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Novel prodrug approach to photodynamic therapy: Fmoc solid-phase synthesis of a cell permeable peptide incorporating 5-aminolaevulinic acid

Mark J. Dixon,[a] Ludovic Bourré,[b] Alexander J. MacRobert[b] and Ian M. Eggleston[a,*]

[a]*Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, UK*
[b]*National Medical Laser Centre, Division of Surgical and Interventional Sciences, Royal Free and University College Medical School, University College London, Charles Bell House, 67-73 Riding House Street, London W1W 7EJ, UK*

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Abstract—The first example of the synthesis of a peptide incorporating 5-aminolaevulinic acid (5-ALA) using standard Fmoc solid-phase chemistry is reported. The synthesised peptide contains residues 52–58 of the cell-permeable peptide Penetratin and represents a prototype for the enhanced topical delivery of 5-ALA using such oligopeptide vectors. Effective intracellular conversion of the peptide to the endogenous photosensitiser, protoporphyrin IX, is observed in PAM212 cells, thus demonstrating the potential of this approach for the development of novel peptide prodrugs for use in photodynamic therapy.

Photodynamic therapy (PDT) is an emerging technique used in the treatment of a variety of human disorders. It relies on the combined use of light, molecular oxygen and a light-activated photosensitising drug to selectively destroy diseased tissue or pathogenic organisms.[1] One such approach is based upon the administration of 5-aminolaevulinic acid (5-ALA, Fig. 1), a naturally occurring compound that in mammalian cells is metabolised via the haem biosynthetic pathway to the porphyrin photosensitiser protoporphyrin IX (PpIX, Fig. 1).[2] Following topical administration of 5-ALA and accumulation of PpIX in the target tissue, administration of red laser light activates PpIX leading to the generation of cytotoxic reactive oxygen species. Currently, the main clinical application of 5-ALA-PDT is the treatment of basal cell carcinomas, although there is significant potential for its use in the treatment of a wide range of other skin conditions, including viral warts, acne vulgaris, psoriasis, actinic keratoses, Bowen's disease, squamous cell carcinoma (SCC) and cutaneous T cell lymphoma, as well as the visualisation and treatment of early tumours in hollow organs such as the bladder.[2,3]

A limitation to the use of 5-ALA in PDT results from its low lipid solubility, a consequence of its zwitterionic character at physiological pH, which leads to poor penetration through biological barriers such as cellular membranes. This problem has partly been addressed through the synthesis of lipophilic ester prodrugs that provide improved cellular uptake and are metabolised into PpIX following the action of non-specific intracellular esterases.[4] A more recent development concerns the preparation of peptide-based 5-ALA prodrugs, and we[5] and others[6] have described the synthesis and evaluation of short 5-ALA peptide derivatives in which the amino and carboxyl functions of the latter are masked, thereby providing improved physical properties and the potential for cell line specific ALA release, according to which peptidases are expressed. However, despite considerable current interest in the use of peptide-targeted delivery of photosensitising agents for PDT,[7] the well-documented instability of 5-ALA and its esters[8] under basic conditions has so far limited applications in this area to relatively simple peptides.

A novel and potentially powerful means of enhancing topical ALA delivery is to conjugate 5-ALA to a cell-penetrating oligopeptide vector, and while some examples of the covalent attachment of porphyrin-type photosensitisers to synthetic targeting peptides have been described,[9] no such derivative containing 5-ALA has

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* Corresponding author. Tel.: +44 01225 383101; fax: +44 01225 386114; e-mail: ie203@bath.ac.uk

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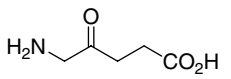
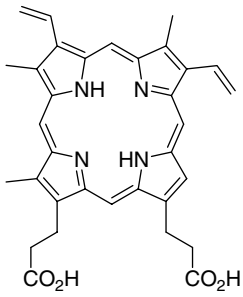


5-ALA



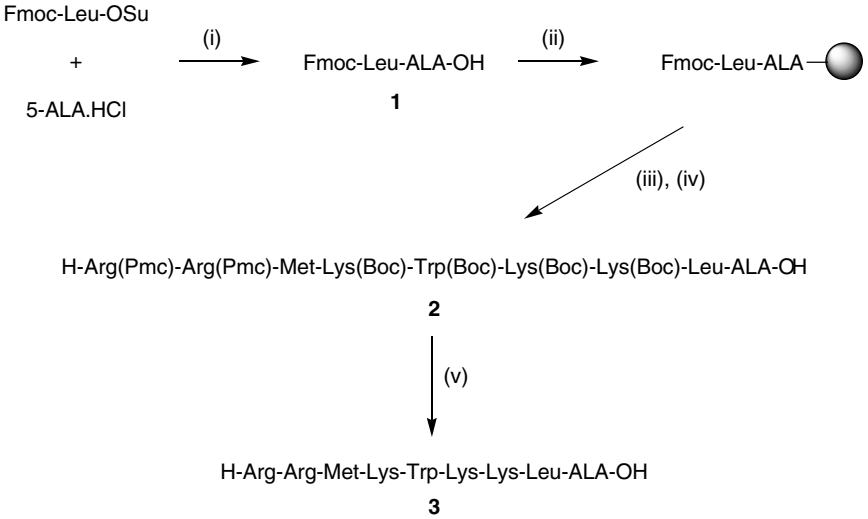
PpIX

Figure 1. 5-Aminolaevulinic acid and protoporphyrin IX.

yet been examined. Cell-penetrating peptides (CPPs) are characterised by their ability to not only self-translocate intracellularly, but also transport a wide range of cargo, including proteins, nucleic acids and nanoparticles,[10] as well as otherwise poorly absorbed small drug molecules.[11] As such, they offer a very attractive approach for improved ALA delivery. As a proof of principle, we decided to attempt the solid phase synthesis of a 5-ALA nonapeptide, in which a dipeptide prodrug is fused to the C-terminal heptapeptide (Arg-Arg-Met-Lys-Trp-Lys-Lys) from the third helix of the *Antennapedia* transcription factor.[12]

The key dipeptide starting material, Fmoc-Leu-ALA-OH (**1**, Scheme 1), was prepared using a novel acylation method recently developed by us,[5b] in which 5-ALA is slowly released from its hydrochloride salt in the presence of a preformed active ester species, and thus immediately acylated before competing dimerisation reactions (to give pyrazines) can intervene. Thus, slow addition of a solution of DIPEA in THF to a mixture of 5-ALA.HCl and the succinimido ester of Fmoc-Leu in THF solution (in which 5-ALA.HCl is insoluble) gave the desired dipeptide in 58% yield.[13] **1** was then loaded onto 2-chlorotrityl chloride polystyrene resin by treating the resin with a solution of the dipeptide and DIPEA in DCM/DMF (1:1) for 60 min. (Scheme 1). As the Leu-ALA unit effectively constitutes a tripeptide mimic, our approach was expected to avoid any risk of intramolecular lactamisation and concomitant elimination from the resin, analogous to diketopiperazine formation, that might occur upon initial Fmoc deprotection. The 2-chlorotrityl linker is in any case known to suppress unwanted cyclisation during Fmoc assembly.[14] The mild cleavage conditions employed with this linker (typically 1% TFA in DCM) also offered the additional advantage of allowing a two-step cleavage/side-chain deprotection strategy to be used, allowing the discrimination of side products resulting from the peptide assembly and deprotection steps of the synthesis.

Assembly of the desired peptide sequence was performed using standard Fmoc chemistry,[13] with Fmoc deprotection being achieved through treatment with piperidine/DMF (1:4) for 4×3 min, and couplings accomplished with PyBOP[15]/HOBt activation for 45 min. The protected peptide **2** was then obtained by cleavage of the resin with TFA/DCM (1:99) for 10×2 min. Although Berger et al. have reported[6a] that the use of Fmoc protection was unsatisfactory for the preparation of ALA-containing peptides, HPLC analysis of the crude product of the cleavage showed essentially a single species, suggesting that the solid-phase peptide assembly had proceeded remarkably smoothly (Fig. 2) and without complications due to the keto function of 5-ALA.[16] Removal of side chain protection from **2** was achieved by treating the latter with a cleavage cocktail of TFA/thioanisole/DCM/water (16:2:1:1) for 2 h (Scheme 1). HPLC and ESMS analysis of the crude deprotected product **3** revealed that the desired peptide



Scheme 1. Reagents and condition: (i) DIPEA, THF, −5 °C, 58%; (ii) 2-chlorotrityl chloride polystyrene resin, DIPEA, DCM/DMF; (iii) Fmoc solid phase peptide synthesis; (iv) TFA/DCM (1:99), 10×2 min; (v) TFA/thioanisole/DCM/water (16:2:1:1), 2 h (see Ref. 13 for full details).

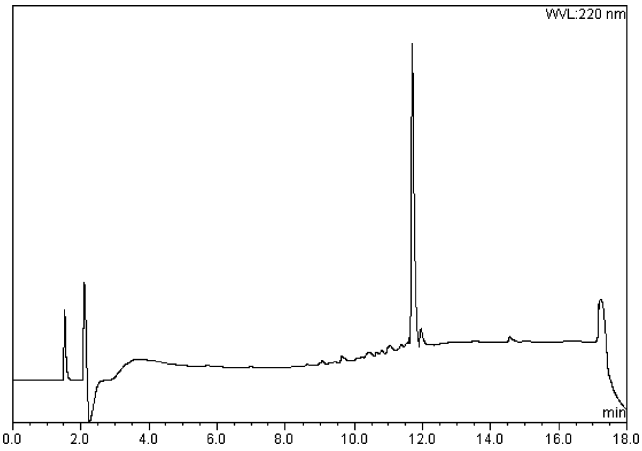


Figure 2. HPLC trace of crude protected peptide **2** following cleavage from the resin.

was the major product with a purity of >90% (Fig. 3, lower trace) and a yield of 62%. A portion of the crude product was purified to homogeneity by semi-preparative HPLC to give pure **3** for biological studies (Fig. 3, upper trace).

The generation of PpIX from the purified prodrug **3** was evaluated in a model cell line (PAM 212 transformed murine keratinocytes).[17] Cells were exposed to **3** for up to 48 h at 37 °C, at a range of concentrations from 0.005 to 0.5 mM. Fluorescence measurements of intracellular PpIX levels were recorded after 4, 5, 7, 24 and 48 h with excitation at 405 and 635 nm emission.[18] As shown in Figure 4 PpIX production on treatment with **3** was both concentration- and time-dependent, with high levels of fluorescence being attained at a prodrug concentration of 0.5 mM after 48 h. Although the overall levels of PpIX fluorescence obtained under these conditions were approximately one half that obtained with ALA itself as reference, following subtraction of the background reading, this demonstrates for the first time the feasibility of using a CPP construct for the intracellular delivery of 5-ALA.

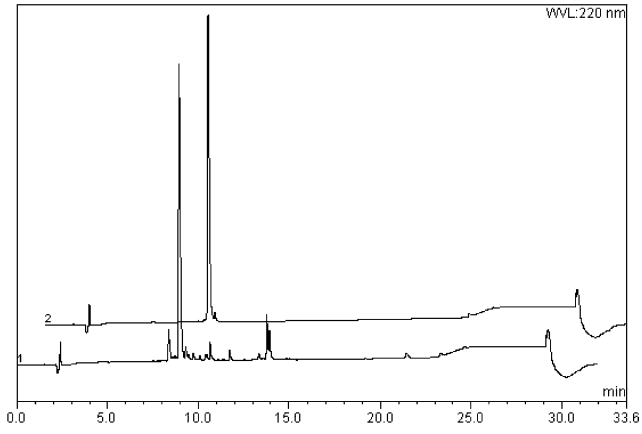


Figure 3. HPLC traces of crude (lower trace) and purified (upper trace) deprotected peptide **3**.

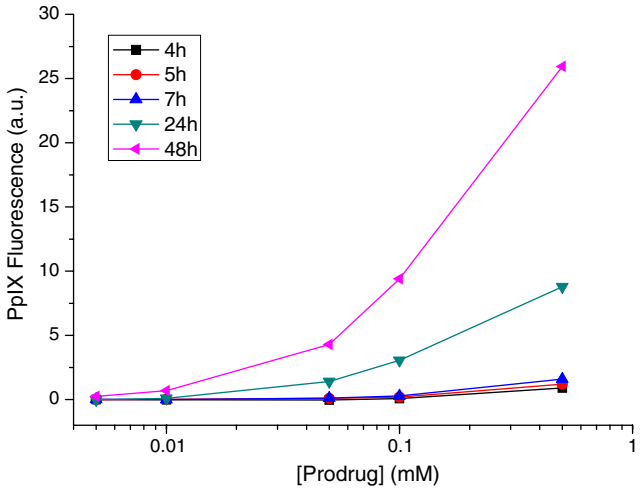


Figure 4. Time- and concentration-dependent production of PpIX on exposure of PAM212 cells to **3** (see Ref. 18 for full details).

The low levels of PpIX fluorescence at all concentrations examined up to 7 h compared to longer timescales (24 or 48 h) suggest that the limiting factor in PpIX production from **3** in this system may be the release of 5-ALA after incorporation of the latter. The mechanism of uptake of CPPs and their cargo has been the topic of considerable discussion in the literature,[10,19] however recent studies indicate that for antennapedia-mediated peptide delivery in particular,[20] endocytic processes are predominantly involved. In this case, the rate of release of the CPP here from the endosome may determine the efficiency with which it can be processed by cytoplasmic peptidases. As a preliminary test of the contribution of endocytic processes to the uptake of **3**, the latter was incubated with PAM212 cells as above at 4 °C compared to 37 °C.[17] A significant reduction in PpIX production was observed, which is consistent with uptake by an energy-dependent process, since all endocytic uptake processes (both clathrin-dependent and independent), along with micro- and macropinocytosis, are blocked at low temperature.[10,17]

In conclusion, we have achieved the synthesis of a 5-ALA-containing CPP construct by standard Fmoc-solid phase peptide synthesis and shown that such a molecule can be converted in vitro to PpIX. This shows the potential of this approach for the development of novel agents for 5-ALA-based PDT. We are currently examining other peptide vectors in this context and the possibility of their use for transport of multiple 5-ALA cargoes.

Acknowledgments

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13. *Experimental conditions, general information*: NMR spectra were acquired on a Bruker Avance DPX 300 spectrometer, operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C. All coupling constants (J values) were measured in Hertz. Low-resolution ES mass spectra were recorded on an ABI Mariner TOF Electrospray Ionisation mass spectrometer in the University of Dundee Post-Genomics and Molecular Interactions Centre. High-resolution ES mass spectra were recorded at the EPSRC National Mass Spectrometry Service (University of Wales, Swansea). Analytical RP-HPLC was performed on a Dionex HPLC system equipped with a Dionex Acclaim 3 μm C-18 (150 × 4.6 mm) column with a flow rate of 1 mL/min. Semi-preparative RP-HPLC was performed on a Dionex HPLC system equipped with a Phenomenex Gemini 5 μm C-18 (250 × 10 mm) column with a flow rate of 2.5 mL/min. Mobile phase *A* was 0.1% TFA in water, mobile phase *B* was 0.1% TFA in acetonitrile. Gradient 1 was $T = 0$ min, $B = 5\%$; $T = 10$ min, $B = 95\%$; $T = 15$ min, $B = 95\%$; $T = 15.1$ min, $B = 5\%$. Gradient 2 was $T = 0$ min, $B = 5\%$; $T = 20$ min, $B = 60\%$; $T = 22$ min, $B = 95\%$; $T = 27$ min, $B = 95\%$; $T = 27.1$, $B = 5\%$; $T = 32$ min, $B = 5\%$. Gradient 3 was $T = 0$ min, $B = 5\%$; $T = 20$ min, $B = 30\%$; $T = 22$ min, $B = 95\%$; $T = 27$ min, $B = 95\%$; $T = 27.1$ min, $B = 5\%$; $T = 32$ min, $B = 5\%$.
Synthesis of **1**: A suspension of 5-ALA.HCl (0.50 g, 3 mmol) and Fmoc-Leu-OSu (1.34 g, 3 mmol) in dry THF (40 mL) was cooled to −5 °C under argon. A solution of DIPEA (0.52 mL, 3 mmol) in dry THF (20 mL) was slowly added over 120 min, then the reaction mixture was stirred overnight under cooling. The reaction mixture was concentrated in vacuo, the residue taken into EtOAc (50 mL) and washed with H_2O (3 × 50 mL). The organic layer was extracted into 5% $NaHCO_3$ (3 × 25 mL), the aqueous layer acidified to pH 4 with conc. citric acid and extracted into EtOAc (3 × 50 mL). The EtOAc extracts were combined, dried ($MgSO_4$) and concentrated in vacuo to yield a white solid. This solid was recrystallised from hexane/EtOAc to give **1** as white crystals (0.81 g, 58%). R_f: 0.33 ($CHCl_3$/MeOH/AcOH, 45:4:1). δ_H (300 MHz, $(CD_3)_2SO$): 0.90 (6H, m, $CH(CH_3)_2$), 1.40–1.68 (3H, m, $CH_2CH(CH_3)_2$, $CH_2CH(CH_3)_2$), 2.41 (2H, m, $COCH_2CH_2$), 2.72 (2H, m, $COCH_2CH_2$), 3.88–4.09 (3H, m, $CHCH_2CH(CH_3)_2$, $NHCH_2CO$), 4.18-4.31 (3H, m, CO_2CH_2CH, CO_2CH_2CH), 7.32 (2H, t, J 7.0, 2 × ArC*H*), 7.43 (2H, t, J 7.0, 2 × ArC*H*), 7.58 (1H, d, J 8.0, N*H*), 7.75 (2H, d, J 7.0, 2 × ArC*H*), 7.9 (2H, d, J 7.0, 2 × ArC*H*), 8.22 (1H, m, N*H*). δ_C (75 MHz, $(CD_3)_2SO$): 21.4 (CH_3), 23.1 (CH_3), 24.2 (CH), 27.6 (CH_2), 34.0 (CH_2), 40.7 (CH_2), 46.7 (CH), 48.4 (CH_2), 53.0 (CH), 65.6 (CH_2), 120.1 (CH), 125.4 (CH), 127.1 (CH), 127.6 (CH), 140.7 (C), 143.8 (C), 156.0 (C), 172.7 (C), 173.7 (C), 205.5 (C). RP-HPLC (analytical, gradient 1, $\lambda = 220$ nm): $R_t = 9.6$ min (100%). MS (ES+) *m/z*: 467.3 (100%) $[M + H]^+$. HRMS (ES+) $C_{26}H_{31}N_2O_6$ (calcd) 467.2177; (found) 467.2180 $[M + H]^+$.
Synthesis of **2** and **3**: Loading of the resin was achieved by treating 2-chlorotrityl chloride polystyrene resin (200 mg, 0.34 mmol) with a solution of **1** (159 mg, 0.34 mmol) and DIPEA (238 μL, 1.36 mmol) in DCM/DMF (v/v, 1:1, 2 mL) for 60 min. Unreacted sites on the resin were capped by treatment with a solution of DCM/MeOH/DIPEA (17:2:1). Fmoc deprotection was achieved by treatment with piperidine/DMF (v/v, 1:4, 3 mL) for 4 × 3 min. Peptide couplings were performed by treatment with a solution of Fmoc-amino acid (2 equiv), PyBOP (1.9 equiv), HOBt (2 equiv) and DIPEA (4 equiv) in DCM/DMF (v/v, 3:1, 2 mL) for 45 min. Solid phase reactions were monitored by use of a qualitative Kaiser test.[21] Cleavage from the resin was achieved by treatment with TFA/DCM (v/v, 1:99, 2 mL) for 10 × 2 min. The cleavage solutions were immediately neutralised by addition to pyridine/MeOH (v/v, 1:9, 20 mL), concentrated to approximately 2 mL and precipitated from ice-cold water (30 mL). The precipitate was washed with ice cold water (2 × 30 mL) and dried in vacuo to yield **2** as a white solid (285 mg, 63% based on loading of first residue). RP-HPLC (analytical, gradient 1, $\lambda = 220$ nm): $t_R = 11.69$ min.
Compound **2** (285 mg, 0.13 mmol) was dissolved in TFA/thioanisole/water/DCM (16:2:1:1, 10 mL) and stirred at room temperature for 2 h. The solvent was removed in vacuo and the resulting residue partitioned between water (20 mL) and Et_2O (20 mL). The aqueous layer was washed with Et_2O (3 × 20 mL) and lyophilized to yield a white solid (158 mg, 0.081 mmol, 62% assuming .6 TFA salt).
Approximately 20% of the crude material was purified by semi-preparative RP-HPLC (gradient 3, $\lambda = 220$ nm): $t_R = 18.40$ min to give **3** as a white solid (15 mg, 7.7 nmol). RP-HPLC (analytical, gradient 2, $\lambda = 220$ nm): $t_R = 8.95$ min. MS (ES+) *m/z*: 315.4 (100%) $[M + 4H]^{4+}$, 420.2 (59%) $[M + 3H]^{3+}$, 629.9 (25%) $[M + 2H]^{2+}$, 1258.8 (5%) $[M + H]^+$. HRMS (ES+) $C_{57}H_{101}N_{19}O_{11}S$ (calcd) 629.8819; (found) 629.8818 $[M + 2H]^{2+}$. Amino acid

analysis (mole ratios): ALA 0.99, Met 0.98, Leu 1.04, Lys 2.98, Arg 2.01, Trp not determined.
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18. *Porphyrin pharmacokinetics*: PAM 212 Cells were seeded into gamma-sterilised 96-well plates at a density of 5×10^4 cells per well. After 48 h, the culture medium was removed, and the wells were washed with PBS. The cells were incubated with freshly prepared solutions of **3** at 0.005, 0.01, 0.05, 0.01, and 0.5 mM. Serum-free medium (100 μL) 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. Fluorescence readings were taken at 4, 5, 7, 24, and 48 h after addition of **3**, and the plates were incubated at 37 °C between measurements. The fluorescence signal from each well was measured using a Perkin-Elmer LS-50B spectrofluorimeter, with excitation at 405 nm and 635 nm emission, with slit widths set to 10 nm and an internal 530 longpass filter used on the emission side. The mean fluorescence was calculated after subtraction of the control values (i.e., without addition of ALA). For temperature studies, PAM212 were seeded in two 96-well plates, as before, and treated with **3** (0.3mM in clear media, 100 μL/well). The plates were wrapped in foil and incubated at 37 and 4 °C, respectively, for 12 h, whereupon the prodrug solution was removed. The cells were rinsed in PBS and clear media (100 μL) were added to each well. Generation of PpIX fluorescence was then monitored as before.
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