure 7. PpIX production and intracellular ALA concentration after incubation of PAM12 keratinocytes (A) and Caco-2 cells (B) with 1 mM concentration of selected drugs.

in this cell line. The same dipeptides also accumulated 3−7 times more efficiently in Caco-2 cells compared to ALA (see Supporting Information and Figure 7B), notwithstanding the lower level of PpIX fluorescence observed relative to equimolar ALA.

Phototoxicity. The phototoxicities of the prodrugs that gave rise to the most significant enhancements in PpIX fluorescence were also evaluated in PAM212 keratinocytes. As shown in Figure 8, the cells were irradiated with blue light (2.5 J/cm^2) after 4 h of incubation with three doses of the selected prodrugs, as well as ALA and ALA-Hex, for comparison. In agreement with the data from the PpIX fluorescence experiments, the dipeptides that exhibited a marked enhancement of PpIX fluorescence also exhibited high phototoxicity. At 1 mM, the phototoxicity of the dipeptides ALA-Hex and ALA were comparable. However, at 0.1 mM, the cell survival observed after exposure to ALA was 11%, while dipeptides **4b** (Ala), **4f** (Leu), **4k** (Met), **4m** (Phe), **4u** (Glu), and the hexyl ester **7a** were as effective as ALA-Hex, reducing the cell viability to only 3%. Furthermore, when the cells were exposed to 0.01 mM ALA, no decrease in cell viability was observed, but compounds **4k**, **4m**, and **7a** still retained high phototoxicity. It is important to underline that under the conditions adopted in all these experiments, the dipeptides displayed no dark toxicity (data not shown).

Conclusions

We have synthesized and characterized a series of ALA-containing peptide prodrugs of general structure Ac-Xaa-ALA-

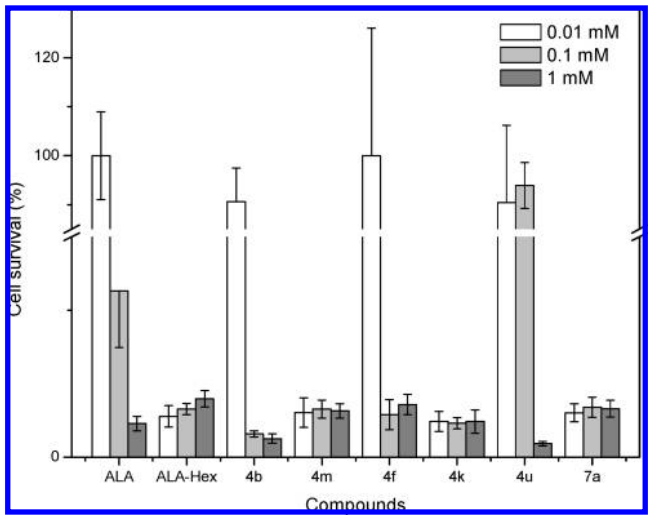
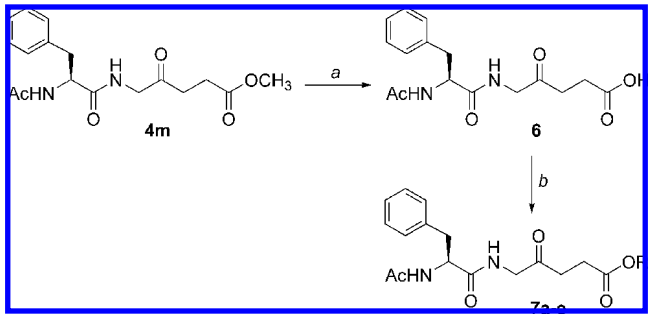


Figure 8. Phototoxicity after incubation with 0.01 mM (white), 0.1 mM (light-gray), and 1 mM (gray) of ALA, ALA-Hex, **4b**, **4f**, **4k**, **4m**, **4u**, and **7a** in PAM212 keratinocytes. Incubation time was 4 h. Irradiation was performed with blue light (2.5 J/cm^2). Cell viability was assessed by MTT assay (see Experimental Section for details).

Scheme 2[a]



[a] Reagents and conditions: (a) LiOH, CH_3OH/H_2O (2:3), rt; (b) ROH, PyBroP, DIPEA, DMAP, CH_2Cl_2, rt.

OR, where Xaa is an α-amino acid, that may be selected so as to provide an appropriate balance of lipophilicity and water solubility. The compounds were obtained in excellent yields and display significantly higher stability in aqueous solution compared to ALA itself. The lipophilicities of the new compounds were quantified by RP-HPLC methods, and the metabolization of the former to produce PpIX upon uptake in PAM212 keratinocytes was evaluated by fluorescence spectroscopy. This revealed a lack of apparent correlation between prodrug lipophilicity and efficiency of PpIX production, which could be rationalized by evaluating the intracellular accumulation of the compounds independently from their metabolic fate. Our method of chemical derivatization and quantification of ALA from intact/partially metabolized prodrugs allows for the first time a distinction to be drawn between prodrugs, which are not internalized in a given cell line, and those which are efficiently internalized but poorly metabolized to PpIX. This should provide a valuable tool for rationalizing the efficiency of other ALA-containing prodrugs. The results obtained herein across three different cell lines confirm that the incorporation of ALA into a short peptide derivative is an effective general approach for increasing cellular delivery of ALA. Several derivatives show significantly elevated cellular accumulation in PAM212 keratinocytes compared to ALA and are indeed able to induce PpIX production at concentrations where ALA is not effective. Future studies will attempt to validate these promising results in an in vivo setting. We are currently applying the synthetic and analytical methods described to fine-tune the structures of our ALA prodrugs to target specific or elevated expressed protease activities[51] and effect selective ALA release in normal or malignant tissue for diagnostic or therapeutic purposes.

Experimental Section

General Remarks. Chemical reagents were purchased from Sigma-Aldrich, Fluka, Acros, Lancaster, and Novabiochem. Anhydrous CH_2Cl_2 was obtained by distillation from calcium hydride, and anhydrous THF and Et_2O were distilled from sodium/benzophenone. All other solvents were purchased from Fisher Scientific and used as received. Analytical TLC was performed using silica gel 60 F_{254} precoated on aluminum sheets (0.25 mm thickness). Column chromatography was performed on silica gel 60 (35−70 μ) from Fisher Scientific. Melting points were recorded on a Reichert−Jung Thermo Galen Kofler block and are uncorrected. ^{1}H and ^{13}C NMR were recorded using a JEOL Delta spectrometer at 270 MHz (^{1}H) and 68 MHz (^{13}C) or on a Varian Mercury-VX spectrometer at 400 MHz (^{1}H) and 100 MHz (^{13}C). Chemical shift values are given in ppm (δ). *J* values are given in Hz. HPLC analyses were performed on a Dionex Ultimate 3000 system (Dionex, UK). The system consisted of a LPG-3400 pump fitted with an internal vacuum degasser, a WPS-300SL semi-preparative autosampler equipped with a 130 μL loop, a TCC-3000 column compartment, a VWD-3400 variable wavelength detector, and a RF-2000 fluorescence detector. The separations were performed on a Gemini 5 μ C18 110A column, 150 mm × 4.6 mm (Phenomenex, UK), equipped with a SecurityGuard C18 (ODS) 4 mm × 3.0 mm ID guard column (Phenomenex, UK), at 35 ± 0.1 °C, with a flow rate of 0.7 mL/min. All compounds submitted to biological analysis had a purity ≥95% as judged by HPLC. High resolution mass spectrometry was performed using a Bruker MicroTOF autospec ESI mass spectrometer. Elemental analyses were performed on an EAI CE-440 instrument. Linear regression analysis was performed using the software package Origin 8 (OriginLab Corporation). clogP values of the compounds were obtained using the software package ChemDraw 11.0.1, (CambridgeSoft).

General Procedure for the Synthesis of Urethane-Protected Dipeptides. *tert*-Butoxycarbonyl-L-phenylalanyl-5-aminolaevulinic Acid Methyl Ester (3m).[36] A solution of Boc-L-Phe-OSu (4.00 g, 11.00 mmol) in dry THF (100 mL) was treated with **1a** (2.00 g, 11.00 mmol), and the resulting suspension was cooled at 0 °C under N_2. A solution of DIPEA (2.00 mL, 11.00 mmol) in dry THF (40 mL) was added by slow infusion with a syringe pump at a rate of 1.49 mL/h. Stirring was continued overnight, and then the solvent was evaporated. The crude product was dissolved in ethyl acetate, preadsorbed onto silica, and purified by flash chromatography (3:1 ethyl acetate/hexanes). Recrystallization from CH_2Cl_2/hexane gave a white solid (4.19 g, 95%); mp 82−85 °C (lit.,[36] 83−85 °C).

General Procedure for the Synthesis of *N*-Acetylated Dipeptides. Acetyl-L-phenylalanyl-5-aminolaevulinic Acid Methyl Ester (4m).[36] Compound **3m** (1.50 g, 3.80 mmol) was treated with 4 M HCl in 1,4-dioxane (19 mL), and the resulting solution was stirred at room temperature for 40 min. The solvent was evaporated, and the residue was dried under vacuum. A suspension of the crude hydrochloride salt thus prepared in dry CH_2Cl_2 (20 mL) was cooled to 0 °C under a N_2 atmosphere and DIPEA (1.45 mL, 8.36 mmol) was added, followed by acetic anhydride (0.77 mL, 7.65 mmol). The mixture was allowed to attain room temperature with stirring overnight and then the solvent was evaporated. The crude product was dissolved in CH_3OH, preadsorbed onto silica, and purified by flash chromatography (30:1 ethyl acetate/CH_3OH). Recrystallization from ethyl acetate gave a white solid (900 mg, 70%); mp 127−130 °C (lit.,[36] 126−128 °C).

General Procedure for the Synthesis of *N*-Acetyl-L-phenylalanyl-5-aminolaevulinic Acid Esters. Acetyl-L-phenylalanyl-5-aminolaevulinic Acid Hexyl Ester (7a). A solution of **4m** (50.0 mg, 0.15 mmol) in water (3 mL) and CH_3OH (2 mL) was treated with LiOH (11.0 mg, 0.45 mmol), and the resulting mixture was stirred at room temperature for 1 h. Dowex (H^+) ion-exchange resin was then added to the mixture until pH 7 was attained. The resin was filtered off, and the solvent was evaporated. The crude acid **6** was dissolved in dry CH_2Cl_2 (5 mL), and the resulting solution was treated with PyBroP (70 mg, 0.15 mmol), DIPEA (78 μL, 0.45 mmol), and DMAP (2 mg, 0.01 mmol), followed by hexan-1-ol (28 μL, 0.22 mmol). The mixture was allowed to stir at room temperature overnight and then the solvent was evaporated. Purification by flash chromatography (30:1 ethyl acetate/CH_3OH = 30/1) gave **7a** as a white solid (19.5 mg, 31%); mp 94−96 °C. ^{1}H NMR (400 MHz, CD_3OD) δ: 7.29−7.18 (5H, m, Ph), 4.66 (1H, dd, *J* 8.8, 5.2, NHC*H*(R′)CO), 4.05 (2H, t, *J* 6.4, OCH_2), 4.02 (2H, br, NHCH_2CO, OCH_2), 3.17 (1H, dd, *J* 13.8, 5.2, C$H_A$$H_B$Ph), 2.87 (1H, dd, *J* 13.8, 8.8, C$H_A$$H_B$Ph), 2.69 (2H, t, *J* 6.9, COCH_2CH_2-CO_2R), 2.56 (2H, t, *J* 6.9, $COCH_2CH_2CO_2R$), 1.89 (3H, s, CH_3CO), 1.63−1.59 (2H, m, CH_2 hexyl), 1.38−1.29 (6H, m, CH_2 hexyl), 0.91 (3H, s, CH_3 hexyl). ^{13}C NMR (100 MHz, CD_3OD) δ: 204.7, 174.4, 174.1, 173.2, 138.5, 130.2, 129.4, 127.7, 65.8, 56.1, 38.9, 35.1, 32.6, 29.7, 28.7, 26.7, 23.6, 22.4, 14.3. ESI-HRMS$^+$: calcd for $C_{22}H_{33}N_2O_5$, 405.2384; found, 405.2371 $[M + H]^+$.

Determination of Chromatographic Lipophilicity. The chromatographic descriptor for lipophilicity φ_0 was determined in isocratic conditions, using mixtures of either CH_3CN or CH_3OH and 50 mM aqueous ammonium formate. The isocratic retention factor (log *k*) was calculated using the equation $\log k = (t_R - t_0)/t_0$. The dead time (t_0) was measured by injecting a solution of uracil according to the recommendations of the manufacturer. The retention time (t_R) was measured in duplicate, and the average value was used to calculate log *k*. For each compound, six values of log *k* were determined using six different mobile phase compositions, whose percentages of organic solvent (φ) were chosen so that $-1.5 < \log k < 0.6$. The isocratic runs consisted of 5 min of equilibration with the chosen φ prior to the injection, followed by 15 min of isocratic flow, a 0.1 min gradient ramp to 95% of organic solvent, a 5 min wash in the same conditions, a 0.1 min ramp back to the initial conditions, and finally 2 min of re-equilibration. The flow rate was 1.0 mL/min. The values of log *k* were plotted versus the percentage of organic solvent in the mobile phase, and linear regression was applied to calculate the values of the slope (*S*), the intercept (log k_w), and the linear correlation (R^2).

Cell Culture. The spontaneously transformed murine keratinocyte cell line, PAM212 (obtained from Prof. R. Groves, King's College, London) was cultured in RPMI-1640 medium (Gibco BRL, Life Technologies Ltd., Paisley, UK) containing L-glutamine (2 mM) and phenol red, supplemented with 10% fetal calf serum (Sigma-Aldrich Ltd.), and penicillin and streptomycin (500 units/mL, and 0.5 mg/mL, Gibco BRL). Human Caucasian lung carcinoma, A549 cells were cultured in Ham's F12K (Sigma-Aldrich Ltd.) containing L-glutamine (2 mM) and phenol red, supplemented with 10% fetal bovine serum (Sigma-Aldrich Ltd.), and penicillin and streptomycin (500 units/mL, and 0.5 mg/mL, Gibco BRL). Human colonic adenocarcinoma, Caco-2 cells were cultured in EMEM (Sigma-Aldrich Ltd.) containing L-glutamine (2 mM) and phenol red, supplemented with 10% fetal bovine serum, 1% nonessential amino acids (Sigma-Aldrich Ltd. Sigma-Aldrich Ltd. Sigma-Aldrich Ltd.) and penicillin and streptomycin (500 units/mL, and 0.5 mg/mL, Gibco BRL). The cells were routinely grown as monolayers in 75 cm^2 culture flasks (TPP, Helena Bioscience, Gateshead, UK), at 37 °C, under a 5% CO_2 atmosphere until confluent.

Fluorescence Pharmacokinetics. Cells were seeded into γ-sterilized 96-well plates (Orange Scientific, Triple Red Laboratory Technologies, Long Crendon, UK) at a density of 5×10^4 cells per well for 48 h. After removing the culture medium, the wells were washed with phosphate buffered saline (PBS) and incubated with freshly prepared solutions of ALA or ALA peptide derivatives: 0.1 mL of serum-free medium containing varying prodrug concentrations was added to a designated series of wells. Each plate contained control wells with cells but without added drug for determination of the background reading, and reference wells containing cells incubated with the same ALA concentrations. For drug incubation, serum-free medium was used because serum is known to cause release of PpIX from cells, thus resulting in loss of fluorescence signal.[52]

The fluorescence signal from each well was measured with a Perkin-Elmer LS 50B fluorescence spectrometer (Perkin-Elmer, Beaconsfield, UK) coupled to an automated plate reader, using 410 nm excitation and 635 nm emission wavelengths with slit widths set to 10 nm and the internal 530 nm long-pass filter used on the emission side; spectral scans were recorded between 600 and 750 nm to check for presence of any porphyrins other than PpIX.[26] PpIX concentration was calculated from a standard curve of PpIX. The mean fluorescence intensity (expressed in arbitrary units) was calculated after subtraction of the control values. Intensity calibrations were performed using rhodamine B embedded in a Perspex disk as a standard.

Photodynamic Treatment. Cells were seeded into 96-well plates at a density of approximately 5×10^4 cells per well. Following incubation for 48 h, the cells were washed with PBS, and 0.1 mL of solutions containing the appropriate drug at a concentration were added to the wells and incubated for 4 h. The plates were then irradiated using a LumiSource lamp (PCI Biotech, Oslo, Norway), emitting a uniform field of low-power blue light (peak output centered on 420 nm) over an area of 14 cm × 32 cm at a fluence of 1.25 J/cm^2. Immediately following irradiation, the medium was replaced and cells were incubated for a further 24 h. Cytotoxicity was determined using the MTT assay: cells were incubated with medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (1 mg/mL dissolved in full RPMI-1640 medium) for 3 h. The insoluble end product (formazan derivatives) was dissolved in DMSO (0.1 mL) after removing the medium. UV absorption was quantified at 570 nm using a 96-well plate reader (MR 700 Dynatech, Dynex, Worthing, UK). The mean cell survival was calculated for each prodrug at every concentration tested and expressed as a percentage of control (incubated with the compounds but not irradiated) cell survival values. Dark toxicity was determined by assessing the cell survival after incubation with the drugs without exposing the cells to irradiation.

Determination of Intracellular PpIX Content. Cells were seeded into 100 mm Petri-dishes at a density of 5×10^4 cells per well for 48 h at 37 °C. The culture medium was removed and the cells were washed with PBS. The cells were then incubated with the appropriate dose of ALA or of the selected dipeptides for 4 h and washed with PBS. CelLytic (Sigma-Aldrich) (1 mL) was added and incubated for 15 min at room temperature, and the cells were mechanically scraped. Cell extracts were centrifuged at 1800 *g* for 10 min to remove the cell debris, and the supernatant containing the PpIX was collected. The content of PpIX in the cell lysates was determined by fluorescence as reported above. The protein content of the cells was determined using a bicinchoninic acid protein determination kit (Sigma-Aldrich).[53]

Determination of Intracellular ALA Accumulation. Solutions. Stock solutions of ALA and ALA esters in deionized water were prepared daily from powders stored at 4 °C. Working solutions were obtained after dilution of the stock solutions using class A glassware. The two ranges of concentrations investigated were 0−5 μg/mL (0−30 μM) and 5−100 μg/mL (30−603 μM). Acetylacetone reagent was prepared by mixing water, absolute ethanol, and acetylacetone in a ratio of 11/6/3 by volume, and 10% formaldehyde reagent was obtained by dilution of commercial 37% v/v aqueous solution in water. Both the solutions were stored at 4 °C.

Preparation of Calibration Samples. For the determination of ALA after hydrolysis of the prodrugs, the calibration samples were prepared by spiking 370 μL of cell lysate (prepared as reported above; see porphyrin extraction) with 30 μL of the appropriate working solution, and then 200 μL of this mixture were diluted to

2 mL with 4 M aqueous HCl in a 16 mm Ø Greenhouse reaction tube. The tubes were transferred into the Greenhouse reactor preheated at 100 °C and refluxed for 3 h under stirring. The samples were then cooled in an ice bath and were subsequently neutralized by addition of equal quantities of solid $NaHCO_3$ until neutral to litmus. Then 50 μL of each sample were used for the derivatization test. For each range of concentrations, two samples were prepared for recovery evaluation purposes.

Derivatization Procedure. First, 50 μL of calibration sample were added to 3500 μL of acetylacetone reagent and 450 μL of 10% formaldehyde solution in a Greenhouse reaction tube equipped with a magnetic stirrer. The tubes were placed in the Greenhouse reactor preheated at 100 °C and stirred for 10 min. The reactor chamber was wrapped with foil in order to protect the tubes with the reaction mixture from light. The samples were then cooled in an ice bath in the dark for 2 h, transferred into HPLC vials, and kept in the autosampler at room temperature until the analysis was performed.

HPLC-Fluorescence Method. The mobile phase consisted of 0.1% acetic acid in water (solvent A) and 0.1% acetic acid in CH_3OH (solvent B). The composition of the mobile phase was as follows: −5.0 to 0.0 min at 60% solvent B, 0.0 to 6.0 min at 60% solvent B, 6.0 to 6.1 min 60 to 95% solvent B, 6.1 to 12.0 min at 95% solvent B, 12.0 to 12.1 min 95 to 60% solvent B. The flow rate was 0.7 mL/min. $\lambda_{exc} = 370$ nm and $\lambda_{em} = 460$ nm were used for the detection, and the fluorescence detector was set on high sensitivity. The peak corresponding to 2,6-diacetyl-1,5-dimethyl-7-(2-carboxyethyl)-3-*H*-pyrrolizine eluted at 4.71 min. The injection volume was 20 μL. The total time required for the analysis was 12.1 min.

Method Validation. The methods developed were validated for linearity, accuracy, and precision, limits of detection, and quantification (see Supporting Information). The linearity was verified over the range 60−600 μM. For every curve, nine calibration standards (in duplicate) were generated as described above on six consecutive days. The samples were then analyzed by HPLC (in duplicate) as reported above, and the detector response (peak area) was plotted against the concentration of the analyte. Linear regression analysis was applied to calculate the slope, the intercept, and the linear correlation (R^2). The intraday precision was evaluated by analyzing three different samples within the concentration range for six times in the same day. Interday precision was determined at the same concentrations over three days. The limit of detection (LOD) and the limit of quantification (LOQ) were determined mathematically from the standard curve equations. The LOD and LOQ were obtained by multiplying the standard deviation (SD) of the intercepts by 3.3 and 10, respectively, and dividing by the slope.

Determination of ALA Concentration in Cell Lysates. Cell lysates were prepared as reported above (see porphyrin extraction). Then 200 μL of lysate were added to 1800 μL of 4 M aqueous HCl. The resulting solution was refluxed for 3 h and then it was cooled in an ice bath. The solutions were neutralized as described above, and 50 μL were submitted to the derivatization reaction and HPLC detection. The concentration of ALA in the sample was calculated from the detector response (peak area) and the calibration curve obtained as described above.

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Supporting Information Available: Characterization data for compounds **2a**, **2b**; **3a**−**l**, **3n**−**3u**, **4a**−**l**, **4n**−**v**, **7b**−**7e**. Detailed experimental procedures and characterization data for compounds **5r**−**5t**. Measured cellular PpIX and ALA content in PAM212, A549, and Caco-2 cells after incubation with prodrugs. Validation parameters for the HPLC-fluorescence method. This material is available free of charge via the Internet at http://pubs.acs.org.

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