Synthesis and Evaluation of Unsaturated Alkyl Esters of 5-Aminolevulinic Acid as Precursors to Protoporphyrin IX

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5-Aminolevulinic acid (ALA), a non-fluorescent drug, has been used as a precursor to the fluorescent photosensitizer protoporphyrin IX (PpIX) in photodynamic therapy (PDT).[1-3] PpIX converts, under irradiation of visible light, triplet oxygen ($^3O_2$) to singlet oxygen ($^1O_2$), which initiates the PDT causing the death of abnormal cells. Hence the efficient penetration of ALA into skin would result in increased accumulation of PpIX and better PDT. Specifically, the limited bioavailability of PpIX is due to hydrophilicity of ALA. To address this limitation of uptake and distribution of ALA, the drug has been converted into its esters to increase its lipophilic nature. ALA esters from saturated aliphatic alcohols of C6-C8 chain were, when applied to skin, found to increase the temporal amount of PpIX in tissue than ALA free acid.[4-6] Methyl, hexyl, octyl, and other esters[7] of ALA were synthesized and investigated as promising precursors to PpIX *in vitro* and *in vivo* as well.

However, there are only a limited number of synthetic methods and procedures for ALA esters available. Simple esters such as methyl and hexyl esters were prepared by heating ALA and thionyl chloride or hydrochloric acid in corresponding alcohols. Higher alkyl esters were prepared through three step reactions which include amino functional group protection, esterification and deprotection. ALA was treated with Boc anhydride and the resulting N-protected ALA was converted to ester by DMAP, EDCI, and alcohol. Acidic hydrolysis of the resulting N-Boc ester gave the ALA ester hydrochloride.[7-9]

As the importance of PDT increases, new efficient synthetic methodology for a variety of esters of ALA is necessary. Although synthesis and increased bioavailability of ALA-esters from saturated hydrocarbon alcohols have been reported, esters of ALA with unsaturated alcohols have never been reported in the literature to the best of our knowledge. Thus our research interest was focused on the study of π-bond effects to bioavailability of the esters and developing a short and convenient, practically, one-pot procedure for ALA esters.

Experimental Section

Representative procedure. To a solution of DMF (3 drops) in thionyl chloride (1 mL) was added ALA·HCl (200 mg, 1.19 mmol) at room temperature (rt). The resulting reaction mixture was stirred for 12 h and evaporated under reduced pressure to give reactive intermediate. To the residue was added 5-hexen-1-ol (0.5 mL) and stirred for 1.5 h at rt and evaporated under reduced pressure and purified silica gel column chromatography to give 265 mg (89%) of 5-hexenyl 5-aminolevulinate hydrochloride as yellow oil. (MeOH : CH_2Cl_2, 1 : 8), 89%.

^{1}H NMR (300 MHz, DMSO-d_6): δ 8.33 (s, 3H), 5.85-5.71 (m, 1H), 5.04-4.92 (m, 2H), 3.99 (t, J = 6.5 Hz, 2H), 3.93 (s, 2H), 2.78 (t, J = 6.6 Hz, 2H), 2.53 (t, J = 6.6 Hz, 2H), 2.02 (q, J = 7 Hz, 2H), 1.56 (quintet, J = 6.7 Hz, 2H), 1.38 (quintet, J = 5.1 Hz, 2H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 202.66, 172.05, 138.41, 115.02, 63.92, 46.52, 34.26, 32.69, 27.56, 27.08, 24.55.

MTT viability assay. A431 cells, an epidermal squamous cell carcinoma cell line, obtained from Korean cell line bank (KCLB No. 80005), were seeded into 96 well plate, and treated with ALA and ALA esters in variable concentrations for 24 hours to check cellular toxicity of ALA unsaturated esters. Colorimetric (MTT) kit for cell survival and proliferation (CHEMICON International Inc., USA) was used following manufacturer's protocol.[10] Briefly, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) solution 100 μL was added to each well in a concentration of 0.5 mg/mL, and plates were incubated at 37 °C for 2 hours. The resulting formazan crystals were dissolved by the addition of 100 μL of DMSO (dimethylsulfoxide) and absorbance was read at 570 nm in ELISA reader (Molecular Device, USA). The results are shown in Figure 1.

Measurement of cellular PpIX synthesis. UV/Vis absorption spectra were taken using Uvikon (model 943) spectrophotometer with a 1.0 nm spectral bandwidth. The steady-state fluorescence emission spectra were obtained on a Varian Cary Eclipse spectrofluorometer with 10 nm slits at room temperature. The absorption spectrum of 2.8×10^{-5} M of PpIX in methanol-water mixture solution (50% v/v) containing 1 M $HClO_4$ revealed very sharp absorption maximum peak at the 408 nm and the molar extinction coefficient of the compound was determined to be 2.7×10^4 $M^{-1}cm^{-1}$ in the solvent.

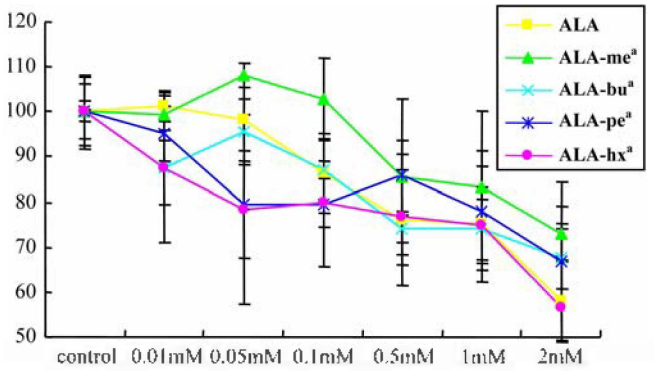


Figure 1. The cell survival of A431 cell and cytotoxicity of ALA and its unsaturated alkyl esters. [a]ALA-me: ALA methyl ester. ALA-bu: 3-butenyl 5-aminolevulinate hydrochloride (entry 6 in Table 1). ALA-pe: 4-pentenyl 5-aminolevulinate hydrochloride (entry 7). ALA-hx: 5-hexenyl 5-aminolevulinate hydrochloride (entry 8). MTT assay was performed in triplicate and cell survival was expressed as percentage.

A431 cells were subcultured in Dulbecco's modified Eagle Medium (Bio-whittaker, Walkersville Inc., MD, USA) containing 10% fetal bovine serum (FBS), 100 units/mL penicillin and 10 ug/mL streptomycin. The cells were grown and incubated in 75 cm^2 flask (Falcon, NJ, USA) at 37 °C in a humidified atmosphere containing 5% CO_2, and subcultured two times per week. Approximately 1×10^6 cells were seeded into 10 cm^2 dishes (Falcon), and cultured for 48 hours. Subsequently, the cells were washed twice with serum-free culture medium, and further incubated in serum-free culture medium containing different concentration of ALA and its esters for 4 hours without exposure to light. The cells were then washed twice with PBS and brought into a solution containing 1 M $HClO_4$ in 50% methanol by scraping with a cell scraper.[4] After 5 min incubation, the cell debris was removed by centrifugation (13,000 rpm/min, 10 min), and the supernatant was collected into new tubes without light exposure. The fluorescence spectra of PpIX of

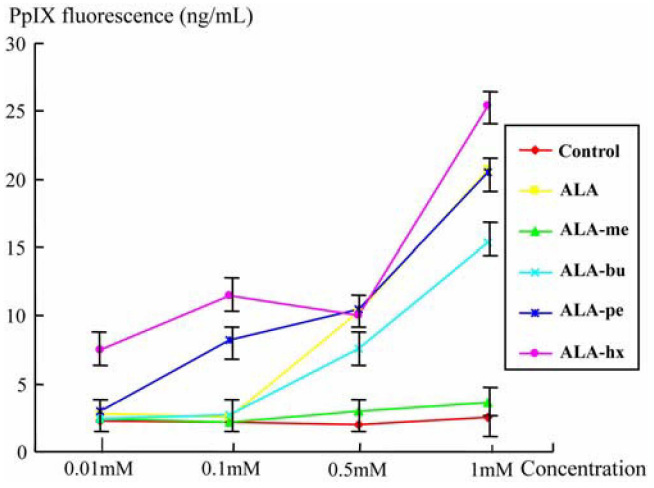
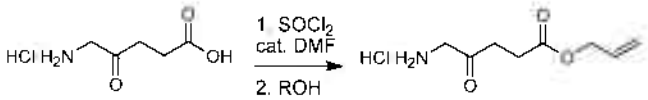


Figure 2. PpIX synthesis in A431 cell from unsaturated alkyl esters of ALA and ALA.

sample at the 408 nm as the excitation wavelength were measured in the range of 573 nm to 800 nm. The quantity of PpIX synthesized in A 431 cell was determined from the area under the emission curve of fluorescence spectra and the results are shown in Figure 2.

Results and Discussion

Vilsmeier-type reactions were widely used for the preparation of reactive intermediate acid chloride from a variety of carboxylic acids for further elaborations.[11] Among many modified procedures, one of the mildest and most efficient way of generating acid chloride is to use excess thionyl chloride with catalytic amount of N,N-dimethylformamide (DMF).[12]



Hence, we decided to apply this method to synthesize ALA esters. To verify the efficiency of this method, saturated long chain alkyl esters, which are reported to literature, were prepared, and the isolated yields were compared with the literature (entries 1-4 of Table 1). It is found that saturated alkyl esters could be prepared in good yields without protecting and deprotecting amino group.

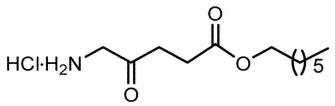
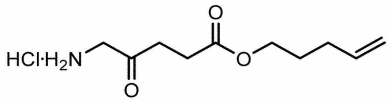
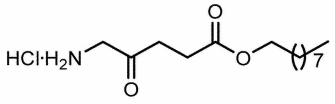
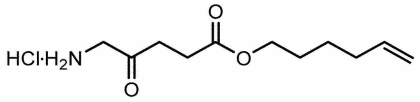
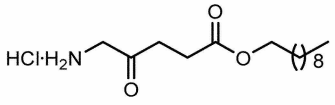
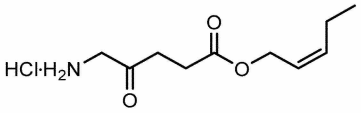
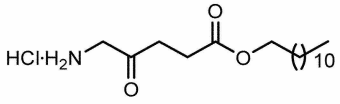
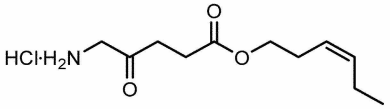
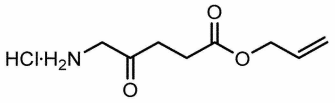
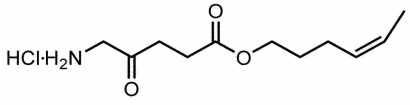
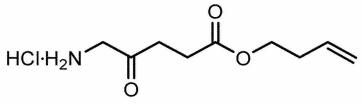
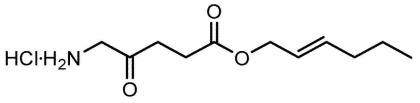
With various unsaturated alcohols such as such as allylic alcohols, homoallylic alcohols and alcohols with *trans* and *cis* double bond, ALA was conveniently converted into corresponding esters in excellent to moderate yields using only thionyl chloride and catalytic DMF in practically one-pot reaction (entries 5-12 in Table 1).

It is noteworthy that our method for ALA esters, which is very simple and popular, is very convenient and effective compared to the literature procedure for long saturated alkyl esters which needed amino group protection and deprotection.

MTT viability assay. Cellular toxicity of ALA unsaturated esters was investigated by measuring cell survival using A431 cells, an epidermal squamous cell carcinoma cell line, obtained from Korean cell line bank (KCLB No. 80005). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide) solution 100 μL was added to each well,[10] and absorbance of the resulting formazan crystals, after dissolving by the addition of 100 μL of DMSO (dimethylsulfoxide), was read at 570 nm in ELISA reader (Molecular Device, USA). The percentage of cell survival was calculated from the absorbance ratio between treated well and control well. MTT assay was performed in triplicate and the results are shown in Figure 1. In dilute concentrations (0.01-0.1 mM) there is a slight decrease in cell survival in ALA unsaturated esters well compared to ALA and ALA-me, however, at higher concentrations (0.5-2 mM) ALA unsaturated esters are not so toxic as ALA and ALA-me.

Measurement of cellular PpIX synthesis. After A431

Table 1. ALA Esters Synthesized

entry	ester	yield (%)	entry	ester	yield (%)
1		84[a]	7		47
2		73[a]	8		89
3		66[a]	9		68
4		68[a]	10		72
5		73	11		90
6		77	12		70

[a]yields reported in Ref. 8 are 83%, 74%, 70%, and not reported , respectively.

cells were subcultured in Dulbecco's modified Eagle Medium, incubated in 75 cm^2 flask (Falcon, NJ, USA) at 37 °C in a humidified atmosphere containing 5% CO_2, and subcultured two times per week, approximately 1×10^6 cells were seeded into 10 cm^2 dishes (Falcon), and cultured for 48 hours and washed twice with serum-free culture medium. The cells were further incubated in serum-free culture medium containing different concentration of ALA and its esters for 4 hours without exposure to light. The fluorescence spectra of PpIX of the cells, after appropriate treatment, at the 408 nm as the excitation wavelength were measured in the range of 573 nm to 800 nm. The quantity of PpIX synthesized in A 431 cell was determined from the area under the emission curve of fluorescence spectra and the results were showed in Figure 2.

ALA-me and butenyl ester (ALA-bu) show less production of PpIX in A 431 cell than ALA even though the lipophilicity is increased after etherification. Gaullier[4] *et al.* reported similar results that ALA methyl ester produced less PpIX than ALA in WiDr, NHIK 3025, and V79 cells.

Hexenyl ester (ALA-hx) increased the PpIX accumulation in A431 cell compared to ALA and ALA-me in broad range of concentrations. Pentenyl ester shows the similar results to ALA-hx. Based on MTT assay on A 431 cell, *vide ante*, ALA -hx and ALA-pe are not more toxic than ALA above 0.5 mM concentration. Even though experimentations for toxicity of esters and accumulation of PpIX were performed only on A431 cell, these results suggest that unsaturated alkyl ester ALA-hx has slightly enhanced cellular uptake and is readily hydrolyzed to get into the hem biosynthetic pathway to produce photosensitizer PpIX on cells. Hence, we propose ALA-hx would be an alternative photosensitizer for PDT using PpIX.

In conclusion, a convenient and efficient one-pot method for synthesis of unsaturated alkyl esters of ALA in good yields has been developed, and this methodology can be suitable for large scale synthesis. MTT viability assay showed the absence of severe cytotoxicity of the alcohols released intracellularly from the synthesized esters and the fluorescence spectral measurement of PpIX produced in A431 cell confirms that the enhanced cellular uptake and rapid hydrolysis of unsaturated alkyl esters of ALA to ALA and production of photosensitizer PpIX.

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Supporting Information: The table of cell survival of A431 cell with ALA and its unsaturated alkyl esters, ^1H, and ^{13}C NMR spectra of unsaturated alkyl esters of ALA are available on request to the corresponding author; jnoh@chonnam.ac.kr, (fax)−82-62-530-3389.

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