

Study of the stability of the 5-aminolevulinic acid tyrosine ester in aqueous solution

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Abstract

Photodynamic therapy based on photoactivable porphyrins (PAPs) can treat various dermatological conditions. The side-effects as well as the non-selective or insufficient accumulation of PAPs in the targeted tissues limit performances. We studied the stability in solution at different temperatures (21 °C; 4 °C), different pH values (7.5; 2.0), and as a function of time of 5-aminolevulinic acid's Tyrosine-ester, a molecule presenting interesting properties to selectively produce PAPs in blood vessels after topical application. Solutions of this precursor can be kept up to 24 h at refrigerated temperatures and under acidic pH. At room temperature or physiological pH, they must be prepared minutes before their use.

Keywords: 5-Aminolevulinic acid, stability, 5-aminolevulinic acid derivatives, photodynamic therapy, tyrosine ester

Introduction

Photodynamic therapy (PDT) based on the topical application of 5-aminolevulinic acid (ALA) or some of its derivatives, as exogenous precursors of fluorescent and photoactivable porphyrins (PAPs), such as protoporphyrin IX (PPIX – for the sake of clarity, the abbreviation « PPIX » will be used throughout instead of « photoactivable porphyrins (PAPs), such as protoporphyrin IX »), is widely used in various fields of medicine for cancerous and non-cancerous applications. This modality of treatment (or detection) has numerous advantages, such as the facts that it is a local treatment, that neither the precursors nor the PPIX are toxic in the absence of light, and that the accumulation of PPIX is somewhat selective for certain hyperplastic, or hyper-metabolic tissues.

Due to these properties, PPIX-based PDT is a treatment of choice for various dermatological conditions, such as Actinic Keratosis (AK), basal cell carcinoma (BCC), as well as other skin pathologies¹.

Despite these clear advantages, the limited specific production of PPIX within the tissues to be destroyed, in particular in blood vessels when treating conditions such as telangiectasia, port-wine stain, or rosacea, limits the performance of PDT treatment. To overcome this problem, one strategy is to target cells present in the wall of skin blood vessels. The concept is that if a given precursor demonstrates selectivity for these cells, they will biosynthesize PPIX preferentially compared to other cells in the vicinity². This approach is interesting not only for oncologic applications³ but also for treatment of any condition of vascular origin, as is the case for the conditions mentioned above.

We synthesized one new ALA derivative, ALA-tyrosine-ester (ALA-Tyr). Based on preliminary results obtained in a separate in vivo study, this molecule presents an improved vascular selectivity compared to ALA. Since it is likely that only free ALA participates in the biosynthesis of PPIX⁴, the stability of the ester bond has an impact on the bioavailability of ALA in the targeted cell after the precursor has been internalized. Thus, we study ALA-Tyr's stability properties. We also present results obtained in similar solutions with free ALA. We investigate the stability of ALA-Tyr in solution at two different temperatures (21°C and 4°C), and at two different pH values (2.0 and 7.5), and at various time points between 1 h and 24 h. It is noteworthy that under these conditions, free ALA^{5,6} and ALA-Tyr are both unstable⁷, but degrade via different pathways: ALA-Tyr degrades through the hydrolysis of the ester bond, whilst free ALA dimerizes, forming 2,5-dicarboxyethyl-3,6-dihydropyrazine (DHPY), and 2,5-dicarboxyethylpyrazine (PY) after oxidation in the presence of molecular oxygen⁸.

Results and Discussion

The structure of the 5-aminolevulinic acid derivative 5-ALA-Tyr ester (**1**) is shown in Figure 1.

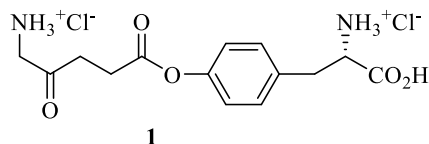


Figure 1. Structure of 5-ALA-Tyr ester (**1**).

N-BocALA (**3**) was synthesized according to published methods, starting from commercially available 5-aminolevulinic acid hydrochloride (**2**)⁹ (see Figure 2).

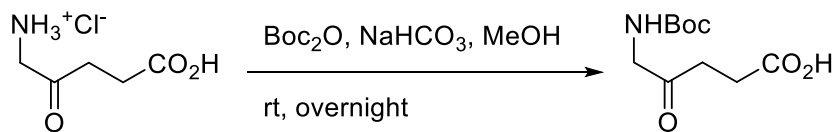


Figure 2. Synthesis of N-BocALA (**3**).

5-ALA-Tyr ester (**1**) was synthesized starting from N-BocALA (**3**) and commercially available Tyr-OBu^t (**4**) according to published method (see Figure 3).¹⁰

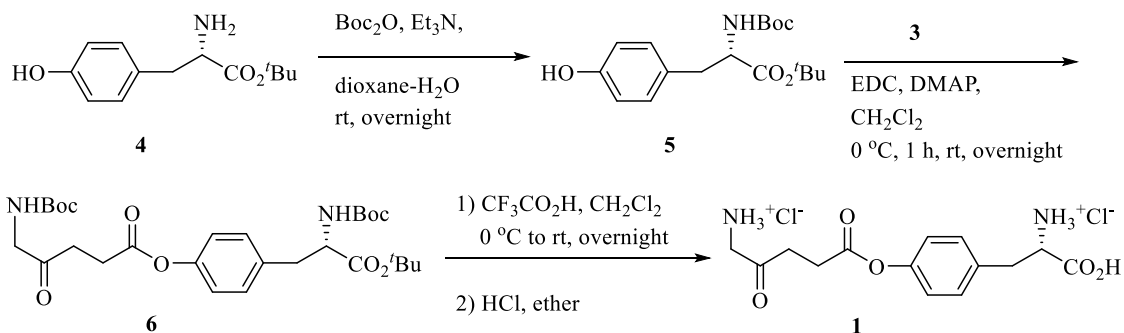


Figure 3. Synthesis of 5-ALA-Tyr ester (**1**).

Figure 4 shows the chromatogram of the ALA-Tyr ester in solution (10 mg/ml in PBS, further diluted for the measurement to 1 mg/ml, see Experimental Section) at two different time points ($t = 0$ and $t = 24$ h) and under various storage conditions. For the sake of readability, a diagonal offset was applied to the $t = 24$ h chromatogram both horizontally and vertically. Individual peaks are identified with markers.

Figure 4a shows the chromatograms of the ALA-Tyr solution stored at temperature $T = 21^{\circ}\text{C}$ and at $\text{pH} = 7.5$. Figure 4b shows the chromatograms of the solution stored at 4°C and at $\text{pH} = 2$. We chose these two sets of conditions because they represent the two extremes in terms of stability of the ALA-Tyr solution. Please note that a scaling factor was applied to Fig. 4b to compensate for different daily calibration parameters.

On Figure 4a, it can be noted that the intensity of the peak corresponding to ALA-Tyr ester decreases after 24 h (upper chromatogram). A concomitant increase in the intensity of the peak corresponding to ALA can be observed. A similar increase in intensity can be observed for the peak corresponding to free Tyrosine. These observations confirm our hypothesis that the ALA-Tyr ester is hydrolyzed into ALA and free Tyrosine over 24 h at temperature $T = 21^{\circ}\text{C}$ at $\text{pH} = 7.5$. Some additional peaks are residues from the synthetic process. They are not detected with other analytical methods, and only appear in the chromatogram due to their good ionization properties. It is however noteworthy that the peak corresponding to BOC-protected ALA-Tyr ester (third peak from left) follows the same evolution and decreases, whilst the peak

corresponding to BOC-protected free Tyrosine (second peak from left) increases, showing that the ester-bond of the BOC-protected moieties are also hydrolyzed over time.

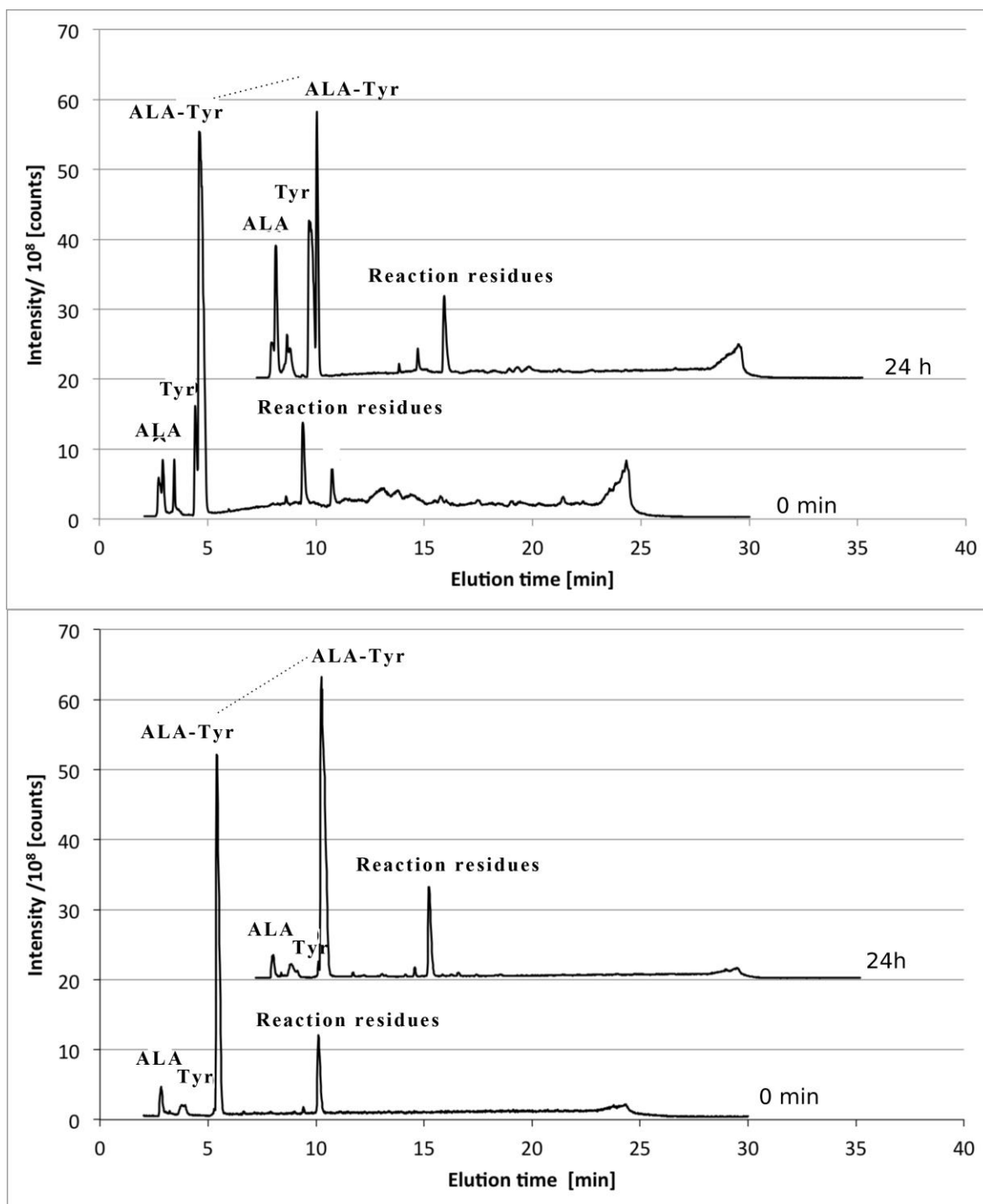


Figure 4. Chromatograms of 1mg/mL ALA-Tyr solutions at different storage conditions. A (top) : stored at pH 7.5 and room temperature during 24h ; B (bottom) : stored at pH 2 at 4 °C during 24h.

On Figure 4b, we observe a very different situation. Some free Tyrosine can be detected after 24 h, but in minute amounts only (approximately 5%). Generally speaking, the chromatogram at 24 h shows that these storage conditions keep the precursor solution in a much more stable state.

Figure 5 shows a more general view of the kinetics of the hydrolysis of the ester bond of ALA-Tyr ester under four pH and temperature storage conditions, namely pH = 7.5 & temperature T = 21°C (see also Fig. 4a); pH = 7.5 & temperature T = 4°C; pH = 2.0 & temperature T = 21°C; pH = 2.0 & temperature T = 4°C (see also Fig. 4b).

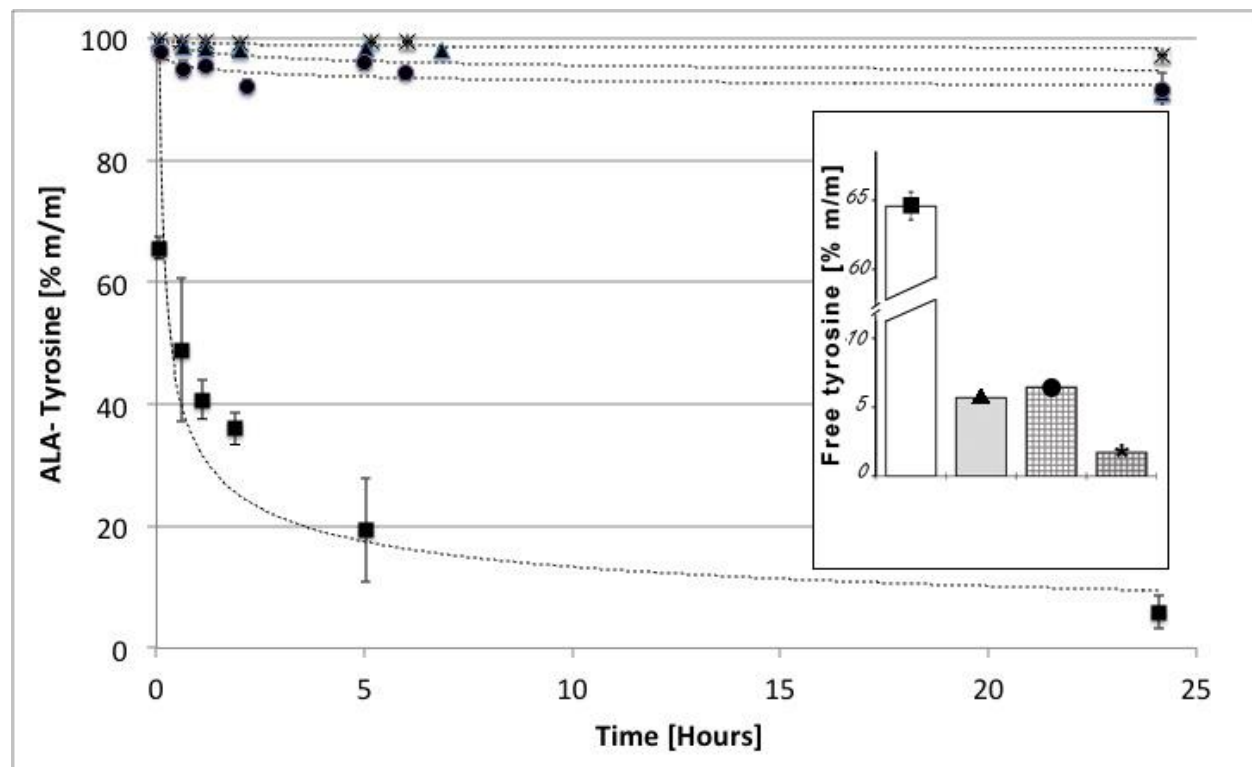


Figure 5. Kinetic profiles of ALA-tyr hydrolyse in different conditions of storage. ■ pH 7.5 at room temperature ; ● pH 7.5 at 4 °C ; ▲ pH 2 at room temperature ; * pH 2 at 4 °C.

Inset : percentage of free tyrosine at 24 h (vertical scale varies in the inset). Results are significant to $p \leq 0.05$.

The measurement reported on Fig. 5 is the percentage of ALA-Tyr in solution relative to its initial concentration. More than 90% of the original quantity of ALA-Tyr remains after 24 h in the stable solutions (those labeled * and ▲), whereas a smaller percentage of ALA-Tyr ester (approximately 90% and <10% of the original quantity, respectively) remains in the other two solutions (those labeled ● and ■). It can be seen from Figure 5 that both temperature and pH have a large impact on the stability of ALA-Tyr in solution. The storage conditions pH = 7.5 & temperature T = 21°C (curve labeled ■, see also Fig. 4a) are the least favorable for ALA-Tyr ester's stability, whilst pH = 2.0 & 4°C (curve labeled *, see also Fig. 4b) are the most

favorable. The inset gives a magnified view of the measurement at 24 h, and shows the percentage of free Tyrosine under the same conditions. Both pH and temperature have an impact on the stability of ALA-Tyr ester in solution. The two parameters are not completely independent. The inset shows that at temperature $T = 21^{\circ}\text{C}$, keeping the $\text{pH} = 2.0$ limits the percentage of free Tyrosine to approximately 5% of its original quantity after 24 h. Lowering the temperature to 4°C allows to bring the percentage of free Tyrosine to approximately 7% of its original quantity. The results shown in the inset are significant ($p < 0.05$). Figure 5 shows that, amongst conditions chosen because they reflect frequent clinical practice (solutions prepared fresh or prepared the day before; physiological or acidic pH; solutions stored in a fridge or at room temperature; etc), the sets of conditions showed differences of limited magnitude in several cases.

Figure 6 shows the chromatogram of an ALA solution stored at temperature $T = 21^{\circ}\text{C}$ over time ($t = 0$ until $t = 24$ h) (10 mg/ml in PBS, further diluted for the measurement to 2.5 mg/ml, see Experimental Section). Two peaks are noteworthy: the peak corresponding to ALA-ALA dimer PY, appears and grows over time; the peak corresponding to ALA decreases over time, albeit in a slightly less clear-cut manner.

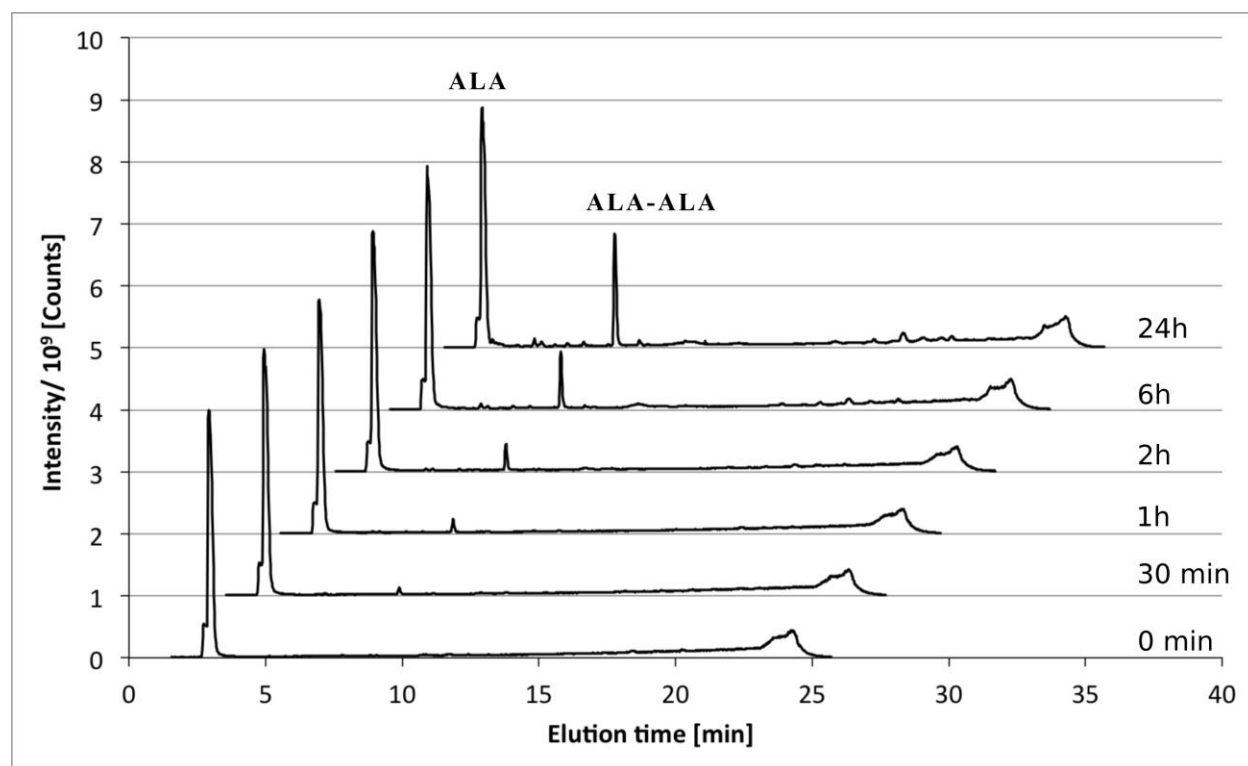


Figure 6. Chromatograms of 2.5 mg/mL ALA solution stored at pH 7.5 and room temperature after 0 min , 30 min , 1 h, 2 h, 6 h and 24 h.

Table 1 summarizes the retention times of ALA and some of its derivatives on our LC-MS

system. The retention times give an idea of the relative hydrophobicity of the various derivatives, with longer retention times corresponding to a higher hydrophobicity.

Table 1. Retention times of various ALA derivatives of interest for PDT treatments measured with our LC-MS system

Derivative	Retention time on our system [min.]
ALA	2.9
ALA Methyl-ester	3.7
ALA-ALA dimer (PY)	3.8
ALA Tyrosine-ester	5.2
ALA Hexyl-ester	14.8

Our results demonstrate that the parameters we studied (temperature and pH of the storage solution) affect the stability of ALA-Tyr in solution. This is in good agreement with previous results reported for ALA^{6, 11} or some of its esters¹², and shows that both ALA and ALA-Tyr solutions can be kept for up to 24 hours if they are refrigerated and their pH = 2, but must otherwise be prepared within minutes before their use.

Conclusions

We measured the hydrolysis of the ester bond in solutions of ALA-Tyr. The end-point for the degradation of the derivatives was the appearance of free Tyrosine, and the disappearance of ALA-Tyr ester. Both pH and temperature have an impact on the stability of ALA-Tyr ester in solution. At temperature $T = 21^{\circ}\text{C}$, keeping the pH = 2.0 limits the hydrolysis of the ester bond of ALA-Tyr ester to approximately 10 % after 24 h (see Fig. 5). Conversely, lowering the temperature to 4°C allows to bring the hydrolysis rate of ALA-Tyr ester at less than 10 % after 6 h at neutral pH (vs. $> 80\%$ at temperature $T = 21^{\circ}\text{C}$, see Fig. 5). Thus, the impact of keeping the pH at acidic value is larger than the impact of lowering the temperature.

Based on these results, we can conclude that it is necessary to keep the solutions of ALA or ALA-Tyr refrigerated, and to use them within 24 h of their preparation. This is particularly important for solutions at neutral (physiological) pH.

Experimental Section

General. Reagents and solvents were purchased from commercial sources (Aldrich, Acrôs, Merck, Fluka, Fluorochem and VWR international). Reagents were used without further purification unless otherwise noted. All reactions were performed under argon (or nitrogen). When needed, glassware was dried overnight in an oven ($T > 100^{\circ}\text{C}$) or under vacuum with a

heat gun ($T > 200$ °C). When solvents were indicated as dry, they were either purchased as such or distilled prior to use or dried by a passage through a column of anhydrous alumina or copper using a Puresolv MD 5 from Innovative Technology Inc., based on the Grubbs' design¹³. Flash column chromatography was performed using Silicycle silica gel: 230-400 mesh (40-63 μm) silica. Reactions were monitored using Merck Kieselgel 60F₂₅₄ aluminium plates. TLC's were visualized by UV fluorescence (254 nm) then one of the following: KMnO_4 , phosphomolybdic acid, ninhydrin, vanillin. NMR spectra were recorded on a Brüker AvanceIII-400, Brüker Avance-400 or Brüker DPX-400 spectrometer at room temperature, ^1H frequency is at 400.13 MHz, ^{13}C frequency is at 100.62 MHz. Chemical shifts (δ) were reported in parts per million (ppm) relative to residual solvent peaks rounded to the nearest 0.01 for proton and 0.1 for carbon (ref: CDCl_3 [^1H : 7.26, ^{13}C : 77.2], CD_3OD [^1H : 3.31, ^{13}C 49.0], d_6 -DMSO [^1H : 2.50, ^{13}C 39.5]). Coupling constants (J) are reported in Hz to the nearest 0.1 Hz. Peak multiplicity was indicated as follows s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br (broad). Attribution of peaks was done using the multiplicities and integrals of the peaks. The accurate masses were measured by the mass spectrometry service of the EPFL by ESI-TOF using a QTOF Ultima from Waters or APPI-FT-ICR using a linear ion trap Fourier transform ion cyclotron resonance mass spectrometer from Thermo Scientific.

***N*-BocALA (5-((*tert*-butoxycarbonyl)amino)-4-oxopentanoic acid) (3).**⁹ A suspension of 5-ALA hydrochloride (**2**) (5.00 g, 29.8 mmol), NaHCO_3 (15.04 g, 179.0 mmol), and di-*tert*-butyl dicarbonate (6.51 g, 29.8 mmol) in dry MeOH was stirred at room temperature overnight. The solid was removed by filtration, and the solvent was evaporated. The oily residue was dissolved in water, and the aqueous solution was acidified with 10% aqueous KHSO_4 to pH 2. The mixture was extracted with EtOAc. The organic layers were washed with brine, dried over Na_2SO_4 , filtered, and evaporated. The oily residue was purified by column chromatography on silica gel ($\text{CH}_2\text{Cl}_2/\text{MeOH} = 10/1$) to give *N*-BocALA (**3**) (6.24 g, 90%) as a white solid, mp 72-74 °C (lit.⁸ 74-77 °C). The spectroscopic data of compound **3** were identical to those reported in the literature⁹.

***N*-Boc-Tyr(OH)-OBu' ((*S*)-*tert*-butyl 2-((*tert*-butoxycarbonyl)amino)-3-(4-hydroxy-phenyl)propanoate) (5).** To a stirred solution of Tyr-OBu' (**4**) (5.0 g, 21.0 mmol) in dioxane and water (42 ml, 0.5 M, dioxane/water = 1/1) were added Et_3N (3.27 g, 4.5 mL, 32.3 mmol, 1.54 equiv) and $(\text{Boc})_2\text{O}$ (5.56 g, 24.4 mmol, 1.14 equiv). The reaction mixture was stirred at rt overnight. After confirming the completion of the reaction by TLC, the reaction mixture was acidified to pH 3 by adding 10% aqueous KHSO_4 , and extracted with EtOAc. The organic layers were washed with brine, dried (Na_2SO_4) and evaporated. Column chromatography on silica gel (petroleum ether/EtOAc = 20/1 then 2/1) to yield *N*-Boc-Tyr(OH)-OBu' (**5**) (6.82 g, 96%) as a white solid, mp 106-107 °C (lit.¹² 112.8-113.0 °C (*n*hexane/ CH_2Cl_2)). The spectroscopic data of compound **5** were identical to those reported in the literature¹⁴.

***N*-Boc-5-ALA-Tyr-OBu' ester ((*S*)-4-(3-(*tert*-butoxy)-2-((*tert*-butoxycarbonyl)amino)-3-oxopropyl)phenyl 5-((*tert*-butoxycarbonyl)amino)-4-oxopentanoate) (6).** To a stirred solution

of *N*-Boc-Tyr(OH)-OBu^t (**5**) (2.26 g, 6.70 mmol), *N*-BocALA (**3**) (1.55 g, 6.70 mmol, 1.0 equiv) and DMAP (8.1 mg, 0.067 mmol, 0.01 equiv) in dry CH₂Cl₂ (135 mL, 0.05 M) at 0 °C was added EDC (1.54 g, 8.04 mmol, 1.2 equiv). The reaction mixture was stirred at 0 °C for 1 h and was warmed to rt. Stirring was continued overnight. Water was added and the organic layer was separated. The aqueous layer was extracted with EtOAc. The combined organic layers were washed with 1 M HCl, H₂O, saturated NaHCO₃ and brine, successively. The organic phases were dried (Na₂SO₄), and evaporated. The crude product was purified by flash column chromatography on silica gel (petroleum ether/EtOAc = 5/1 then 1/1) to give *N*-Boc-5-ALA-Tyr-OBu^t ester (**6**) as white foam (3.17 g, 86%).

¹H NMR (CDCl₃) δ 7.09 (br d, *J* = 8.3 Hz, 2H), 6.91 (br d, *J* = 8.3 Hz, 2H), 5.30 (br s, 1H), 5.03 (br d, *J* = 7.9 Hz, 1H), 4.36-4.31 (m, 1H), 3.98 (br d, *J* = 4.7 Hz, 2H), 2.95 (br d, *J* = 5.8 Hz, 2H), 2.79-2.74 (m, 4H), 1.36 (s, 9H), 1.34 (s, 9H), 1.31 (s, 9H).

¹³C NMR (CDCl₃) δ 204.4, 171.1, 170.8, 155.7, 155.1, 149.5, 134.2, 130.5, 121.3, 82.1, 79.8, 79.7, 54.9, 50.3, 37.8, 34.3, 28.3, 27.9.

HRMS (ESI+) *m/z* (M + H)⁺ calculated for C₂₈H₄₃N₂O₉ 551.2969. Found: 551.2965.

5-ALA-Tyr ester hydrochloride ((*S*)-5-(4-(2-ammonio-2-carboxyethyl)phenoxy)-2,5-dioxopentan-1-aminium chloride) (1**).** To a solution of *N*-Boc-5-ALA-Tyr-OBu^t ester (**6**) (1.55 g, 1.0 mmol) in CH₂Cl₂ (5 mL) at 0 °C was added TFA (5 mL) and the reaction mixture was stirred at rt overnight, then poured into a solution of HCl-diethyl ether. The precipitate was filtered and washed rapidly with diethyl ether to give 5-ALA-Tyr ester hydrochloride (**1**) as a hygroscopic white solid, mp 174 °C-dec.

¹H NMR (DMSO-*d*₆) δ 7.31 (br d, *J* = 8.5 Hz, 2H), 7.06 (br d, *J* = 8.5 Hz, 2H), 4.07 (br t, *J* = 6.2 Hz, 1H), 4.00 (s, 2 H), 3.16-3.06 (m, 2H), 2.93 (t, *J* = 6.5 Hz, 2H), 2.82 (t, *J* = 6.5 Hz, 2H).

¹³C NMR (DMSO-*d*₆) δ 202.6, 171.0, 170.3, 149.5, 132.8, 130.6, 121.6, 53.3, 46.5, 35.0, 34.4, 27.4.

HRMS (ESI+) *m/z* (M + H)⁺ calculated for C₁₄H₁₉N₂O₅ 295.1294. Found: 295.1294.

Anal. Calcd. for C₁₄H₂₀Cl₂N₂O₅: C, 45.79; H, 5.49; N, 7.63. Found: C, 45.49; H, 5.73; N, 7.34.

Preparation of solutions.

Solutions were prepared by dissolving the selected product (ALA or ALA-Tyr) at a concentration of 10 mg/ml in PBS. The pH of the solution was adjusted with a solution of NaOH 1N if necessary. Solutions were stored either at temperature T = 21 °C or at T = 4 °C according to the needs of the study. Just before measurement, 50 μl were sampled from the solution and diluted 10x with water. A sample of 100 μl of this last solution was injected into the column.

Detection of degradation products.

The degradation products of the ALA derivatives were monitored by reverse-phase liquid chromatography, coupled to a mass spectrometer (Autoprep system (Waters)), equipped with pumps (2525 binary gradient module), column fluidics organizer, sample manager (2767), PDA detector and ZQ4000 ESI-MS.

The ionization mode was electrospray, positive mode, cone voltage was set at 25 volts, the source temperature was 100 °C, the mass range was *m/z* 150-1000 in 2 sec. The mobile phase

was a water/acetonitrile (ACN) gradient: A: H₂O 99.89 % - HCOOH 0.1 % - TFA 0.01 % ; B: CH₃CN 90 % - , H₂O 9.89 % - HCOOH 0.1 % - TFA 0.01 %, varying along a 30 min gradient (*t* = 0 min: 98 % water and 2 % ACN; *t* = 20 min: 40 % water and 60 % ACN; *t* = 21 min: 100 % ACN; *t* = 22min: return to initial conditions). The column was Vydac C18 5µm (218TP54), 4.6x250 mm and flow rate was 1 mL/min.

Under those analytical conditions, more hydrophobic compounds are retained for longer times. ALA derivatives solutions were analyzed at various time points after their preparation (0, 30, 60, 120 minutes, 6 h and 24 h). All measurements were performed twice and are expressed as the mean value of the two measurements with 67% CI.

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