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CHEMICAL SYNTHESIS OF A "GSA-PYRROLE" AND ITS REACTION WITH EHRLICH'S REAGENT

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Abstract A rational chemical synthesis of 4-acetyl-2-(2-carboxyethyl)-5-methylpyrrole (**5**), the product formed when glutamate-1-semialdehyde (GSA, **2**) is reacted with acetylacetone in the first step of the quantitative analysis for GSA in biological media Rates of reaction of the GSA-pyrrole (**5**) with Ehrlich's reagent (the second step in GSA quantitation) are compared with the rates of the reactions of well-characterized "ALA-pyrroles" (**3**) and (**4**) Pyrrole (**5**) reacts more slowly with Ehrlich's reagent, and extinction coefficients for the corresponding Ehrlich's adducts were determined to be 46,000 for (**3**) and 865 for (**5**) These observations resolve the discrepancies observed in earlier quantitations of GSA and allow more accurate determinations of it in biological materials

Introduction

δ-Aminolevulinic acid (**1**, ALA) is the key acyclic building block in the biosynthesis of hemes, chlorophylls and bacteriochlorophylls, vitamin B_{12}, and factor 430 [1] Two pathways to ALA have been identified in Nature The most established ("classical") approach is the so-called "Shemin" pathway[2] (Figure 1) wherein glycine and acetate pro-

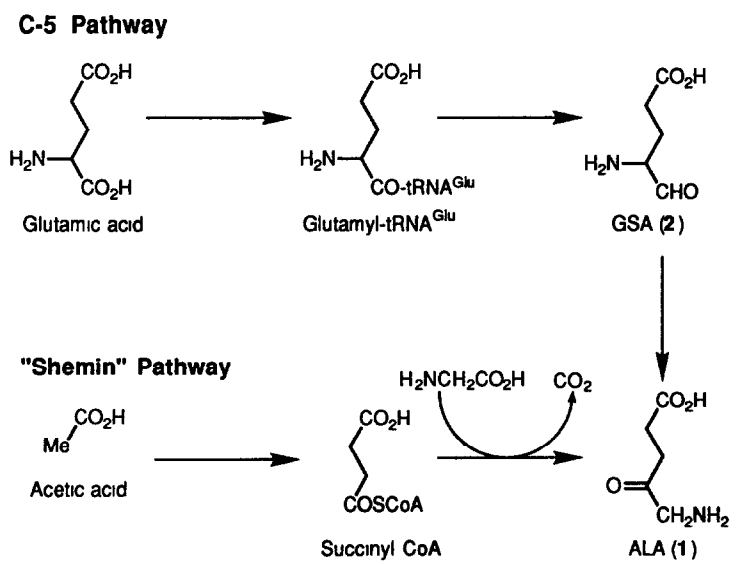


Figure 1 The two pathways for biosynthesis of ALA (**1**)

vide the carbons and nitrogen atom of ALA More recently, the so-called C-5 pathway[3] was identified (Figure 1), and in this glutamic acid is used as the carbon source Glutamate-1-semialdehyde (**2**, GSA) has been shown[4] to be the immediate biosynthetic precursor of ALA in plants and many bacteria, and thus is of central importance in the biosynthesis of various chlorophylls

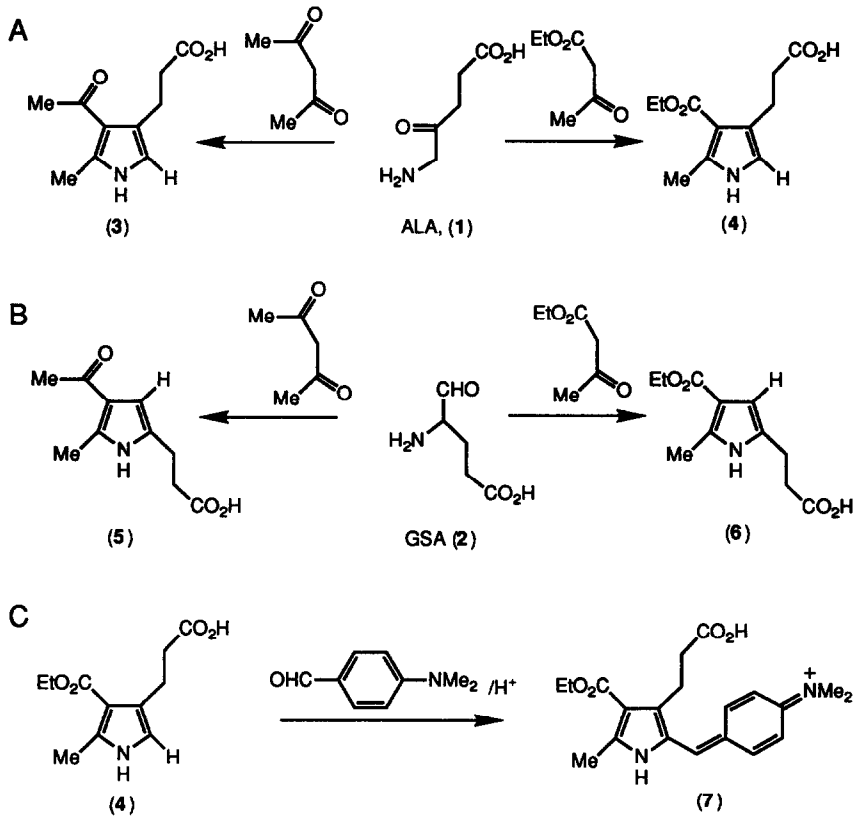


Figure 2 Products from the reaction of A, ALA with acetylacetone and ethyl acetoacetate, B, GSA with acetylacetone and ethyl acetoacetate, and C, ALA-pyrrole (**4**) with Ehrlich's reagent.

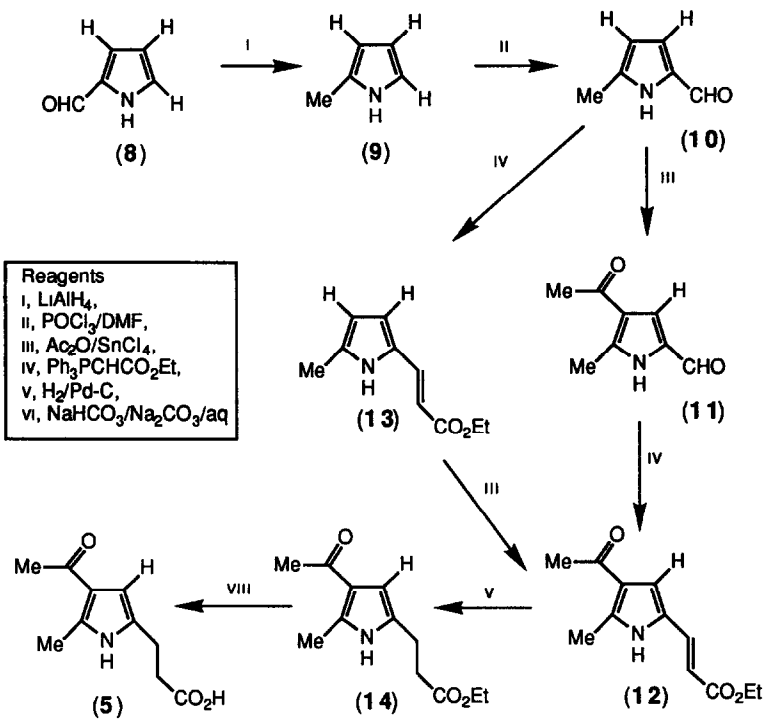
Quantitation of ALA in biological samples is accomplished[5] by the reaction of ALA with acetylacetone or ethyl acetoacetate to give (Figure 2) the ALA-pyrroles (**3**) or (**4**), respectively Pyrroles (**3**) and (**4**) are then measured quantitatively via the color test using Ehrlich's reagent (p-dimethylaminobenzaldehyde) [6] Analysis and determination of GSA is difficult due to the low yields and complicated synthetic processes required for its chemical synthesis,[7] and because of the lack of clear and reliable data for its quantification The former problem has recently been overcome with the development of a new synthetic route to GSA by Gough et al [8] The quantitative analysis of GSA in biological material has been performed, as with ALA, by its *in situ* condensation with acetylacetone or ethyl acetoacetate (Figure 2B), and subsequent colorimetric assay (Ehrlich) of the resulting pyrroles (**5**) or (**6**), respectively [5] Figure 2C shows the structure of a typical Ehrlich adduct (**7**) from ALA-pyrrole (**4**) Nevertheless, the Ehrlich reaction conditions in the GSA sequence lead to the non-enzymatic formation of ALA,[9] which also reacts in the Ehrlich test[10] and thus leads to erroneous quantification Furthermore, slow reaction of GSA-pyrroles with Ehrlich's reagent have led to the use of a

scaling factor in the GSA estimate [9] We have therefore undertaken the development of a chemical synthesis of the well-defined GSA-pyrrole (**5**), as well as some related compounds, and we present results of the reaction and quantification of these substances using the Ehrlich test

Results and Discussion

Synthesis of the GSA-pyrrole,(**5**)

The pyrrole (**5**) was synthesized as shown in Scheme I Commercially available 2-formylpyrrole (**8**) was reduced with lithium aluminum hydride to give a 74% yield of 2-methylpyrrole (**9**). Vilsmeier formylation ($POCl_3$/DMF) of (**9**) gave a 64 5% yield of 2-formyl-5-methylpyrrole (**10**) which was acetylated under Friedel-Crafts conditions with acetic anhydride to give the 4-acetyl-2-formyl-5-methylpyrrole (**11**) in 72% yield. Wittig reaction of (**11**) with (carbethoxymethylene)-triphenylphosphorane gave the acrylate (**12**) in 88% yield Alternatively, the Wittig reaction could be carried out first on pyrrole (**10**) to give the acrylate (**13**) (93%), and Friedel-Crafts acetylation of (**13**) then gave (**12**) in 73% yield Catalytic hydrogenation of the acrylate (**12**) gave the pyrrole-2-propionic ester (**14**) in 77% yield, and simple hydrolysis afforded the corresponding GSA-pyrrole (**5**) in 60% yield



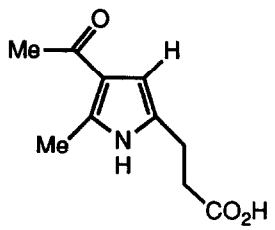
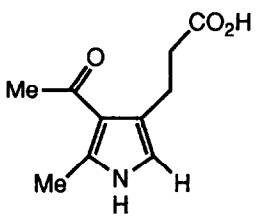
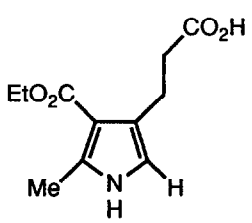
Scheme I Synthetic routes to GSA-pyrrole (**5**)

Reaction of (**5**) *with Ehrlich's Reagent*

The Ehrlich test uses the reaction of pyrroles with p-dimethylaminobenzaldehyde in acid, leading to a bright red color which can be used for quantification using spectrophotometry Since ALA (**1**) is not a pyrrole, it must first be converted quantitatively into a pyrrole, this is done very conveniently by condensation with acetylacetone or ethyl acetoacetate While the characterization of ALA in this way normally poses no problem, the quantification of GSA (**2**)

has been performed in a similar fashion, but with no standardization and no clear view on what was happening chemically. Moreover, differences in the quantifications with ALA and GSA should certainly be expected because ALA-pyrroles are 2-(or 5-) unsubstituted, whereas GSA-pyrroles are 3-(or 4-) unsubstituted.

Table I Spectroscopic properties of Ehrlich reaction products of the synthetic pyrroles

Compound	Time (min) for complete reaction	λ_{max} (nm)	Extinction coefficient
 GSA-pyrrole (**5**)	45	486	865 ± 44
 ALA-pyrrole (**3**)	1 5	554	46,100 ± 2,800
 ALA-pyrrole (**4**)	20-30	470	15,200 ± 2,300 in acid only

In order to characterize the synthetic pyrroles, their quantitative determination with Ehrlich's reagent[6] was investigated Our results are given in Table I, and reveal drastic differences in the reaction products Pyrrole (**3**), derived from ALA and acetylacetone, reacted very fast with Ehrlich's reagent to form the characteristic pink product described in the literature,[10] (Figure 3) The absorption coefficient determined for the Ehrlich adduct was found to be 46,100 ± 2,800, which is about 20-25% smaller than values determined by reaction of ALA with acetylacetone followed by the Ehrlich's test [5,9] Reaction was complete within 80 sec

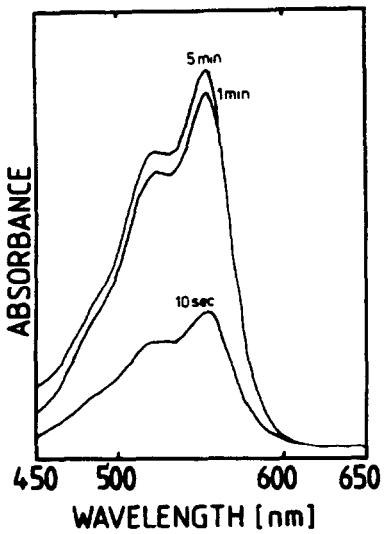


Figure 3 Absorption spectrum of ALA-pyrrole (3) reacted with Ehrlich's reagent after different reaction times (as specified)

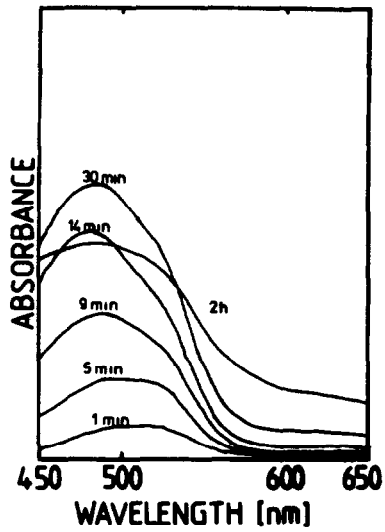


Figure 4, Absorption spectra of GSA-pyrrole (5) reacted with Ehrlich's reagent after different reaction times (as specified)

For presumably both electronic and steric reasons, the GSA-pyrrole (5), which reacts at the 3-position (rather than 2-), reacts considerably more slowly (Figure 4) This result is in agreement with the results of Treibs and Herrmann for other 3-(or 4-) unsubstituted pyrroles [12] They proposed a dipyrromethene structure for the reaction product, but obtained it only after considerably longer reaction times and under more drastic conditions than for 2-(or 5-) unsubstituted pyrroles In the case of GSA-pyrrole, this product is immediately formed but completion of the reaction takes time For practical purposes the absorbance should be measure after 40 min, since after longer reaction times some decomposition is observed Addition of water to the reaction mixture leads to broadening of the peak and a decrease in extinction at 486 nm down to about 50% The absorption maximum of the product, at 486 nm, is shifted almost 70 nm to shorter wavelengths compared with (3) Similar results were obtained by Mauzerall and Granick[5] for the Ehrlich reaction with ethyl 2,4-dimethylpyrrole-5-carboxylate (4) The weak pink color of the Ehrlich pigment from (5) is in agreement with observations of Gough et al [8] for the GSA-pyrrole (5) formed by condensation of GSA with acetylacetone

For GSA-pyrrole (5) an extinction coefficient of 865 ± 44 at λ_{max} 486 nm was determined. The extinction coefficients of all three pyrrole Schiffs bases determined were linear over an absorbance unit range of 0-0 8. The data for the GSA-pyrrole (5) clearly show the problems associated with the in situ condensation and subsequent Ehrlich reaction used so far Wang et al [10] and Kannangara and Schouboe[11] gave a l_{max} at 553 nm for the GSA-pyrrole (6) formed with ethyl acetoacetate and even determined a E_{mM} of 1. These results are clearly inconsistent with our findings with pure GSA-pyrrole (5) and therefore must be due to the formation of ALA and its detection via pyrrole formation as already suggested by Gough et al [8] The only pyrrole in our series which gives an absorption maximum at 553 nm is that from ALA

Surprising results were obtained with pyrrole (4), which can be formulated as the ethyl acetoacetate adduct of

ALA In pure glacial acetic acid and 4N $HClO_4$ an Ehrlich adduct with λ_{max} 470 nm and an extinction coefficient of 15,200 ± 2,300 is formed. If the pyrrole is dissolved in water instead, and then mixed with Ehrlich's reagent, a pink color product absorbing at 553 nm is formed initially The optical spectrum is identical with that obtained for the Ehrlich adduct of (3). Nevertheless, this species is transformed within 30 min into the compound absorbing at 470 nm in a process characterized by an isosbestic point at 490 nm (Figure 5) Since most of the data on ALA determination and quantification were performed upon the condensation product obtained by reaction of ethyl acetoacetate with ALA prior to the Ehrlich reaction, our results point to some critical factors in the ALA quantification method. Clearly, the amount of ALA determined in the Ehrlich test is very sensitive to both incubation time and the reaction conditions [12]

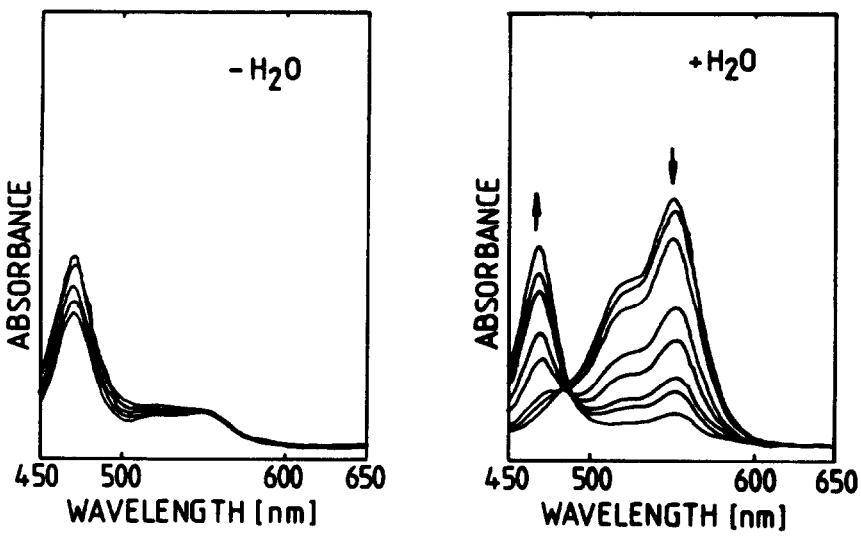


Figure 5: Absorption spectra of ALA-pyrrole (4) reacted with Ehrlich's reagent after different times, in the presence and absence of added water

Experimental

Melting points were measured on a Thomas/Bristoline microscopic hot stage apparatus and were uncorrected Silica gel 60 (70-230 and 230-400 mesh, Merck) or neutral alumina (Merck; usually Brockmann Grade III, i.e deactivated with 6% water) were used for column chromatography Preparative scale thin layer chromatography was carried out on 20 x 20 cm glass plates coated with Merck G 254 silica gel (2 mm thick). Analytical thin layer chromatography was performed using Merck 60 F254 silica gel (precoated sheets, 0 2 mm thick). Unless stated otherwise, proton NMR spectra were obtained in deuteriochloroform solution at 300 MHz using a General Electric QE300 spectrometer; chemical shifts are expressed in ppm relative to residual chloroform (7 258 ppm). Elemental analyses were obtained from MidWest Analytical Laboratory Electronic absorption spectra were measured in dichloromethane solution using a Hewlett-Packard 8450A spectrophotometer. For the standard Ehrlich test used in this work, the pyrroles were dissolved in glacial acetic acid/water (1.1, vol/vol) and an equal amount of modified Ehrlich's reagent (4N $HClO_4$)[6] was added. Absorbances were measured as indicated in Table I

2-Methylpyrrole (9): To a dry 1 liter round bottom flask equipped with a condenser at 0°C under argon containing 450 mL of tetrahydrofuran was added $LiAlH_4$ (23 94 g, 0 631 mol). This mixture was stirred for 10 minutes and 2-formylpyrrole (8) (20 0 g, 0 210 mol) dissolved in 150 mL of tetrahydrofuran was added dropwise over a period of 30 min After the addition was completed the dropping funnel was replaced with a condenser and the solution was refluxed for 36 h At this point the mixture was cooled to 0°C and ca 25 mL of water was added cautiously Subsequently, 4 N NaOH (25 mL) was added slowly, followed by water (100 mL) The gray-white lithium salts were filtered and the filtrate was concentrated under vacuum The light brown residue was dissolved in dichloromethane (50 mL) and washed with H_2O (2 x 25 mL), brine (25 mL) and then dried over anhydrous sodium sulfate The

dichloromethane was removed under vacuum and the light brown oil was distilled under reduced pressure with the fraction between 90-95°C 25 mm (lit bp 146-149 °C 760 mm[13]; 148°C 755mm[14] collected to give 12.65 g (0 156 mol, 74% yield) of a clear colorless oil (12 65 g, 0 1559 mol, 74%)· δ_H 8.00 (br s, 1H, NH), (d, J=3 Hz, 1H, H-3), 6 37 (q, J=3 Hz, 1H, H-5), 6 14 (br s, 1H, H-4), 2 44 (s, 3H, CH_3).

2-Formyl-5-methylpyrrole (10): Phosphorus oxychloride (15 2 mL, 0.163 mol) was added dropwise to N,N-dimethylformamide (16.03 mL, 0.207 mol) at -50°C in a dry ice-acetonitrile bath. Dichloroethane (50 mL) was added and the mixture was kept at -50 °C for 15 min The mixture was allowed to warm to 0°C. A solution of 2-methylpyrrole (9) (12.00 g, 0.148 mol) in dichloroethane (50 mL) was then added, and the mixture was kept at 0°C for 1 h The light green solution was allowed to warm to room temperature, and then refluxed for 30 min. At this time a solution of sodium acetate (100 65 g, 0.739 mol) in 300 mL water was added and the solution was refluxed for 1 h Upon cooling, the solution was washed with a saturated solution of sodium bicarbonate (4 x 100 mL). The organic layer was removed and the aqueous layer was extracted with diethyl ether (3 x 100 mL). The combined organic layers were washed with saturated sodium bicarbonate (2 x 200 mL), brine (2 x 200 mL) and dried over sodium sulfate The solvent was removed under vacuum, and the resulting residue was chromatographed on silica gel (elution with 100% dichloromethane) and collected to give 10 41 g (95 39 mmol, 64 5%) of the title compound as a white powder Mp 72-74°C (lit [15] mp 70°C); δ_H 11 09 (br s, 1H, NH), 9 33, (s, 1H, CHO), 6.92, (t, J=3 Hz, 1H, H-3), 6.06 (t, J=3 Hz, 1H, H-4), 2.38 (s, 3H, CH_3) Anal Calcd for C_6H_7NO C, 66 04, H, 6 47; N, 12 84 Found: C, 65 77, H, 6 43, N, 12 75

2-(2-Ethoxycarbonylvinyl)-5-methylpyrrole (13): 2-Formyl-5-methylpyrrole (**10**) (1 516 g, 13.89 mmol) was dissolved in *p*-xylene (100 mL) containing (carbethoxymethylene)-triphenylphosphorane (5 324 g, 15.28 mmol) and the mixture was refluxed for 12 h under nitrogen. At this time the *p*-xylene was removed under vacuum and the resulting residue was chromatographed on silica gel (elution with 100% dichloromethane) The product was collected to give 2 31 g (12 89 mmol, 93%) of the title compound. Mp 101-103°C, δ_H 8 73 (br s, 1H, NH), 7 49 (d, J=16 Hz, 1H, C<u>H</u>CHCO$_2$Et), 6 44 (t, J=3 Hz, 1H, H-3), 5 95 (d, J=16 Hz, CHC<u>H</u>CO$_2$Et), 5 91 (t, J=3 Hz, 1H, H-4), 4 23 (q, J=7 Hz, 2H, C<u>H</u>$_2$CH$_3$), 2 31 (s, 3H, CH_3), 1 31 (t, J=7 Hz, 3H, CH$_2$C<u>H</u>$_3$) Anal Calcd for $C_{10}H_{13}NO_2$ C, 67 02, H, 7 31, N, 7 82 Found C, 66 97, H, 7 23, N, 7 69

4-Acetyl-2-formyl-5-methylpyrrole (11): 2-Formyl-5-methylpyrrole (**10**) (1 20 g, 10 99 mmol) was dissolved in a solution of dichloromethane (100 mL) and acetic anhydride (1 68 g, 16 49 mmol) Distilled tin(IV) tetrachloride (6 01 g, 23 09 mmol) was added dropwise over a period of 5 min and the mixture was then stirred at room temperature for 8 h The dark solution was poured into ice-water (100 mL) and the organic layer was washed with saturated sodium bicarbonate solution (2 x 100 mL), saturated sodium chloride solution (2 x 100 mL) and then dried over anhydrous sodium sulfate The solvent was removed under vacuum and the residue was chromatographed on a column of silica gel (elution with dichloromethane) to give 1 20 g (7 95 mmol, 72% yield) Mp 146-147°C (sublimes at about 80°C), δ_H 10 81 (br s, 1H, NH), 9 43 (s, 1H, CHO), 7 31 (s, 1H, H-3), 2 66 (s, 3H, CH_3), 2 46 (s, 3H, $COCH_3$) Anal Calcd for $C_8H_9NO_2$ C, 63 56, H, 6 00, N, 9 27 Found· C, 63 53, H, 6 00, N, 9 38

4-Acetyl-2-(2-ethoxycarbonylvinyl)-5-methylpyrrole (12): *(a) From Pyrrole (11)* - 4-Acetyl-2-formyl-5-methylpyrrole (**11**) (0 80 g, 5 29 mmol) was dissolved in *p*-xylene (50 mL) containing (carbethoxymethylene)-triphenylphosphorane (2 03 g, 5 82 mmol) and the mixture was heated under reflux for 12 h under nitrogen After this time the *p*-xylene was removed under vacuum and the residue was chromatographed on a column of silica gel (elution with 2% methanol in dichloromethane) to afford 1 02 g (4 65 mmol, 88%) of the title pyrrole Mp 198-200°C, δ_H 9 53 (br s, 1H, NH), 7 47 (d, J=16 Hz, 1H, 2-C<u>H</u>CHCO$_2$Et), 6 79 (d, J=3 Hz, 1H, H-3), 6 07 (d, J=16 Hz, 1H, 2-CHC<u>H</u>CO$_2$Et), 4 24 (q, J=7 7 Hz, 2H, C<u>H</u>$_2$CH$_3$), 2 59 (s, 3H, CH_3), 2 41 (s, 3H, $COCH_3$), 1 32 (t, 3H, CH$_2$C<u>H</u>$_3$) Anal Calcd for $C_{12}H_{15}NO_3$ C, 65 14, H, 6 83, N, 6 33 Found C, 65 05, H, 6 83, N, 6 51

(b) From Pyrrole (13) - 2-(2-Ethoxycarbonylvinyl)-5-methylpyrrole (**13**) (0 80 g, 4 46 mmol) was dissolved in a solution of dichloromethane (40 mL) and acetic anhydride (0 911 g, 8 92 mmol), and the mixture was cooled to 0°C Distilled tin(IV) tetrachloride (2 32 g, 8 92 mmol) was added dropwise over a period of 5 min The solution was stirred at 0°C for 30 min and then poured into ice water (100 mL) The organic layer was washed with saturated sodium bicarbonate (2 x 50 mL), saturated sodium chloride (2 x 50 mL) and then dried over sodium sulfate The solvent was removed under vacuum and the resulting residue was chromatographed on silica gel (elution with 2% methanol in dichloromethane) The title compound was collected to give 0 67 g (3 26 mmol, 73%), identical in all respects with the material obtained from method *(a)*

4-Acetyl-2-(2-ethoxycarbonylethyl)-5-methylpyrrole (14): 4-Acetyl-2-(2-ethoxycarbonyl-vinyl)-5-methylpyrrole (**12**) (0 131 g, 0 592 mmol) was dissolved in dry tetrahydrofuran containing 10% Pd on activated carbon (25 mg), and hydrogenated at room temperature and atmospheric pressure until hydrogen uptake had ceased The mixture was filtered through a Celite pad, and the solvent was removed under vacuum The resulting residue was chromatographed on a silica gel (elution with 2% methanol in dichloromethane) and was collected to give 0 102 g

(0 458 mmol, 77%) of the title compound Mp 122-124°C, δ_H 9.12 (br, s 1H, NH), 6 17 (d, J=3 Hz, 1H, H-3), 4 14 (q, J=7 Hz, 2H, $C\underline{H}_2CH_3$), 2.82 (t, J=7 Hz, 2H, $C\underline{H}_2CH_2CO_2Et$), 2 61 (t, J=7 Hz, 2H, $CH_2C\underline{H}_2CO_2Et$), 2.48 (s, 3H, CH_3), 2 35 (s, 3H, $COCH_3$), 1 25 (t, J=7 Hz, 3H, $CH_2C\underline{H}_3$) Anal Calcd. for $C_{12}H_{17}NO_3$: C, 64 55, H, 7 67, N, 6 27 Found C, 64 39, H, 7 68; N, 6 46

4-Acetyl-2-(2-carboxyethyl)-5-methylpyrrole (5): 4-Acetyl-2-(2-ethoxycarbonylethyl)-5-methyl-pyrrole (**14**) (0 23 g, 1 12 mmol) was dissolved in tetrahydrofuran (5 mL) and a solution of sodium carbonate (3.00 g) and sodium bicarbonate (3.00 g) dissolved in water (20 mL) was added. The mixture was heated at reflux for 3 days The solvent was removed under vacuum and to the resulting residue was added 10% hydrochloric acid (25 mL) This solution was extracted with dichloromethane (3 x 25 mL). The resulting organic layer was washed with water (2 x 25 mL), saturated sodium chloride (2 x 25 mL), and then dried over anhydrous sodium sulfate. The solvent was removed under vacuum and the resulting solid was washed with cyclohexane to give 0.12 g (0.669 mmol, 60%) of the title compound as a white powder. Mp 186-189°C, δ_H 6 05 (s, 1H, H-3), 2 66 (t, J=7 Hz, 2H, $C\underline{H}_2CH_2CO_2H$), 2 44 (t, J=7 Hz, 2H, $CH_2C\underline{H}_2CO_2H$), 2 32 (s, 3H, CH_3), 2 21 (s, 3H, $COCH_3$) Anal Calcd. for $C_{10}H_{13}NO_3$: C, 61 52; H, 6 72, N, 7 18 Found C, 61 52, H, 6 82, N, 7 25

4-Acetyl-3-(2-carboxyethyl)-5-methylpyrrole (3): This pyrrole was made according to the literature procedure[5] from ALA and acetylacetone. It had mp 195°C (Lit [5] 194-195°C). δ_H (d_6-DMSO) 6 12 (d, J=2 1 Hz, 1H, H-2), 2 69 (t, J=7 Hz, 2H, $C\underline{H}_2CH_2CO_2H$), 2 51 (t, J=7 Hz, 2H, $CH_2C\underline{H}_2CO_2H$), 2 36 (s, 3H, CH_3), 2 22 (s, 3H, $COCH_3$) Anal Calcd. for $C_{10}H_{13}NO_3$ C, 61 52, H, 6 72, N, 7.18 Found C, 61 27, H, 6 79; N, 7 21

Ethyl 3-(2-Carboxyethyl)-5-methylpyrrole-4-carboxylate (4): This pyrrole was prepared according to the literature procedure[5] from ALA and ethyl acetoacetate It had mp 165°C (Lit.[5] 164-165°C) δ_H (d_6-DMSO) 11 93 (s, 1H, CO_2H), 10 93 (br s, 1H, NH), 6 39 (s, 1H, H-2), 4 12 (q, J=7 Hz, 2H, $OC\underline{H}_2CH_3$), 2 77 (t, J=7 Hz, 2H, $C\underline{H}_2CH_2CO$), 2 40 (t, J=7 Hz, 2H, $CH_2C\underline{H}_2CO$), 2 34 (s, 3H, CH_3), 1 23 (t, J=7 Hz, 3H, $OCH_2C\underline{H}_3$) Anal Calcd for $C_{11}H_{15}NO_4$ C, 58 64, H, 6 72, N, 6 22 Found C, 58.39, H, 6 77, N, 6 33

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