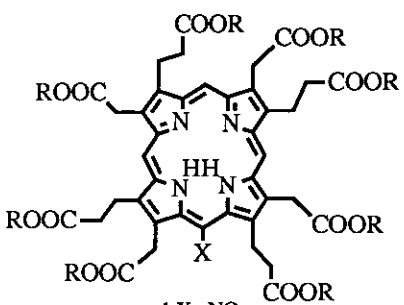
ISOLATION OF NOVEL MESO-SUBSTITUTED UROPORPHYRINS FROM CULTURES OF PSEUDOMONAS

Wen Lin, Robert S. Burkhalter, and Russell Timkovich*

Department of Chemistry, University of Alabama
Tuscaloosa, Alabama 35487-0336, U.S.A.

Abstract---Two novel and unusal porphyrins have been isolated from the pool of porphyrin metabolites in cultures of Pseudomonas. They correspond to substitution of a meso proton in uroporphyrin by a nitro group or a chlorine atom. The purified compounds have been characterized by ^{1}H-nmr, mass spectrometry, and visible spectroscopy to establish their structure.

When aminolevulinic acid (ALA) is added to anaerobic cultures of Pseudomonas, tetrapyrrole biosynthesis is tremendously stimulated to the point where porphyrinogens are actually secreted into the surrounding culture medium by the cells and then undergo air oxidation to aromatic porphyrins.[1] From such cultures we have recently purified and characterized uroporphyrin, porphyrins decarboxylated at one to three acetate positions, and coproporphyrin, and have determined the geometrical isomers that are produced. [2] In the course of that work, some unusual compounds were discovered including two novel porphrins (1 and 2) with substituted meso positions that are described in this communication.



1 X= NO_2
2 X= Cl

a R= H
b R= CH_3

The porphyrin mixture containing **1a** or **2a** was collected from cultures of Pseudomonas mendocina (ATCC 25411) or Pseudomonas stutzeri strain Zobell (formerly P. perfectomarius[3] ATCC 14405) respectively and fractionated as described previously.[2] After mineral acid esterification to **1b** and **2b**, the compounds were purified on silica gel hplc columns. **1b** eluted with a capacity factor of 8.33 in chloroform:hexane 8:2 with 0.025% pyridine, while **2b** eluted with a capacity factor of 21.7. These compounds are not produced in great quantitites. The final isolated yields were 5 nanomoles of **1b** from one liter cultures of P. mendocina and 8 nanomoles of **2b** from the Zobell strain, whereas, these same organisms secreted 4.4 and 3.1 micromoles respectively of uroporphyrin per liter of culture.

The ^{1}H-nmr spectra were obtained in deuterated chloroform at 298 ^{o}K and were referenced versus residual protic chloroform at 7.26 ppm. The outstanding characteristic of each spectrum was the presence of only three meso singlet resonances. (500 MHz, **1b**: meso (s, 1H each), 10.376, 10.365, and 10.221 ppm; acetate , 5.139 (s, 4H), 5.126 (s, 2H), and 4.818 (s, 2H) ppm; propionate (t, J=7 Hz, 2H each), 4.447, 4.433, 4.398, 4.061, 3.371, 3.366, 3.325, and 3.087 ppm; methyl esters (s, 3H each), 3.856, 3.811, 3.805, 3.798, 3.784, 3.711, 3.688, and 3.668 ppm; NH (br, 1H each), -3.42 and -3.65 ppm. **2b**: meso (s, 1H each), 10.232, 10.215, and 10.021 ppm; acetate (s, 2H each), 5.217, 5.161, 5.118, and 5.077 ppm; propionate (t, J=7 Hz, 2H each except where noted), 4.529, 4.425, 4.415, 4.398, 3.358, 3.347, and 3.290 (4H) ppm; methyl esters (s, 3H each), 3.843, 3.796, 3.791, 3.784, 3.775, 3.703, 3.693, and 3.676 ppm; NH (br, 2H), -3.00 ppm.) The NH resonances of **1b** were unusual in demonstrating at room temperature two peaks. It has been well documented that in most porphyrins, rapid tautomerization averages the two protons into a single resonance even in unsymmetrical porphyrins.[4] Meso-nitrated protoporphyrin IX methyl ester was prepared as described previously. [5] Three products were purified by silica hplc corresponding to NO_2 substitution at three of the four inequivalent meso positions. These compounds also had two NH resonances (5-nitro, -3.75 and -3.85 ppm; 15-nitro, -3.56 and -3.77 ppm; 20-nitro, -3.76 and -3.93 ppm.). It is unlikely that tautomerization has been slowed by the meso substitution. It is more plausible that the two resonances represent a splitting of the tautomers into two classes were the chemical shift difference is significant versus the exchange rate so that resolution can be seen at room temeprature. For example, the two states may represent when the protons are on the two nitrogens closest to the substituted meso or the two furthest away. The porphyrin skeleton is inherently asymmetrical because of the single meso substitution, but, it was determined to be the type I isomer by means of rotating frame nuclear Overhauser enhancement measurements.[6] Each meso singlet gave an enhancement to both an acetate and a propionate substituent, which is only possible for the type I isomer . Visible spectra of the esters in chloroform (band maxima (relative absorbance): **1b**; 407(100), 506(9.4), 539(5.2), 575(4.7), and 630(2.9) nm. **2b**; 411(100), 508(8.4), 542(2.5), 581(2.9), and 632(0.8) nm.) had phyllo-type characteristics, which is typical for meso substituted porphyrins.[7]

For the quantitites available, the substituents "X" could only be identified by detailed mass spectrometric studies. High resolution molecular ion mass determinations (determined by liquid secondary ion mass spectrometry, LSIMS, also known as fast atom bombardment) were in complete agreement with the assigned structures (**1b**: obs. 988.344, σ= 0.002, for the monoprotonated molecular ion; calc., 988.346 for $C_{48}H_{54}N_5O_{18}$; **2b**: obs. 977.323, σ= 0.002 and 979.320, σ= 0.003 for the monoprotonated molecular ions; calc., 977.322 for $C_{48}H_{54}N_4O_{16}{}^{35}Cl$ and 979.319 for $C_{48}H_{54}N_4O_{16}{}^{37}Cl$). Other mass spectral characteristics add further evidence to the assigned structures. In electron impact mass spectra, the chloro-compound (**2b**) gave the isotope distribution expected for a mono-Cl compound, and fragmented readily with loss of Cl and HCl. **1b** underwent a peculiar reaction in LSIMS spectra in some liquid matrices. In matrices containing thiol-compounds (thioglycerol, or "magic bullet",[8] the 988 protonated molecular ion during the lifetime of the sample on the LSIMS probe tip, decreased in intensity and was replaced by an ion with m/z of 974 (exact mass 974.360, σ= 0.005; calc. 974.367 for $C_{48}H_{56}N_5O_{17}$). We believe this is due to *in situ* reduction of $-NO_2$ to -NHOH by reducing equivalents from the matrix. Non-thiol matrices (nitrobenzyl alcohols and glycerol) did not produce this reaction. The nitro-derivatives of protoporphyrin IX methyl ester underwent the same conversion during LSIMS in the thiol matrices.

It is difficult to explain the genesis of these meso-substituted porphyrins. It seems unlikely that they are mainstrearm anabolic intermediates. It is more likely that they are side reactions occuring during porphyrin biosynthesis highly stimulated by exogenous aminolevulinic acid. The cells were grown anaerobically on high concentrations of nitrate to sustain dissimlatory denitrification. However, the nitronium cation should not be present at sufficient levles for direct attack on the porphyrin, since the cultures were at neutral pH. However, denitrification does produce nitrite, NO, and other complicated oxides of nitrogen as intermediates, and there could exist a multi-step reaction involving attack by some such species on porphyrin or porphyrinogen, either intra or extracellularly, followed by later oxidation to a nitro group. Small levels of chlorine or some other such activated form may be produced from culture medium chloride by side reactions of denitrifications, leading to the chloro-porphyrin.

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