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Synthesis and evaluation of new 5-aminolevulinic acid derivatives as prodrugs of protoporphyrin for photodynamic therapy†

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Protoporphyrin IX (PpIX) is used as a photosensitizer in the photodynamic diagnosis (PDD) and photodynamic therapy (PDT) of cancer and is synthesized intracellularly from 5-aminolevulinic acid (5-ALA) precursors. Thirteen novel 5-ALA derivatives were designed and synthesized appropriately with tailored hydrophilicity and lipophilicity. The generation of PpIX was detected and their antitumor activity *in vitro* and *in vivo* was also investigated. It was shown that compounds **9b–c**, **11b–c** and **13a** displayed a characteristic long wavelength absorption peak at 593 nm after 5 h incubation in mice fibrosarcoma S180 cells. After being exposed to 600 nm laser light irradiation, these compounds can inhibit cell proliferation in S180 cells *in vitro*. The growth of S180 cell tumors in Kunming mice was significantly inhibited by these compounds *in vivo*. Among these compounds, **13a** has low dark toxicity and high phototoxicity, which makes it an effective and promising prodrug for PDT.

Introduction

The photodynamic therapy (PDT) of cancer is based on the administration of a photosensitizer with tumor-localizing properties, and subsequent irradiation with light of an appropriate wavelength leading to selective damage to the treated tissue.[1–3] 5-Aminolevulinic acid (5-ALA) is a natural amino acid biosynthesized not only in animals, but also in plant mitochondria. Together with light or laser irradiation, it is used as a photosensitizer precursor as part of fluorescence diagnosis (FD) and photodynamic therapy (PDT) to identify/kill tumor cells, resulting in a new strategy for cancer diagnosis and therapy.[4,5] In mammalian cells, 5-ALA is converted to protoporphyrin IX (PpIX), which is the precursor of hemin and a potent photosensitizer (Fig. 1).[6,7] Meanwhile, in most clinical and preclinical studies, it has been proved that systemic or topical application of 5-ALA is capable to temporarily increase the concentration of PpIX in the target tissues.[8]

A significant drawback of 5-ALA-PDT is the fact that 5-ALA is a zwitterion at physiological pH resulting in low lipid solubility and limiting passage through biological barriers such as cellular membranes.[9,10] To overcome this obstacle, a lot of compounds have been synthesized to improve the uptake of 5-ALA and its selectivity.[11,12] One approach has been adopted, involving the use of more lipophilic 5-ALA derivatives, such as alkyl or ethyleneglycol esters, which are potential substrates for cellular esterases.[13–15] As prodrugs 5-aminolevulinic acid ester derivatives have been widely studied. Methylamino levulinate (MAL) was approved for the treatment of actinic keratosis (AK) and basal cell carcinoma (BCC) in Europe and Australia and hexylaminolevulinate (HAL) has been used to improve the detection of superficial bladder cancer.[16] The use of alkyl esters of 5-ALA results in a nonspecific distribution of 5-ALA in all cell types, but with an increased PpIX production in tumour cells.[17]

An attractive way to obtain 5-ALA prodrugs that have both improved physicochemical properties and can selectively

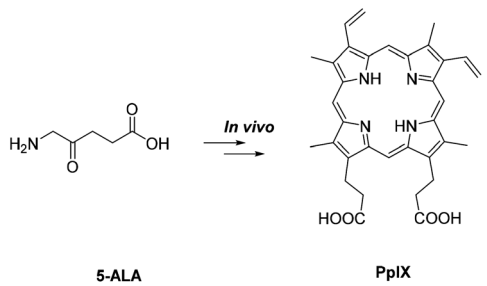

Fig. 1 General principle of 5-aminolevulinic acid metabolism.

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release ALA in specific cell lines is to incorporate 5-ALA into a short peptide derivative.[18,19] Following this approach, upon cellular uptake, 5-ALA release is mediated by the action of cytoplasmic esterases and/or proteases, and it may be possible to design 5-ALA prodrugs that target the disease-dependent levels of a given enzymatic activity. A previous investigation showed that the general structure Ac-Xaa-ALA-OR (Xaa is L-Phe, L-Leu) was the most effective structure to deliver 5-ALA to cells among a series of derivatives, with an enhancement of PpIX and PDT effectiveness.[20–22]

In this context, the syntheses of thirteen new 5-ALA derivatives are described. Their chemical characterization, photophysical properties, and photodynamic activities *in vitro* and *in vivo* are reported.

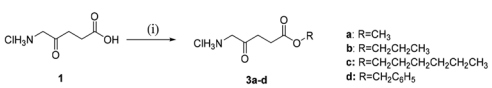
Results and discussion

Synthesis and characterization

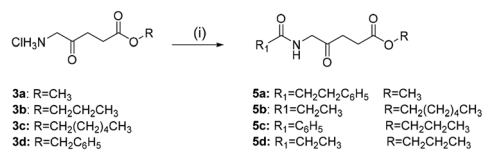
Synthesis of the ALA esters. The desired 5-ALA esters were obtained according to the method previously described.[23] The esters (**3a–d**) derived from 5-aminolevulinic acid (**1**), and methanol, 1-propanol, 1-hexanol, and benzyl alcohol (**2a–d**) were obtained in excellent yields (Scheme 1). In the process of preparation, the major problem was the removal of the corresponding alcohol used in excess, especially high boiling point and water-insoluble alcohol.[24] The characterization of compounds was entirely in accordance with the previous literature.[25]

Synthesis of the amide derivatives of N-terminal modified 5-ALA. The condensation of 5-aminolevulinate esters (**3a–d**), and phenylpropionic acid, propionic acid, and benzoic acid (**4a–c**) to obtain the analogues (**5a–d**) was straightforward (Scheme 2).[26] At room temperature, **5a** was hydrolyzed with LiOH solution to give **6a** (Scheme 3). Compounds **5a–d** and **6a** were both purified using column chromatography and characterized (Fig. S1–S5†). Previous literature studies[27,28] reported that the 5-ALA derivatives formed from three molecular 5-ALA esterification and 5-ALA-dendrimers were both capable of inducing the production of high levels of PpIX. Following the same synthetic method, the two carboxyls of succinic acid were linked with two molecular benzyl 5-ALA to obtain **7a** (Fig. S6†) (Scheme 4).

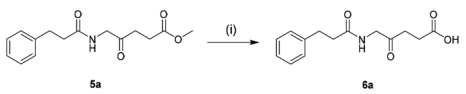
According to the previous literature,[29] Cbz-protected amino acids and Boc-protected amino acids were used for the condensation reaction to prepare **9a–c** (Fig. S7–S9†) (Scheme 5). It was found that the deprotection of the Cbz-group was more difficult than that of the Boc-group. So Boc-protected amino acids (**11a–c**) were synthesized (Scheme 6) (Fig. S10–S12†), and then **11a** was selected to give the mono-deprotected peptide **12a** as trifluoroacetate in good yield. Without purification, the acetylation of **12a** directly provides product **13a** as white powder (Fig. S13†) (Scheme 7).



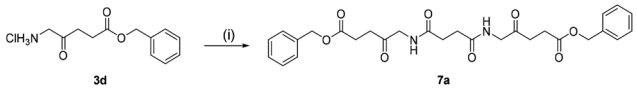
Scheme 1 Reagents and reaction conditions: (i) ROH, $SOCl_2$, 30 °C–70 °C, 2 h–5 h, 75%–96%.



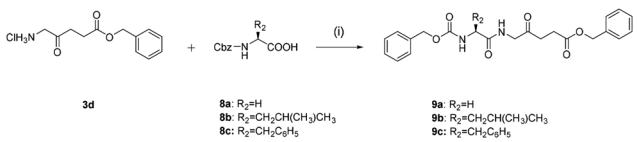
Scheme 2 Reagents and reaction conditions: (i) Acids (**4a–c**), HOBt, EDCI, DIPEA, rt, 5 h.



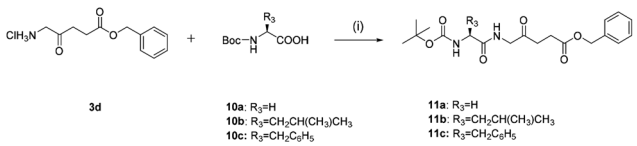
Scheme 3 Reagents and reaction conditions: (i) LiOH, THF, rt, 1 h.



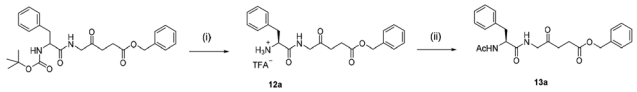
Scheme 4 Reagents and reaction conditions: (i) Succinic acid, HOBt, EDCI, DIPEA, rt, 6 h.



Scheme 5 Reagents and reaction conditions: (i) HOBt, EDCI, DIPEA, rt, 5 h.



Scheme 6 Reagents and reaction conditions: (i) HOBt, EDCI, DIPEA, rt, 6 h.

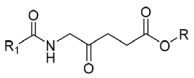


Scheme 7 Reagents and reaction conditions: (i) TFA, rt, 1 h; (ii) acetic anhydride, TEA, 20 h.

UV-visible absorption and fluorescence spectra

5-ALA and its derivatives are frequently used as photosensitizers for PDT, although they are not sensitizers themselves.[30] However, after they are transformed and metabolized into PpIX *in vivo*, the UV-visible absorption and fluorescence spectra can be obtained.[31] The production of PpIX after incubation with ALA and its derivatives was evaluated in mice fibrosarcoma S180 cells, and the results of these experiments are summarized in Table 1. After 5 h of incubation, five compounds (**9b**, **9c**, **11b**, **11c**, and **13a**) displayed photosensitivity characteristics compared to equimolar ALA, which had the characteristic Soret and Q-band absorption at 405 nm (Soret) and 593 nm (Q-band), respectively (Fig. 2a). Meanwhile, when excited at 405 nm, these compounds showed the strongest emission peaks at 635 nm similar to equimolar ALA (Fig. 2b). However, UV-visible absorption and fluorescence were not detected in a parallel series when S180 cells were exposed to the dipeptides **5a**, **5b**, **5c**, **5d**, **6a**, **7a**, **9a**, and **11a** (data not shown). So it can be speculated that in molecules **5a**, **5b**, **5c**, **5d**, **6a**, **7a**, **9a**, and **11a**, the 5-aminolevulinic ester groups are much easier to be metabolized and decomposed by enzymes *in vivo* before 5-aminolevulinic acid was released to form PpIX compared with molecules **9b**, **9c**, **11b**, **11c**, and **13a**.

Table 1 Detectability of PpIX from N-modified 5-ALA derivatives



Code	R_1	R	Detectability of PpIX[a]
5a	$C_6H_5CH_2CH_2$–	$-CH_3$	–
5b	CH_3CH_2–	$-CH_2(CH_2)_4CH_3$	–
5c	C_6H_5–	$-CH_2CH_2CH_3$	–
5d	CH_3CH_2–	$-CH_2CH_2CH_3$	–
6a	$C_6H_5CH_2CH_2$–	–H	–
7a	Bn-ALA-succinyl–	$-CH_2C_6H_5$	–
9a	C_6H_5-CH_2-OCONH-Gly–	$-CH_2C_6H_5$	–
9b	C_6H_5-CH_2-OCONH-Leu–	$-CH_2C_6H_5$	+
9c	C_6H_5-CH_2-OCONH-Phe–	$-CH_2C_6H_5$	+
11a	$(CH_3)_3$C-OCONH-Gly–	$-CH_2C_6H_5$	–
11b	$(CH_3)_3$C-OCONH-Leu–	$-CH_2C_6H_5$	+
11c	$(CH_3)_3$C-OCONH-Phe–	$-CH_2C_6H_5$	+
13a	CH_3CO-Phe–	$-CH_2C_6H_5$	+

[a] "+" means PpIX is detectable. "–" means PpIX is undetectable.

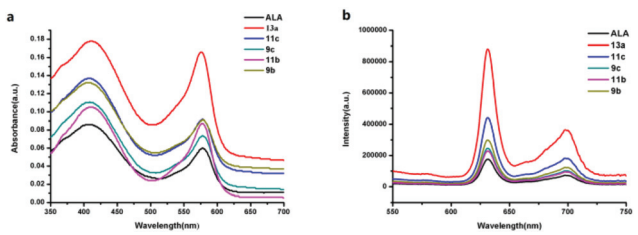


Fig. 2 (a) UV-vis absorption spectrum registered after exposure of S180 cells to 2 mM ALA and its derivatives. The results obtained after 5 h of incubation are shown. (b) Fluorescence spectrum obtained after incubation of S180 cells to 2 mM ALA and its derivatives for 5 h, excitation wavelength is 405 nm.

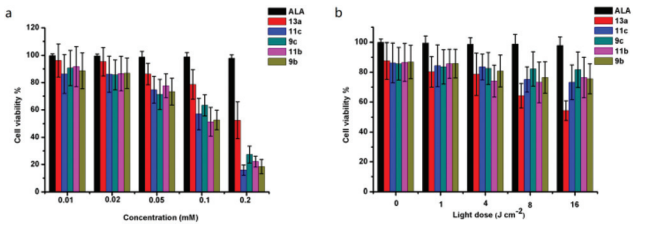


Fig. 3 Dark toxicity and phototoxicity of ALA and its derivatives. (a) Dark toxicity of different concentrations assayed. (b) Light dose dependence effect on the cell viability after incubation with 0.02 mM of ALA or its derivatives for 19 h. Cell survival is expressed as a percentage of the control nonirradiated and exposed to ALA or its derivatives. Data correspond to mean values ± SD from three different experiments.

Cytotoxicity on mice fibrosarcoma S180 cells

The effect of ALA and its derivatives on the viability of cultured S180 cells was evaluated by using the MTT assay. As shown in Fig. 3a, the dark cytotoxicities of **13a**, **11c**, **9c**, **11b**, and **9b** were visibly higher than that of ALA. When the concentration was low (0.01 mM and 0.02 mM), the cell viabilities of **13a**, **11c**, **9c**, **11b**, and **9b** were all more than 80%. However, with the concentration of prodrugs increased to 0.05 mM, 0.1 mM and 0.2 mM, the cell viability decreased gradually to about 20% (Fig. 3a). Among these five compounds, **13a** showed the lowest dark-toxicity. After irradiation with laser light, a significant cytotoxicity was detected. At low concentration (0.02 mM), the cell viability of **13a** was lowest, upon varying the light dose (Fig. 3b). With the increase of light dose, the cell viability of **13a** significantly decreased, but ALA, **11b**, **11c**, **9c** and **9b** had no obvious change.

In vivo photodynamic antitumor potency

The PDT antitumor efficacy of 5-ALA derivatives (**9b–9c**, **11b–c**, **13a**) was evaluated in S180 tumor bearing Kunming mice. By comparing the tumor weight in different groups, the inhibition rates of tumor growth could be calculated. When tumor sizes had reached 5–7 mm in diameter, the mice were given intravenous injection *via* the tail vein at a dose of 15 mg kg^{-1}. After 3 h incubation, the tumor site was irradiated with laser light (600 nm, 180 J cm^{-2}, 250 mW cm^{-2}). After five days of administration, treatment effects became significant. Although compounds **11b**, **11c**, **9b**, and **9c** can induce the production of PpIX *in vivo*, their dark toxicity resulted in tumor fester and mice death (Fig. S14–17†). Among these compounds, **13a** had the most medicinal properties with low dark toxicity and high phototoxicity. The volume of tumors in three sets of parallel control groups was larger than that in the **13a**-PDT group. The volume growth curves of tumors are provided in Fig. 4a, the tumor volume increased about 10-fold for 14 days in the control group. Tumor images are shown in Fig. 4b, and the tumors began to swell after 1 day post PDT. The tumors

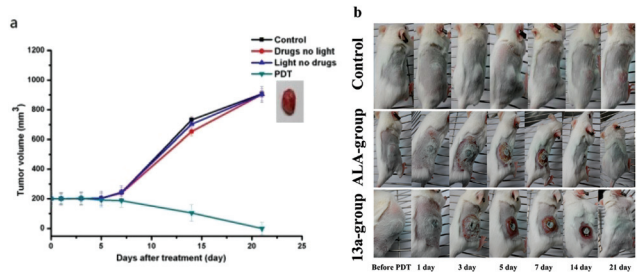


Fig. 4 (a) Tumor volume at different times after **13a**-PDT. The tumors in control groups continued to grow and were significantly larger than in the PDT-treated group after 14 days post-treatment. (b) Images of mice bearing S180 tumors. Before and following PDT for **13a** and ALA at different times and mice with saline were set as the control.

became dark, necrotic and a scar formed at 3 days post PDT. Then the scar of tumors fell off and normal healthy skin was reconstructed 14 days post PDT.

Experimental

Materials and instrumentation

All solvents and reagents were obtained from commercial suppliers and used without further purification unless otherwise stated. Melting points were determined on a Reichert-Jung Thermo Galen Kofler block and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrometer. Chemical shifts were reported as δ ppm values relative to the internal standard tetramethylsilane. ESI-MS spectra were recorded on a Micromass triple quadrupole mass spectrometer. HRMS spectra were recorded on a Bruker Daltonics APEXIII 7.0 tesla FT mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany), which allowed the determination of the molecular formula of the $[M + H]^+$, the $[M + Na]^+$ or the $[M + K]^+$ peak. The solvent was removed by rotary evaporation under reduced pressure, and silica gel chromatography was performed using silica gel H (300–400 mesh). The UV-vis absorption spectrum was recorded on an ultraviolet visible spectrophotometer (Model V-530, Japan). Fluorescence spectra were recorded on a fluorescence spectrophotometer (FluoroMax-4, France). The synthesis of compounds (**3a–d**) was performed according to previous reports.

General procedures for the preparation of the peptide analogues 5a–d *via* coupling of 5-aminolevulinic acid esters 3a–d with acids 4a–c

A total of acids (**4a–c**) (6.1 mmol) in dry DCM (10 mL) were activated over 1 h by the addition of HOBt (0.82 g, 6.1 mmol) and EDCI (1.16 g, 6.1 mmol) at room temperature under N_2. A solution of 5-ALA esters (**3a–d**) (5.5 mmol) in DCM (5 mL) was added, and then DIPEA (2.87 mL, 16.5 mmol) was added. After 5 h stirring at room temperature, the solvent was evaporated under reduced pressure. The crude material was extracted with 3 × 100 mL of ethyl acetate and washed sequentially with 75 mL of 1 M citric acid, 10% $NaHCO_3$, and NaCl saturated water. The organic layer was dried over $MgSO_4$, and the solvent was removed under reduced pressure. The residue was purified by flash chromatography (CH_2Cl_2 : MeOH = 50 : 1) to afford the pure product as a white solid (**5a–d**).

Methyl 4-oxo-5-(3-phenylpropanamido)pentanoate (5a). 66.2% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.21 (t, J = 5.7 Hz, 1H), 7.30–7.15 (m, 5H), 3.93 (d, J = 5.7 Hz, 2H), 3.58 (s, 3H), 2.82 (dd, J = 8.8, 6.8 Hz, 2H), 2.64 (t, J = 6.5 Hz, 2H), 2.47 (td, J = 6.9, 4.2 Hz, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 206.06, 173.09, 172.21, 141.70, 128.72, 128.67, 126.33, 51.84, 48.81, 37.08, 34.28, 31.44, 27.61. HRMS (MALDI-TOF) calcd for $C_{15}H_{20}O_4N$ $[M + H]^+$ 278.1387, found 278.1388.

Hexyl 4-oxo-5-propionamidopentanoate (5b). 65.3% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 6.24 (s, 1H), 4.24 (t, J = 3.1 Hz, 2H), 4.08 (td, J = 6.8, 2.0 Hz, 2H), 2.80–2.73 (m, 2H), 2.69 (d, J = 6.4 Hz, 2H), 2.35–2.24 (m, 2H), 1.62 (q, J = 6.8 Hz, 2H), 1.33 (d, J = 9.7 Hz, 6H), 1.19 (td, J = 7.7, 2.1 Hz, 3H), 0.94–0.81 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 204.11, 173.83, 172.46, 65.09, 49.24, 34.62, 31.40, 29.36, 28.51, 27.84, 25.53, 22.53, 14.00, 9.69. HRMS (MALDI-TOF) calcd for $C_{14}H_{26}O_4N$ $[M + H]^+$ 272.1856, found 272.1857.

Propyl 5-benzamido-4-oxopentanoate (5c). 45.7% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 7.87–7.81 (m, 2H), 7.57–7.51 (m, 1H), 7.47 (dd, J = 8.2, 6.7 Hz, 2H), 6.95 (s, 1H), 4.45 (d, J = 4.5 Hz, 2H), 4.07 (t, J = 6.7 Hz, 2H), 2.84 (dd, J = 7.4, 5.1 Hz, 2H), 2.74 (dd, J = 7.2, 5.0 Hz, 2H), 1.71–1.64 (m, 2H), 0.96 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, CDCl3) δ ppm: 204.08, 172.48, 167.28, 133.72, 131.78, 128.61, 127.08, 66.53, 49.70, 34.71, 27.88, 21.93, 10.36. HRMS (MALDI-TOF) calcd for $C_{15}H_{20}O_4N$ $[M + H]^+$ 278.1387, found 278.1387.

Propyl 4-oxo-5-propionamidopentanoate (5d). 67.5% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 6.22 (s, 1H), 4.25 (d, J = 4.6 Hz, 2H), 4.06 (t, J = 6.7 Hz, 2H), 2.77 (dd, J = 7.4, 5.0 Hz, 2H), 2.70 (dd, J = 7.2, 4.8 Hz, 2H), 2.30 (q, J = 7.6 Hz, 2H), 1.66 (s, 2H), 1.20 (t, J = 7.6 Hz, 3H), 0.96 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, CDCl3) δ ppm: 204.06, 173.77, 172.45, 66.51, 49.26, 34.65, 29.39, 27.83, 21.93, 10.36, 9.71. HRMS (MALDI-TOF) calcd for $C_{11}H_{20}O_4N$ $[M + H]^+$ 230.1387, found 230.1388.

4-Oxo-5-(3-phenylpropanamido)pentanoic acid (6a). To a solution of **5a** (100 mg, 0.36 mmol) in THF (100 ml) was added 1 M LiOH (1.8 ml, 1.8 mmol) at room temperature under N_2, and the solution was stirred at room temperature for 2 h. After evaporation of the solvent, the residue was dissolved in water. Then the pH of the mixture was adjusted to 3 with 1 M HCl, and the product was precipitated. Product **6a** was obtained by filtration (73.3 mg, 77.2%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 7.29 (d, J = 7.7 Hz, 2H), 7.20 (dd, J = 7.6, 5.0 Hz, 3H), 6.31 (s, 1H), 4.18 (d, J = 4.7 Hz, 2H), 2.96 (t, J = 7.9 Hz, 2H), 2.69 (s, 4H), 2.55 (dd, J = 8.9, 6.7 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 204.02, 176.11, 172.60, 140.56, 128.56, 128.31, 126.31, 77.24, 49.23, 37.95, 34.41, 31.48, 27.55. HRMS (MALDI-TOF) calcd for $C_{14}H_{18}O_4N$ $[M + H]^+$ 264.1231, found 264.1230.

Dibenzyl 5,5′-(succinylbis(azanediyl))bis(4-oxopentanoate) (7a). To a solution of succinic acid (0.72 g, 6.1 mmol) in dry DCM (10 ml) were added HOBt (1.64 g, 12.2 mmol) and EDCI (2.32 g, 6.1 mmol) and the solution was stirred at room temperature for 1 h under N_2. A solution of benzyl 5-aminolevulinic acid hydrochloride (**3d**) (3.2 g, 12.5 mmol) in DCM (6 mL) was added and then DIPEA (2.87 mL, 16.5 mmol) was added. After stirring for 6 h, the solvent was evaporated under reduced pressure. The crude material was dissolved with ethyl acetate and washed sequentially with 1 M citric acid (3 × 75 mL), 10% $NaHCO_3$, and NaCl saturated water. The organic layer was dried over $MgSO_4$, and the solvent was evaporated under reduced pressure. Purification by flash chromatography (CH_2Cl_2 : MeOH = 50 : 1) gave the pure product as a white solid (2.0 g, 63.1%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 7.37 (q, J = 6.2, 4.7 Hz, 10H), 6.59 (s, 2H), 5.13 (d, J = 2.2 Hz, 4H), 4.19 (d, J = 3.9 Hz, 4H), 2.74 (q, J = 4.5 Hz, 8H), 2.62 (s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 203.92, 172.25, 172.14, 135.64, 128.62, 128.36, 128.23, 66.68, 49.30, 34.50, 31.29, 27.82. HRMS (MALDI-TOF) calcd for $C_{28}H_{33}O_8N_2$ $[M + H]^+$ 525.2231, found 525.2231.

General procedures for the preparation of the peptide analogues 9a–c *via* coupling of 5-aminolevulinic acid esters with Cbz-protected amino acids 8a–c

A total of Cbz-amino acids (2.0 mmol) dissolved in dry DCM (10 mL) was activated over 1 h by the addition of HOBt (0.28 g, 2.1 mmol) and EDCI (0.39 g, 2.1 mmol) at room temperature under N_2. A solution of benzyl 5-aminolevulinic acid hydrochloride (**3d**) (0.54 g, 2.1 mmol) in DCM (6 mL) was added and then DIPEA (1.1 mL, 6.3 mmol) was added. After 5 h stirring at room temperature, the solvent was removed under reduced pressure. The crude material was extracted with 3 × 100 mL of ethyl acetate and washed sequentially with 75 mL of 1 M citric acid, 10% $NaHCO_3$, and NaCl saturated water. The organic layer was dried over $MgSO_4$, and the solvent was removed under reduced pressure. Purification by flash chromatography (CH_2Cl_2 : MeOH = 50 : 1) gave the product as a white solid (**9a–c**).

Benzyl 5-(2-(((benzyloxy)carbonyl)amino)acetamido)-4-oxopentanoate (9a). 75.4% yield, ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 7.37 (d, J = 7.2 Hz, 10H), 6.70 (s, 1H), 5.45 (s, 1H), 5.14 (d, J = 12.6 Hz, 4H), 4.22 (d, J = 4.3 Hz, 2H), 3.94 (d, J = 5.5 Hz, 2H), 2.75 (s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 203.42, 172.12, 169.03, 156.55, 136.15, 128.59, 128.54, 128.33, 128.22, 128.20, 128.09, 77.21, 67.26, 66.70, 49.04, 44.43, 34.52, 27.88. HRMS (MALDI-TOF) calcd for $C_{22}H_{25}O_6N_2$ $[M + H]^+$ 413.1708, found 413.1707.

Benzyl 5-(2-(((benzyloxy)carbonyl)amino)-4-methylpentanamido)-4-oxopentanoate (9b). 77.8% yield, ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 7.42–7.33 (m, 10H), 6.72 (s, 1H), 5.23–5.17 (m, 1H), 5.13 (d, J = 2.2 Hz, 4H), 4.27 (s, 1H), 4.23–4.14 (m, 2H), 2.75 (s, 4H), 1.68–1.66 (m, 2H), 1.55 (d, J = 9.0 Hz, 1H), 0.96 (d, J = 5.4 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 203.13, 172.16, 170.95, 136.21, 129.25, 128.75, 128.63, 128.56, 128.39, 128.26, 128.22, 128.06, 127.12, 77.26, 67.13, 66.72, 56.11, 49.09, 38.47, 34.44, 27.82. HRMS (MALDI-TOF) calcd for $C_{26}H_{33}O_6N_2$ $[M + H]^+$ 469.2334, found 469.2333.

Benzyl 5-(2-(((benzyloxy)carbonyl)amino)-3-phenylpropanamido)-4-oxopentanoate (9c). 85.3% yield, ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 7.36 (tt, J = 12.7, 6.3 Hz, 10H), 7.29–7.12 (m, 5H), 6.51 (s, 1H), 5.30 (s, 1H), 5.12 (d, J = 8.6 Hz, 4H), 4.50 (d, J = 8.0 Hz, 1H), 4.25–4.03 (m, 2H), 3.11 (d, J = 6.7 Hz, 2H), 2.71 (s, 4H), 1.65 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 203.13, 172.16, 170.95, 136.21, 129.25, 128.75, 128.63, 128.56, 128.39, 128.26, 128.22, 128.06, 127.12, 77.26, 67.13, 66.72, 56.11, 49.09, 38.47, 34.44, 27.82. HRMS (MALDI-TOF) calcd for $C_{29}H_{31}O_6N_2$ $[M + H]^+$ 503.2176, found 503.2177.

General procedures for the preparation of the peptide analogues 11a–c *via* coupling of 5-aminolevulinic acid esters with Boc-protected amino acids 10a–c

A total of Boc-amino acids (2.0 mmol) dissolved in dry DCM (10 mL) was activated over 1 h by the addition of HOBt (0.28 g, 2.1 mmol) and EDCI (0.39 g, 2.1 mmol) at room temperature under N_2. A solution of benzyl aminolevulinic acid hydrochloride (**3d**) (0.54 g, 2.1 mmol) in DCM (6 mL) was added and then DIPEA (1.1 mL, 6.3 mmol) was added. After 6 h stirring at room temperature, the solvent was removed under reduced pressure. The crude material was dissolved with ethyl acetate and washed sequentially with 1 M citric acid (3 × 75 mL) and 10% $NaHCO_3$ (3 × 50 mL). The organic layer was dried over $MgSO_4$, and the solvent was evaporated *in vacuo*. Purification by flash chromatography (CH_2Cl_2 : methanol = 100 : 1) gave the product as a white solid (**11a–c**).

Benzyl 5-(2-((*tert*-butoxycarbonyl)amino)acetamido)-4-oxopentanoate (11a). 80.3% yield, ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 7.43–7.31 (m, 5H), 6.77 (s, 1H), 5.18 (s, 1H), 5.13 (s, 2H), 4.22 (d, J = 4.6 Hz, 2H), 3.86 (d, J = 5.6 Hz, 2H), 2.84–2.66 (m, 4H), 1.48 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 203.39, 172.10, 169.52, 155.91, 135.64, 128.58, 128.32, 128.19, 80.39, 77.21, 66.68, 49.02, 44.22, 34.53, 28.29, 27.87. HRMS (MALDI-TOF) calcd for $C_{19}H_{27}O_6N_2$ $[M + H]^+$ 379.1864, found 379.1864.

Benzyl 5-(2-((*tert*-butoxycarbonyl)amino)-4-methylpentanamido)-4-oxopentanoate (11b). 67.8% yield, ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 7.37 (q, J = 7.1, 6.5 Hz, 5H), 6.82 (s, 1H), 5.13 (s, 2H), 5.05–4.80 (m, 1H), 4.20 (d, J = 4.2 Hz, 3H), 2.76 (dt, J = 12.2, 5.3 Hz, 4H), 1.78–1.61 (m, 2H), 1.47 (s, 9H), 0.97 (dd, J = 5.9, 2.8 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm: 203.79, 173.05, 172.25, 155.70, 135.66, 128.58, 128.31, 128.19, 79.95, 66.61, 52.97, 49.05, 41.44, 34.45, 28.31, 27.77, 24.72, 23.03, 21.84. HRMS (MALDI-TOF) calcd for $C_{23}H_{35}O_6N_2$ $[M + H]^+$ 435.2490, found 435.2490.

Benzyl 5-(2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanamido)-4-oxopentanoate (11c). 85.7% yield, ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 8.31 (d, J = 5.7 Hz, 1H), 7.46–7.14 (m, 10H), 7.04 (d, J = 8.6 Hz, 1H), 5.08 (s, 2H), 4.20 (s, 1H), 3.97 (dd, J = 12.9, 5.4 Hz, 2H), 3.04–2.95 (m, 1H), 2.76 (d, J = 10.8 Hz, 1H), 2.72 (d, J = 7.9 Hz, 2H), 2.56 (d, J = 6.7 Hz, 2H),

1.29 (s, 9H). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm: 206.03, 172.63, 172.54, 155.75, 138.72, 129.65, 128.89, 128.48, 128.44, 128.32, 126.63, 78.47, 65.97, 56.16, 48.93, 37.86, 34.30, 28.59, 27.79. HRMS (MALDI-TOF) calcd for $C_{26}H_{33}O_6N_2$ $[M + H]^+$ 469.2335, found 469.2333.

Benzyl 5-(2-acetamido-3-phenylpropanamido)-4-oxopentanoate (13a). The protected amino acid-ALA derivative (**11c**) (234 mg, 0.5 mmol) was dissolved in a mixture of TFA (1 mL, 13 mmol) and DCM (3 mL) under N_2. After the solution was stirred for 1 h at room temperature, TFA was evaporated under high vacuum. The products (**12a**) were obtained in good purity without further purification. A suspension of the crude trifluoroacetic acid salt thus prepared in dry DCM (10 mL) was cooled to 0 °C under an argon atmosphere and DIPEA (0.18 mL, 1.0 mmol) was added, followed by acetic anhydride (0.1 mL, 0.98 mmol). The mixture was allowed to attain room temperature with stirring overnight and then the solvent was evaporated. The crude product was purified by flash chromatography (CH_2Cl_2 : MeOH = 50 : 1). Recrystallization from ethanol gave a white solid (93.1 mg, 45.4%). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 7.37 (dq, J = 7.0, 4.0, 2.9 Hz, 7H), 7.31 (d, J = 1.6 Hz, 2H), 7.27–7.19 (m, 4H), 6.54 (s, 1H), 6.17 (d, J = 7.8 Hz, 1H), 5.13 (s, 2H), 4.74 (q, J = 7.2 Hz, 1H), 4.21–3.98 (m, 2H), 3.09 (d, J = 6.9 Hz, 2H), 2.70 (s, 4H), 2.20 (s, 2H), 2.00 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm: 203.24, 172.17, 171.20, 170.20, 136.41, 129.21, 128.63, 128.59, 128.33, 128.19, 127.01, 66.67, 54.30, 49.05, 38.33, 34.39, 27.80, 23.06. HRMS (MALDI-TOF) calcd for $C_{23}H_{27}O_5N_2$ $[M + H]^+$ 411.1915, found 411.1914.

Photophysical and photochemical measurements

Absorption and emission spectra. The UV-visible absorption spectrum was recorded on an ultraviolet visible spectrophotometer (Model V-530, Japan). Fluorescence spectra were recorded on a fluorescence spectrophotometer (FluoroMax-4, France). Slits were kept narrow to 1 nm in excitation and 2 nm in emission. All the measurements were carried out at room temperature in quartz cuvettes with a path length of 1 cm. All compounds were dissolved in DMSO, and then wrapped in castor oil to obtain 2 mM of solution after evaporation. After 5 h incubation in the culture, the sample was obtained through the added PBS buffer solution.

In vitro experiments

Cell line and culture conditions. Mice fibrosarcoma S180 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences. All cell culture related reagents were purchased from Shanghai Ming Rong Bio-Science Technology Co., Ltd. Cells were cultured in normal RPMI-1640 culture medium. All media were supplemented with 10% fetal bovine serum (FBS), 100 units per mL penicillin G and 100 μg mL^{-1} streptomycin. All cells were incubated at 37 °C in 5% CO_2 in a humidified incubator.

MTT cell viability assay

S180 cells were cultured in RPMI-1640 medium with 10% (v/v) FBS, collected by centrifugation for 5 min. The cells were harvested and seeded in 96-well plates at 2×10^4 cells per well. The cells were allowed to attach to the bottom of the wells for 24 hours before starting the experiment in 5% CO_2 at 37 °C. The medium containing ALA and its derivatives (**13a**, **11c**, **11b**, **9c**, and **9b**) at different concentrations (0.01, 0.02, 0.05, 0.1, and 0.2 mM) was administered to cells and uptake was allowed for 24 hours to test the dark toxicity. RPMI-1640 medium containing drugs was removed and the cells were washed with fresh PBS before irradiation with different light doses (range from 1 to 16 J cm^{-2}) using an Nd:YAG laser at 600 nm when the phototoxicity was tested at 0.02 mM. The cell viability was evaluated by using the MTT colorimetric assay 24 hours after treatment.

In vivo experiments

***In vivo* PDT efficacy.** Five-week-old Kunming male mice were purchased from Shanghai Slack Laboratory Animal Co., Ltd. The mouse tumor models were set up by the subcutaneous injection of 5×10^6 S180 cells in the flank. When growing to an approximate diameter of 10 mm, tumors were excised and small pieces of the tumor (approximately 1 mm square pieces) were then implanted subcutaneously into the right dorsal area of Kunming male mice. When tumor sizes had reached 5–7 mm in diameter after implantation (14–21 days), 18 mice were randomly divided into three groups: laser radiation group, ALA (15 mg kg^{-1}) + laser radiation group, and **13a** (15 mg kg^{-1}) + laser radiation group followed 5 h later by laser radiation. The mice were restrained in rat holders and exposed to the laser (λ = 600 nm) with a light dose of 180 J cm^{-2}. After PDT, the mice were observed daily for tumor regrowth or tumor cure. Visible tumors were measured using two orthogonal measurements L and W (perpendicular to L), and the volumes were calculated using the formula $V = LW^2/2$ and recorded.

Human & animal welfare

All experiments were performed in triplicate and the data were expressed as mean plus and minus the standard error of the mean. Analysis of variance (ANOVA) and Student's t-test were used to determine the statistically significant difference among different groups when appropriate.

The experiments were carried out in accordance with the guidelines issued by the Ethical Committee of Donghua University.

Conclusions

In summary, thirteen novel ALA derivatives were synthesized and their photodynamic activities were evaluated *in vitro* and *in vivo*. Among these compounds, **5a–5d**, **6a**, **7a**, **9a**, and **11a** are unable to show photosensitivity characteristics, and **13a**, **11c**, **11b**, **9c**, and **9b** are able to metabolize and induce PpIX. After 5 h incubation in S180 cells, compounds **9b–c**, **11b–c** and **13a** display a characteristic long wavelength absorption peak at 593 nm, in accordance with PpIX induced by the control group

5-ALA. Among them, **13a** has the most medicinal properties which possess suitable dark toxicity and high phototoxicity. *In vivo* therapeutic efficacy of PDT using **13a**, after being exposed to 600 nm laser light irradiation, the growth of S180 cell tumors in Kunming mice was significantly inhibited. Therefore, **13a** is a promising antitumor photosensitizer for photodynamic therapy.

Conflicts of interest

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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