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Non-enzymic tetramisation of ethyl 3-(4-ethoxycarbonylmethyl-1*H*-pyrrol-3-yl)propionate with formaldehyde follows a similar course to the non-enzymic tetramisation of porphobilinogen

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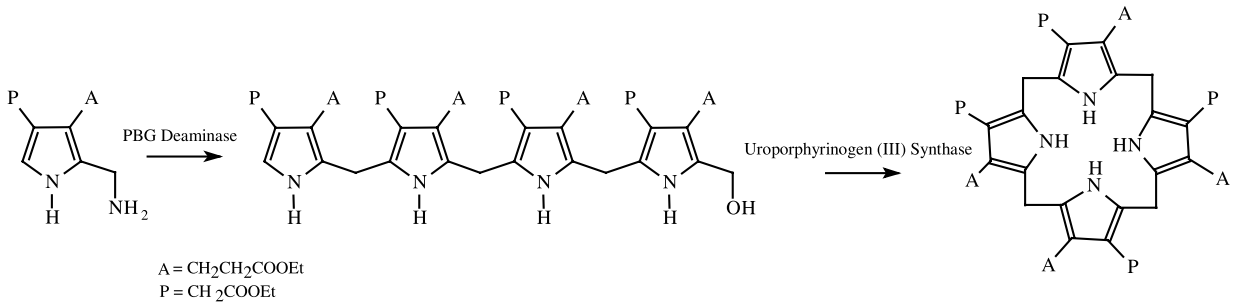
Abstract—The octaethyl esters of uroporphyrins were formed directly from ethyl 3-(4-ethoxy-carbonylmethyl-1*H*-pyrrol-3-yl)propionate and formalin in a yield of ca. 30% with the isomers I, II, III and IV being formed in the ratio 1:1:4:2. Under anaerobic conditions, the colourless uroporphyrinogen esters were formed in a similar ratio. These observations parallel the non-enzymic formation of uroporphyrinogens from the naturally occurring tetrapyrrole precursor, porphobilinogen, highlighting the similarity in both tetramisation and isomerisation reactions. © 2001 Elsevier Science Ltd. All rights reserved.

Porphobilinogen (3-(5-aminomethyl-4-carboxymethyl-1*H*-pyrrol-3-yl)-propionic acid) is the monopyrrolic building unit from which all tetrapyrroles are formed in biological systems.[1] Four molecules of porphobilinogen are transformed into uroporphyrinogen III by the action of two enzymes (Scheme 1); porphobilinogen deaminase[2] that forms a linear 1-hydroxymethylbilane called preuroporphyrinogen from four molecules of porphobilinogen and uroporphyrinogen III synthase[2] that catalyses the cyclisation and rearrangement of preuroporphyrinogen specifically to the uroporphyrinogen III isomer. The latter reaction has attracted much interest over several decades since it involves the isomerism of the terminal pyrrole ring (ring d) of preuroporphyrinogen.

Experiments carried out some 40 years ago demonstrated that any single isomer of uroporphyrinogen, when heated in dilute acid under anaerobic conditions,[3] generated the same ratio of uroporphyrinogen I, II, III and IV isomers in the ratio 1:1:4:2. In basic or neutral conditions, the interconversion of the isomers did not occur significantly. In the presence of dimedone,[4] a formaldehyde scavenger, the yields of uroporphyrinogens were reduced substantially suggesting that the aminomethyl group of porphobilinogen, or its hydroxymethyl equivalent, is freely in equilibrium with formaldehyde. This was also suggested by carrying out the non-enzymic polymerisation of porphobilinogen in the presence of formaldehyde, which again lowered the yield of uroporphyrinogens.



Scheme 1.

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In a related study, it was demonstrated that the natural tetrapyrrole precursor, porphobilinogen, may be transformed non-enzymically into uroporphyrinogens in high yield by heating in dilute acid under anaerobic conditions. A mixture of all four possible isomers of uroporphyrinogen (I, II, III and IV) was formed in the same ratio (1:1:4:2) as in the isomerisation of uroporphyrinogens.

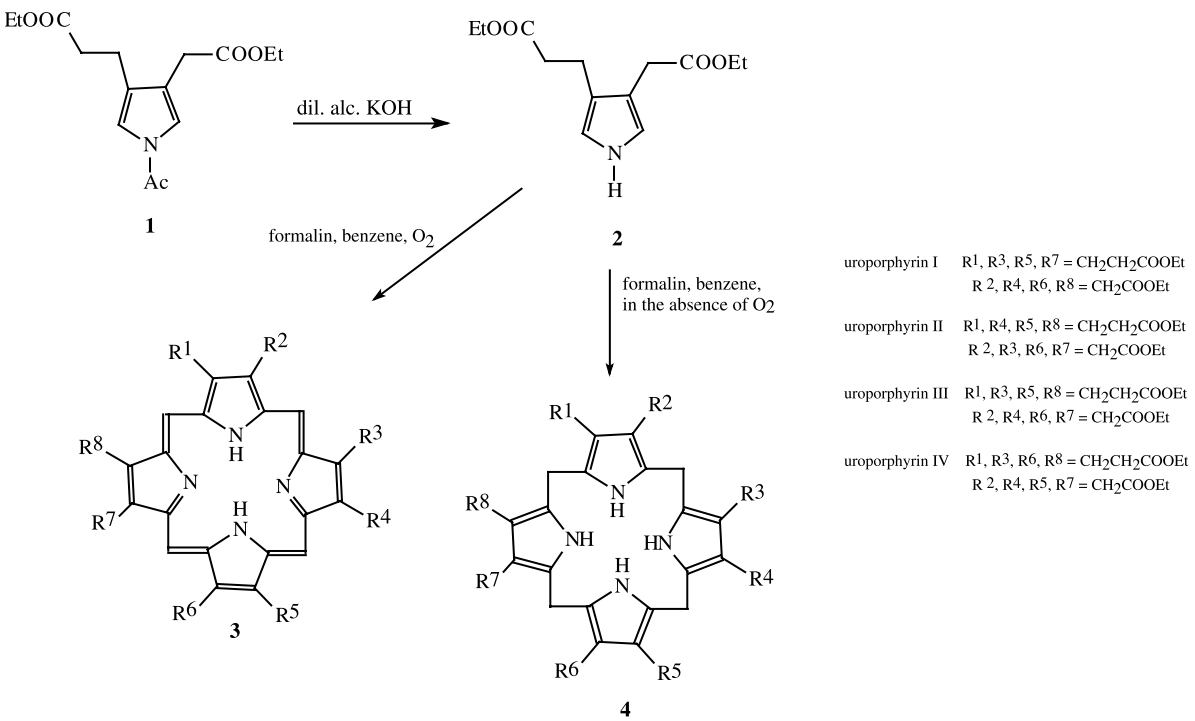
These observations suggested that the bond lability at the pyrrole α-positions was likely to be exploited in the enzyme-catalysed rearrangement of preuroporphyrinogen into uroporphyrinogen III. Any mechanism must take into account the observation that all the enzymic reactions occur with strict steric control with no evidence of randomisation of the paired *meso*-hydrogen atoms.[5] In this paper we report the formation of octaethyl-uroporphyrinogens and octaethyl-uroporphyrins by reaction of ethyl 3-(4-ethoxy-carbonylmethyl-1*H*-pyrrol-3-yl)propionate **2** (opsopyrrole dicarboxylic ester) with formaldehyde to compare the reaction with the equivalent tetramisation of porphobilinogen.

The preparation of the octaethyl-uroporphyrins is shown in Scheme 2. Compound **1** was prepared according to the literature.[6] Compound **2**[7] was then obtained in 60% yield by hydrolysis of **1** in dilute alcoholic KOH for 3–4 hours. This was then refluxed with formalin (37%) and a catalytic amount of *p*-TsOH in benzene, in the dark, under nitrogen and then open to the air. After work-up, crude octaethyl-uroporphyrins **3**,[8] were obtained in ca. 30% yield. Recrystallisation from $CHCl_3$/EtOH, yielded dark brownish-red amorphous crystals.

The *meso*-protons at ca. δ 10.25 and NH protons at δ −3.60 can be clearly observed. The broadness of the *meso*-proton peaks arises since all four isomeric uroporphyrins are present. The ^{13}C NMR (not shown) revealed the structure of the octaethyl-uroporphyrins, in which the *meso*-carbons are at δ 97.8 and the pyrrolic carbons are at δ 144.6, 144.0, 140.9 and 132.9. A small amount of the octaethyl-uroporphyrin mixture was hydrolysed in 2 M NaOH/EtOH at room temperature overnight and the products were purified using an Amberlite IR 120 (H^+ form) column. After evaporation of the solvent, dark reddish-violet crystals were obtained.

These isomers of uroporphyrin free acids were separated by reverse phase HPLC using a 150 mm×3.9 mm Nova-pak C_{18} column connected to a Waters 626 pump and a Waters 600s controller, eluting with 13% v/v acetonitrile and 87% 1 M ammonium acetate buffer at pH 5.16.[9] The uroporphyrin free acid isomers I, II, III and IV were eluted in a ratio of ca. 1:1:4:2.

The chemical formation of uroporphyrinogen esters under anaerobic conditions was also investigated by in situ ^1H NMR. Ethyl 3-(4-ethoxycarbonylmethyl-1*H*-pyrrol-3-yl)propanoate **2**, formalin (37%) and a *p*-TsOH crystal in deuterated chloroform were introduced into a NMR tube and placed in a long Dewar flask containing liquid nitrogen. The NMR tube was connected to a three-way tap to allow the contents to be evacuated and filled with nitrogen gas several times. The NMR tube was then sealed and warmed to 4°C in the spectrome-



Scheme 2.

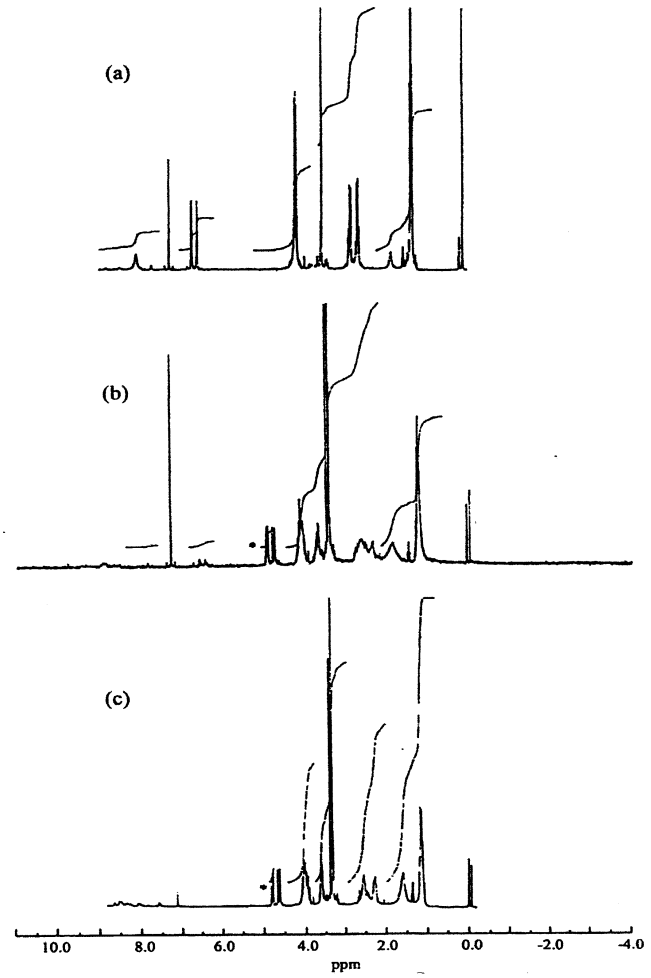


Figure 1. The in situ ^{1}H NMR spectra illustrating the formation of octaethyl uroporphyrinogens from compound **2** and formalin under anaerobic conditions: (a) 0 minutes at 4°C; (b) 100 minutes at 4°C; (c) additional 20 minutes at 25°C. * indicates the impurities from formalin, partly erased for clarity.

ter. The in situ ^{1}H NMR (250 MHz) showed little evidence for the formation of uroporphyrinogen esters at 4°C until after a period of 100 minutes (Fig. 1b). When the temperature was raised to 25°C for another 20 minutes (Fig. 1c), products were observed clearly. The ^{1}H NMR peaks were broad, with ethyl groups at δ 4.25 and δ 1.25, propanoic groups at δ 2.70 and δ 2.42 (both having an upfield chemical shift of ca. 0.06 ppm), the acetic group at δ 3.45 (upfield chemical shift of ca. 0.05 ppm) and the *meso*-protons at δ 3.70. No sign of peaks at ca. δ 10.25 and δ −3.6 were observed (Fig. 1b), indicating that no uroporphyrins had been formed. A ^{13}C NMR spectrum was obtained using the same sample tube immediately after the in situ ^{1}H NMR experiment. From the spectrum (not shown), the structure of the uroporphyrinogens was also revealed in which the *meso*-carbons appear at δ 21.8 and the pyrrolic carbons at δ 125.6, 124.0, 117.0 and 110.0. The ^{13}C NMR results of the chemically synthesised uroporphyrinogens reported here were similar to the ^{13}C NMR data of the uroporphyrinogens I and III, which were prepared enzymatically.[10] The above results show that a mixture of uroporphyrins can be prepared by a relatively simple procedure in yields approaching 30%. By use of HPLC techniques for the separation of uroporphyrin esters and free acids, it is possible to obtain pure isomers on a large scale. The in situ ^{1}H NMR studies clearly show the formation of a mixture of uroporphyrinogen esters from ethyl 3-(4-ethoxycarbonylmethyl-1*H*-pyrrol-3-yl)-propionate **2** and formalin under anaerobic conditions.

The findings indicate that, whether the staring material is porphobilinogen alone or opsopyrrole dicarboxylic ester and formaldehyde, the ratio of isomers is the same and that therefore the mechanism is likely to be the same in both processes, with 1-hydroxymethylbilanes, such as preuroporphyrinogen, as intermediates.

The above study not only provides further insight into the novel reactivity of reduced porphyrinogen macrocyclic systems but, in addition, offers a simple alternative method for the synthesis of all four octaethyl-uroporphyrinogen isomers and their oxidised equivalents.

Acknowledgements

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7. Compound **2** was obtained in 60% yield from the hydrolysis of **1** in dilute KOH/EtOH. δ_H (250 MHz, $CDCl_3$): 8.15 (s, 1H), 6.70 (s, 1H), 6.55 (s, 1H), 4.15 (m, 4H), 3.49 (s, 2H), 2.78 (t, 2H, J=7.0 Hz), 2.56 (t, 2H, J=7.0 Hz), 1.25 (m, 6H).
8. Compound **2** (0.33 g) was then refluxed with formalin (37%, 0.12 ml) and a catalytic amount of *p*-TsOH in benzene (40 ml) using a Dean–Stark apparatus for 4 hours under N_2 in the dark. After this, the solution was stirred in the open air at 20°C overnight, followed by bubbling with O_2 for 2 hours. The resulting solution was washed with 1 M NaOH (2×20 ml), water (1×20 ml), dried with Na_2SO_4 and treated with decolourising charcoal. Filtration and lyophilisation gave **3** (90 mg, 26%). Crystallisation from $CHCl_3$/EtOH gave brownish-red

amorphous crystals (40 mg, 12%). R_f=0.65 (EtOAc:toluene 1:1). UV–vis ($CHCl_3$): λ_{max} (ε)=405.2 (1.65×10^5) [lit.[12] λ_{max} (ε)=405 (2.15×10^5)], 501.2 (1.39×10^4), 534.8 (0.83×10^4), 570.8 (0.6×10^4) and 624.8 (0.36×10^4). δ_H (250 MHz, $CDCl_3$): 10.25 (m, 4H), 5.15 (m, 8H), 4.50 (m, 8H), 4.20 (m, 16H), 3.40 (m, 8H), 1.20 (m, 24H) and −3.60 (s, 2H). δ_C: 173.21, 172.78, 171.11, 144.6, 144.0, 140.93, 132.93, 97.80, 61.09, 60.33, 37.11, 32.69, 21.60, 14.99, 13.89. FAB-MS: m/z (%): 1055 [M+H] (82), 1041 [M+2H−Me] (100), 1027 [M+2H−Et] (70) and 982 [M+H−2Et−Me] (18).

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