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THE SYNTHESIS AND APPLICATIONS OF 5–AMINOLEVULINIC ACID (ALA) DERIVATIVES IN PHOTODYNAMIC THERAPY AND PHOTODIAGNOSIS

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Abstract: A route has been developed to high–purity precursors, *viz.*, ALA esters, to be used in photodiagnosis and photodynamic therapy. Hexyl, butyl and methyl 5–aminolevulinates are similar to the ALA acid in chemical stability and efficacy in producing the appropriate photosensitizer PpIX. Tests carried out on animal models showed the method based on the esters to be the more selective.

Keywords: levulinic acid, bromination, methyl 5–aminolevulinate, butyl 5–aminolevulinate, photodynamic therapy

Photodynamic therapy (PDT) is based on the application of photosensitizers to the afflicted tissue and subsequent activation by light. During the past seven years, 5–aminolevulinic acid (ALA) has been one of the major substances used in PDT and in photodiagnosis (PD) (1–4). When applied exogenously, ALA is selectively metabolized *in situ* in neoplastic tissues to yield the actual photosensitizer, *viz.*, protoporphyrin IX (PpIX), a compound that produces a strong fluorescence which serves as a basis for diagnosis. The accumulation of PpIX in tissues to yield the actual photosensitizer, *viz.*, protoporphyrin IX (PpIX), a compound that produces a strong fluorescence which serves as a basis for diagnosis. The accumulation of PpIX in tissues occurs by avoiding the feedback control in the pathway of hem biosynthesis. Under the action of PpIX and of the visible light radiation at wavelengths coinciding with porphyrin absorption bands and molecular oxygen, singlet oxygen is generated which gives rise to necrosis of tissues. At present, the topical application of ALA is a promising novel way of treating the following superficial skin alterations: basal cell carcinoma (BBC), superficial squamous cell carcinoma (SCC), Bowen's disease, actinic keratoses (AC), acne, psoriasis and hirsutism. ALA has been successfully used for photodetection in urology and gyneacology and has been effective in treating certain viral infection, particularly those resulting in warts. The sensitivity of superficial bladder cancer detection can be increased to almost 95%.

As a hydrophilic compound, pure ALA exhibits several limitations: it penetrates only the epidermis; at physiological pH–values in aqueous media, it condenses rapidly to yield inactive derivatives; thereby the therapy has to be repeated several times. A possible solution to this problem is the use of derivatives of ALA (5–6).

The present study is intended to develop ALA derivatives that are more hydrophobic, *viz.*, ALA methyl, butyl and hexyl esters, to enhance their penetrability into the human skin. The products were prepared in a four–step synthesis, starting from levulinic acid.

The compounds were examined for chemical stability in aqueous media in relation to pH. The derivatives were tested on animal models and exhibited a lower rate of PpIX formation as compared with that of ALA, yet the penetration into the skin was deeper and the selectivity coefficient between the normal and the dysplastic tissue was superior.

A number of methods have been reported to prepare 5–aminolevulinic acid hydrochloride. One method involves a three–step synthesis including

bromination of levulinic acid in methanol (7, 8), reaction of methyl 5–bromolevulinate with potassium phthalimide, and a subsequent hydrolysis of the resulting phthalimide derivative with a 6M hydrochloric acid (9). Another method is to convert the 5–bromo ester into ALA hydrochloride *via* an azide derivative (10). Two routes have also been described to prepare ALA hydrochloride from acid chlorides of monomethyl and monoethyl butanedioates (11, 12). The reported esterification of ALA · HCl involves the reaction of ALA · HCl with anhydrous alcohol and thionyl chloride (6, 8). Esterification of ALA · HCl with methanol has also been described (8).

EXPERIMENTAL

Materials

The following reagents and solvents were used:
– levulinic acid and phthalimide, Aldrich's 98% pure,
– bromine, potassium carbonate, sodium hydrogen carbonate, solvents used as reaction media and for extraction and crystallization, dimethyloformamide, methanol, chloroform, toluene, ethyl acetate, propan–1–ol, and diethyl ether (POCh, Gliwice, PL),
– acetonitrile (Fluka)
– methanol and butan–1–ol, POCh's 99.5% pure, for esterification of ALA · HCl,
– hexan–1–ol, Merck's 98% pure.

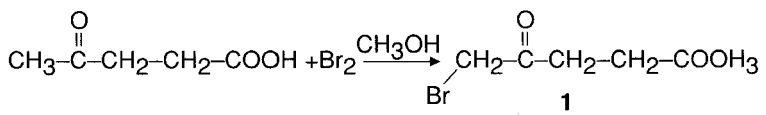
GC and HPLC were used to follow the progress of the reaction. Purity of the ALA · HCl esterification reaction was followed by TLC; chromatograms were developed in a (3:1:0.5 v/v) butan–1–ol–water–acetic acid system. The resulting esters were identified by IR and ^{1}H NMR spectra.

Bromination and esterification of levulinic acid. Methyl 5–brominolevulinate (1)

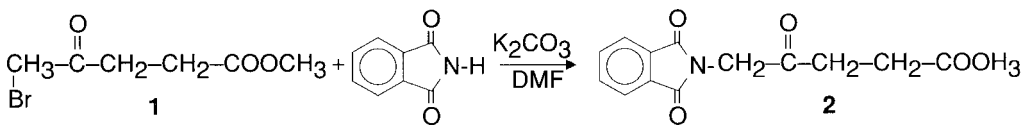
Levulinic acid (660.8 g, 5.697 mol) was placed in a reactor in methanol (6.7 l) and bromine (293 ml, 5.687 mol) was added dropwise with continuous stirring. The resulting solution was stirred at 20–30°C for 3.5 h and then boiled for 2 h under reflux. The mixture was allowed to cool, diluted with water and extracted twice with methylene chloride. The extracts were combined, washed with brine, twice with a saturated $NaHCO_3$ solution and again with brine, and then dried over anhydrous $MgSO_4$. The solvent was distilled off and the remaining crude reaction mixture was separated by rectification at a reduced pressure to yield 420 g (35.7%) of methyl 5–bromolevulinate (**1**) (b.p.=71–72°C; p=0.2 mm Hg).

Reaction of methyl 5–bromolevulinate (1) with phthalimide in the presence of K_2CO_3. Methyl 5–phthalimidolevulinate (2)

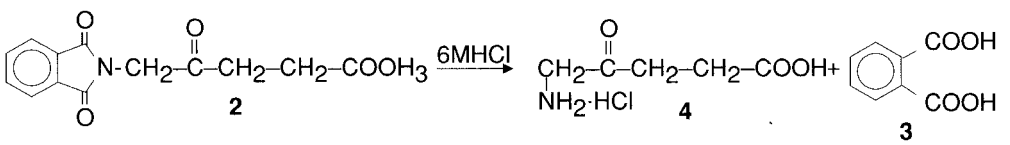
Phthalimide (200.5 g, 1.364 mol) and dimethylformamide (1 l) were placed in a reactor. Potassium



Scheme 1.



Scheme 2.



Scheme 3.

carbonate (250.3 g, 1.814 mol) was added with stirring, the mixture was heated at 40°C for 1.5 h, then allowed to cool to 25°C, and bromoester (**1**) (259 g, 1.239 mol) was added dropwise. The compounds was stirred together at 40°C for 1.5 h. The reaction mixture was diluted with water and then extracted thrice with (3:1 v/v) toluene–ethyl acetate. The extracts were washed with brine, three times with cold (0–2°C) aqueous 1.8% NaOH, again with brine, and then dried over anhydrous $MgSO_4$. The solvent was evaporated and the resulting crude compound (297.5 g) was redissolved in chloroform (350 ml); methanol was added (1 l) and the mixture was cooled to –25°C. The resulting precipitate was collected by filtration at a reduced pressure, washed with cold methanol and dried to yield methyl 5–phthalimidolevulinate (**2**) (220 g; 65%), purity better than 99% (by HPLC).

Hydrolysis of methyl 5–phthalimidolevulinate (2). 5–Aminolevulinic acid hydrochloride (4)

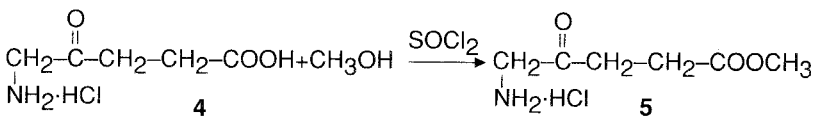
Methyl 5–phthalimidolevulinate (**2**) (200 g, 0.945 mol) was boiled with 6M HCl (3.45 l) for 12 h. Then the mixture was cooled to –25°C. The precipitate *o*–phthalic acid (**3**) was collected by filtration and the filtrate was evaporated to dryness. The residue was redissolved in 2M HCl and stirred with active carbon at 20°C. The carbon was filtered off and the filtrate was evaporated to dryness. The residue was dissolved in water (75 ml) acidified with conc. hydrochloric acid (0.75 ml), propan–1–ol (1.4 l) was added and the mixture was cooled to –25°C. The resulting precipitate was collected by filtration at a reduced pressure and dried to yield 5–aminolevulinic acid hydrochloride (**4**) (116 g, 73.2%), m.p. 153.5–154.0°C, purity 95.5% (by HPLC) (for comparison: Merck's product is 89% pure; Fluka's product is 94% pure).

Preparation of methyl ester of ALA hydrochloride (5)

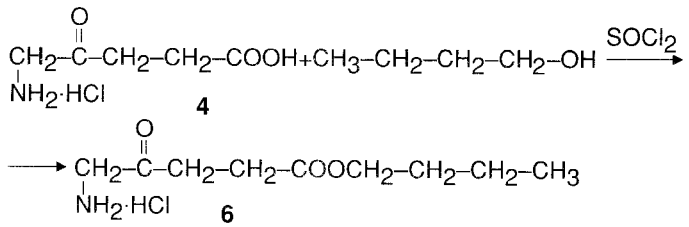
To methanol (120 ml) cooled to –20°C thionyl chloride (33.1 g, 0.278 mol) was added dropwise and then 5–aminolevulinic acid hydrochloride (**4**) (20 g, 0.119 mol) was added. The mixture was stirred magnetically for 30 h at 20°C. The excess of methanol and thionyl chloride was evaporated. The residue was stirred with acetonitrile for 18 h at 20°C. The resulting precipitate was collected by filtration at a reduced pressure, washed with acetonitrile and dried to yield the methyl ester (**5**) (18.7 g; 86.3%), m.p. 121.5–123.0°C [ref. (8), 119.0–121.0°C], purity 100% (by HPLC). IR (KBr), ν: 3438.59 (N–H stretch); 2954.39 (C–H stretch), 1721.39 (C=O stretch), 1593.81 (N–H bending) 1200.78 (C–N stretch) cm^{-1}, ^{1}H–NMR (DMSO, TMS), δ: 2.54 (t, 2H, CH_2); (t, 2H, CH_2); 3.57 (s, 3H, OCH_3); 3.92 (s, 2H, NH_3^+–CH_2C=O); 8.39 (s, 3H, NH_3^+) ppm.

Preparation of butyl ester of ALA hydrochloride (6)

Butan–1–ol (120 ml) was placed in a reaction flask and cooled to –25°C and then thionyl chloride (20 ml, 33.1 g, 0.278 mol) was added dropwise with continuous mechanical stirring. Then ALA hydrochloride (**4**) (20 g; 0.119 mol) was added. The mixture was stirred for 1 h at 20°C and then boiled for 25 min. The mixture was set aside at 20°C. The excess of the alcohol and thionyl chloride was evaporated. The residue was shaken with acetonitrile and left for 5 h at 20°C and for another 5 h at 0°C. The resulting precipitate was collected by



Scheme 4.



Scheme 5.

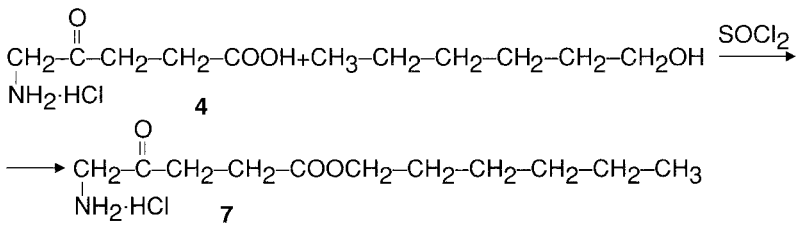
filtration, washed with acetonitrile and dried to yield 16 g of the ester (crop I), m.p. 137.5–138.5°C. The filtrate was evaporated and diethyl ether (30 ml) was added to the residue, shaken and left for 10 days at 0°C. The resulting precipitate was collected by filtration, washed with diethyl ether and dried to yield 5.11 g crystals (crop II), m.p. 139.0–140.0°C. The total yield of crystals of butyl ester (**6**) was 21.05 g (78.9%). IR (KBr), ν: 3446.57 (N–H stretch); 2959.54 (C–H stretch), 1725.48 (C=O stretch), 1589.29 (N–H bending); 1212.39 (C–N stretch) cm^{-1}. ^{1}H–NMR (DMSO, TMS) δ: 0.87 (t, 3H, CH3, J=7.42 Hz); 1.31 (m, 2H, CH_2); 1.53 (m, 2H, CH_2); 2.53 (t, 2H, CH_2, J=6.5 Hz), 2.79 (t, 2H, CH_2, J=6.6 Hz), 3.92 (s, 2H, NH_3^+–CH2–C=O); 3.99 (t, 2H, OCH_2, J=6.6 Hz); 8.37 (s, 3H, NH_3^+) ppm.

Preparation of hexyl ester of ALA hydrochloride (7)

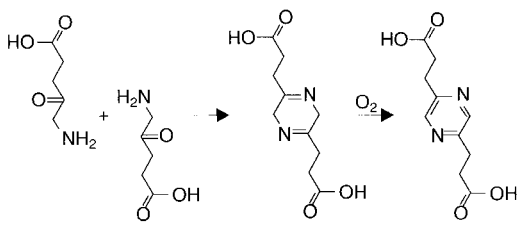
To hexan–1–ol (120 ml) cooled in a flask to –10°C, thionyl chloride (20 ml, 33.1 g; 0.278 mol) was added dropwise and then 5–aminolevulinic acid hydrochloride (**4**) (20 g; 0.119 mol) was added. The compounds were stirred together for 1 h at 20°C and boiled for another 1 h. The mixture was stirred for 24 h at 20°C. The precipitated crude product was collected by filtration and dried, then acetonitrile was added and stirred for 24 h. The resulting precipitate was collected by filtration, washed with acetonitrile, and dried to yield 12.5 g of the hexyl ester (crop I), m.p. 145.0–147.0°C. The filtrate was cooled to –25°C and the precipitated product was collected by filtration and stirred with diethyl ether at 20°C to yield 2 g crystalline hexyl ester (crop II), m.p. 145.0–147.0°C. The total yield of the hexyl ester (7) was 14.5 g (48.2%). IR (KBr), ν: 3252.68 (N–H stretch); 2957.62 (C–H stretch), 1729.99 (C=O stretch), 1590.79 (N–H bending), 1215.10 (C–N stretch) cm^{-1}. ^{1}H–NMR (DMSO, TMS), δ: 0.85 (t, 3H, CH_3, J = 6.96 Hz); 1.26 (m, 6H, $3CH_2$); 1.54 (m, 2H, CH_2); 2.53 (t, 2H, CH_2, J = 6.5 Hz), 2.78 (t, 2H, CH_2, J = 6.6 Hz), 3.92 (s, 2H, NH_3^+–CH_2–C=O); 3.98 (t, 2H, OCH_2, J = 6.68 Hz); 8.39 (s, 3H, NH_3^+) ppm.

***In vivo* experiments**

The effectiveness of formation of PpIX *via* ALA ester derivatives was studied in white mice belonging to BALB/C strain and having a myoma tumor implanted. The tumor was 5–8 mm in diameter. The preparations were prepared in the form of a cream immediately before the experiment, the concentration of the active ingredient being 5–20% (mole contents of individual derivatives were identical). The basis of the cream included eucerin, water, ceryl (hexacosyl) alcohol and like components. Mice were anaesthetized, the area of the tumor and the surrounding area of the healthy tissue were shaved and the cream was applied in a layer. The spots involved were covered with a plastic film or with a special artificial leather which rendered penetration of the preparation into the skin easier. Fluorescence of the resulting PpIX was followed in time by using a fiber–based multichannel spectral analyzer induced by a laser operated at a wavelength of 632 nm (13). This wavelength enables the light to penetrate into the total depth of the tumor and the PpIX concentration to be measured within the whole volume of the tissue. The maximum fluorescence level achieved in 6–9 h after application of ALA and ALA derivatives,



Scheme 6.



Scheme 7.

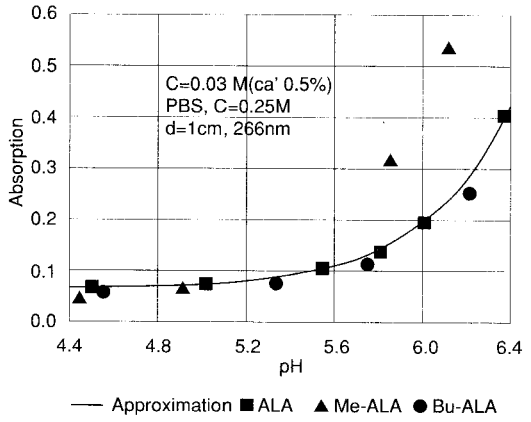


Figure 1. Comparison of the stability of compounds in aqueous solutions at different pH values

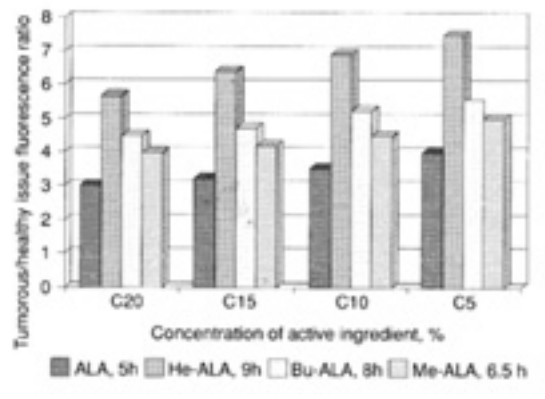


Figure 2. Comparison of the maximum efficiencies of formation of PpIX induced by ALA and ALA esters in relation to concentration of active ingredient

corresponded to the maximum concentration of PpIX. The experiments were made in Pathomorphology Department of Military University of Medicine.

Study on the stability of ALA derivatives in water

Aqueous 0.03 M ALA and 0.03 M ALA ester solutions were prepared in a 0.25 M phosphate buffer. The solutions were deoxygenated by bubbling an argon gas and then absorption spectra were recorded with time with a Lambda 900 spectrophotometer.

RESULTS

One essential problem encountered in the use of ALA in the PDT–PD method is the stability of the compound in aqueous solutions. According to

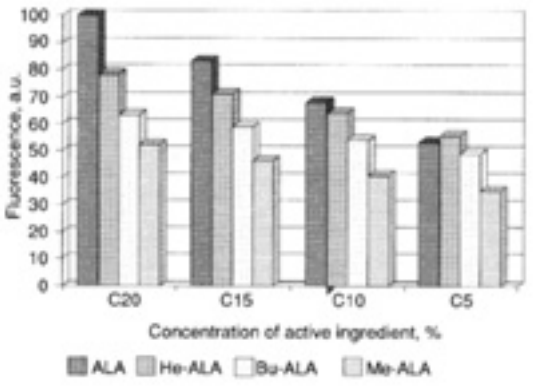


Figure 3. Comparison of the contrast of fluorescent light emitted by the tumorous and healthy tissues

concentration of the acid, in a medium of pH equal to 5.5 two ALA molecules condense to yield 2,5–dicarboxyethyl–3,6–dihydropyrazine, which further becomes oxidized with atmospheric oxygen to yield 2,5–dicarboxyethylpyrazine (14–15). A band appears in the absorption spectrum at about 265 nm.

Figure 1 shows changes of the spectrum occurring with time in relation to pH of the solution. The solutions are stable up to pH of about 5.5. At higher pH, the condensation commences to occur regardless of the structure of the compound prepared. Thus, esterification itself does not affect the stability of the compound in water and, in the case of the methyl ester, even a reduction in stability is quite conspicuous.

The maximum amounts of PpIX synthesized from individual derivatives are shown in Figure 2 in relation to concentration of the active ingredient. Pure ALA is most effective at higher concentrations. As the concentration of the active ingredient is reduced, differences in efficiency are becoming lower and lower and at a concentration of 5% the hexyl ester is the most active. The lower rate at which PpIX is formed by the esters may be interpreted in terms of the hydrolysis which is necessary to yield pure ALA after the compounds have penetrated into the cell membranes. However, it should be emphasized that, in the presence of ester, the contrast in the fluorescence between the tumorous and the healthy tissue is greater (Figure 3). This is the essential feature by which the esters predominate over the pure acid.

CONCLUSIONS

Generally, the level of PpIX synthesized by the esters is slightly lower or close to that produced

by ALA. The amounts of the photosensitizer produced locally are 15–20 times higher than the amounts of porphyrins accumulated in the tumors after venal injection of porphyrins; the absolute amount of PpIX is thus not essential. The greatest advantage of the esters is that they produce a more pronounced contrast.

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