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CHEMICAL OXIDATION OF SYNTHETIC IRON(III)-COMPLEX OF 15-PHENYL PROTOPORPHYRIN IX

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Abstract - Oxidative cleavage of 15-phenyl protohemin IX in pyridine solution in the presence of ascorbic acid (coupled oxidation), followed by esterification of the products with boron trifluoride-methanol rendered only three isomeric biliverdins. These were identified by ^{1}H NMR and MS spectrometry as 10-phenyl biliverdin IXα (**1**), 5-phenyl biliverdin IXβ (**2**) and 15-phenyl biliverdin IXδ (**3**) dimethyl esters. The fact that biliverdin IXγ dimethyl ester derivative is not obtained indicates that oxidation fails to occur in the γ-meso-carbon bearing the phenyl group.

INTRODUCTION

Heme is degraded in general metabolism by a microsomal heme oxygenase, which selectively cleaves the α-meso-carbon to give biliverdin IXα, CO and free iron.[1-3] This reaction occurs at the expense of O_2 and reducing equivalents provided by NADPH and cytochrome P450 reductase.

It is widely accepted that in the first step of the heme oxygenase reaction heme is hydroxylated at the α-meso carbon leading to α-meso-hydroxyheme. The main experimental evidence for this intermediate is the finding that synthetic α-meso-hydroxyheme is converted to biliverdin IXα both by HO-1 (inducible mammalian isoform of heme oxygenase)[4-5] and by a variety of heme oxygenase systems.[6-9] In the second step of the reaction, α-meso-hydroxyheme undergoes loss of the α-meso-carbon bearing the hydroxyl group as CO to give verdoheme. Finally, in the third step, heme oxygenase catalyzes the formation of biliverdin from verdoheme in a reaction that also requires NADPH, cytochrome P450 reductase, and O_2 (Figure 1).[10,11]

On the other hand, Ortiz de Montellano *et al.* reported the oxidation by HO-1 of α-meso-methylmesoheme and γ-meso-methylmesoheme to biliverdins IXα and IXγ respectively without the concomitant formation of CO.[12] These findings established that the α-meso-carbon and the attached

methyl group are eliminated by a mechanism that does not procede *via* the normal α-meso-hydroxyheme intermediate.

On carrying out chemical oxidation (coupled oxidation) of hemin IX all four possible biliverdin isomers IXα, IXβ, IXγ and IXδ are obtained, indicating that oxidative cleavage takes place indifferently on the four meso carbon atoms. This also occurs with other hemins bearing diverse substituents on the porphyrin ring.[13,14]

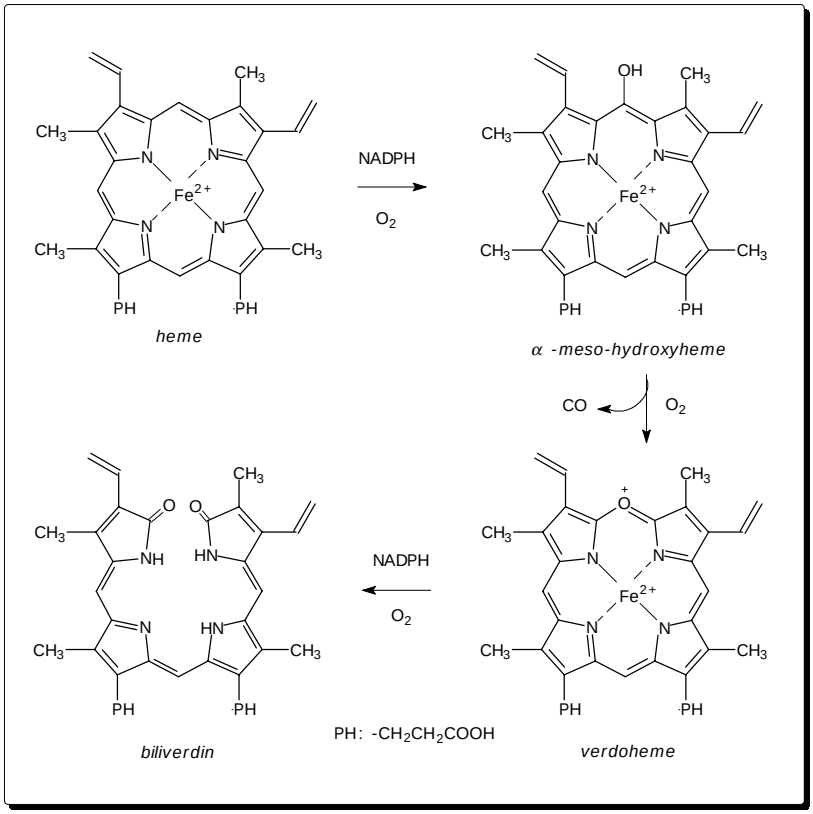


Figure 1. Enzymatic degradation of heme IX to biliverdin IXα

In order to re-examine the above mechanism, we carried out the complete synthesis of 15-phenyl protoporphyrin IX,[15] which possesses a substitution pattern similar to natural substrate hemes of the heme oxygenase, as well as a bulky substituent in the γ-meso position of the porphyrin ring. When the Iron(III)-complex of this porphyrin was subjected to chemical oxidation, three isomeric biliverdins were isolated as methyl esters. All possessed a phenyl group and were assigned to isomers 10-phenyl biliverdin IXα (**1**), 5-phenyl biliverdin IXβ (**2**) and 15-phenyl biliverdin IXδ (**3**) (Figure 2).

EXPERIMENTAL

Preparation of Iron(III)-complex of 15-phenyl protoporphyrin IX dimethyl ester

15-Phenyl protoporphyrin IX dimethyl ester (80 mg, 0.11 mmol) was dissolved in 1.5 mL of pyridine and 80 mL of acetic acid, and 1 mL of aqueous saturated ferrous sulfate was added at once. A stream of

nitrogen was then passed through while the solution was heated at 80ºC for 20 min. The mixture was allowed to stand in air for 20 min to allow auto-oxidation to the ferric complex to occur.

The resultant solution was diluted with chloroform (200 mL), washed with a 25% hydrochloric acid solution (2 x 150 mL) then with a saturated NaCl solution (100 mL) and finally dried (Na_2SO_4) and evaporated to dryness at reduced pressure. Hemin dimethyl ester was purified by dissolution in a small volume of 5% methanol in methylene chloride and filtered through a TLC silica gel packed column (2 x 25 cm) previously washed under slight pressure with the same solvent. The hemin was eluted as a single band by using the same solvent while low pressure was applied. Evaporation of the hemin containing eluates afforded 80 mg (92%) of product. MS: *m/z* (relative intensity), 720 (M^+, 100); VIS spectrum (methylene chloride): λ max 400 nm (ε 67000), 573(7000).

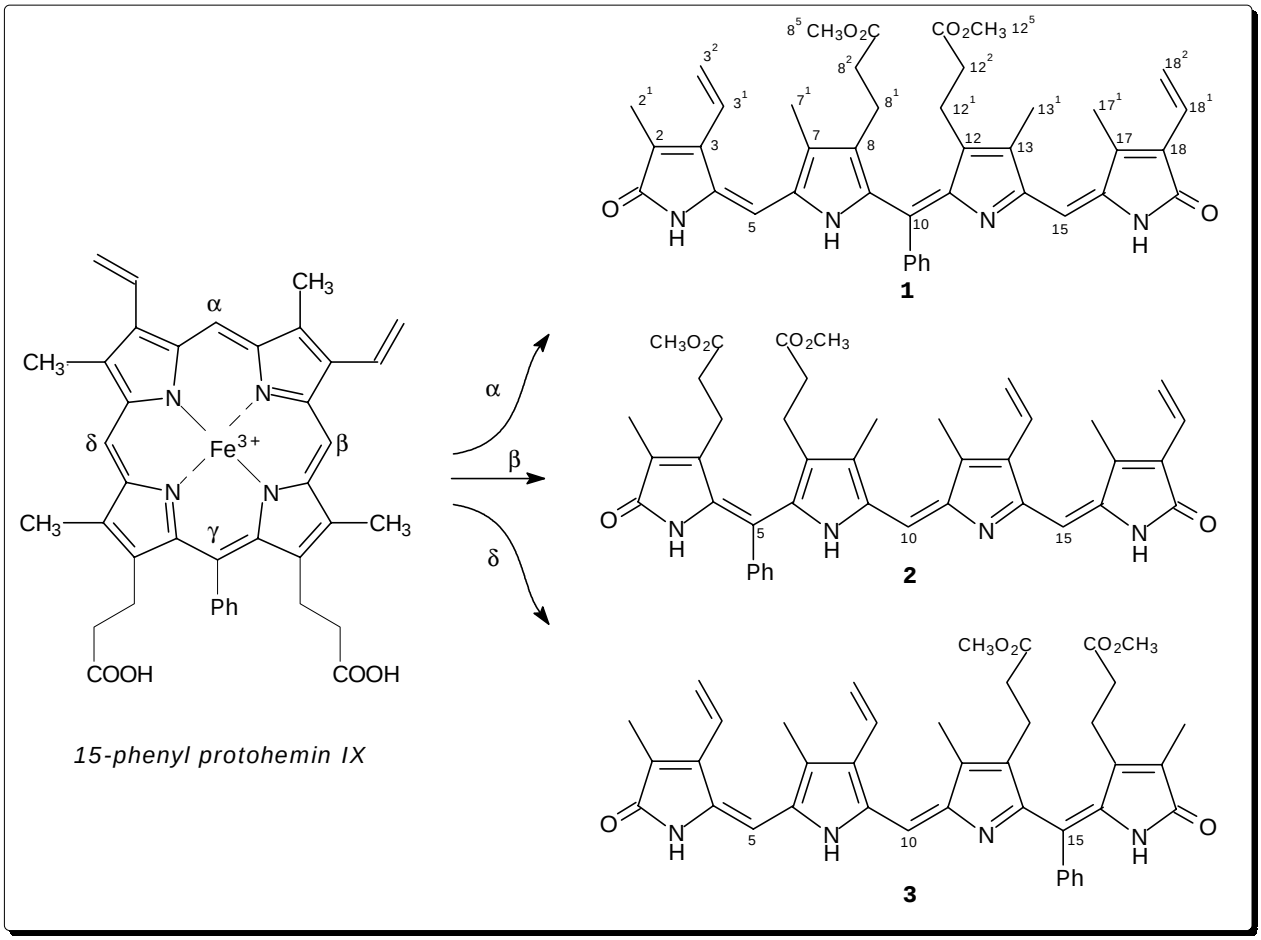


Figure 2. The three biliverdin isomers obtained by chemical oxidation of 15-phenyl protohemin IX after esterification of the reaction products.

Chemical oxidation of hemin to biliverdins

Iron(III)-complex of 15-phenyl protoporphyrin IX dimethyl ester (20 mg, 0.028 mmol) was dissolved in a mixture of 10 mL of tetrahydrofuran, 5 mL of methanol and 2 mL of 1 M potassium hydroxide, and the solution kept in the dark at room temperature for 48 h. The organic solvent was then evaporated under a

stream of nitrogen at 25ºC and the aqueous solution adjusted to pH 7.0 with a 1.5 M monosodium phosphate solution. The Iron(III)-complex of 15-phenyl protoporphyrin IX thus obtained was treated with oxygen-ascorbic acid according to the method of Bonnett *et al.*[13] with slight modification in the separation procedure. Briefly, the hemin solution was diluted with 80 mL of pyridine and 240 mL of water. Oxygen was bubbled through the solution while it was kept at 37ºC during 20 min in the dark. The stream of oxygen was then discontinued and a solution of 240 mg of ascorbic acid in 4 mL of water was added dropwise with vigorous stirring. After completing the addition the solution was further stirred during 3 min and then poured over a mixture of chloroform-ice (100 mL). The chloroform layer was separated and the aqueous solution further extracted with chloroform (2 x 50 mL). Extracts were pooled, dried (Na_2SO_4) and evaporated to dryness.

The residue of verdoheme was dissolved in 20 mL of methanol, 2 M potassium hydroxide in methanol (2 mL) and 14% BF_3-CH_3OH (20 mL) were added under nitrogen. The mixture was heated under reflux during 15 min and kept overnight at 25ºC in the dark. It was then poured over water (30 mL) and extracted with chloroform (3 x 20 mL). Organic extracts were dried (Na_2SO_4), evaporated to dryness at 40ºC and the residue was applied on two 20 x 20 cm TLC silica gel plates. Adsorbed pigments were separated using acetone-methylene chloride (8:92) as solvent. The three main blue bands (Rf=0.74, 0.54 and 0.43) were eluted without delay with acetone and purified by a second TLC using acetone-methylene chloride (8:92) for the three biliverdins. Biliverdin isomers were eluted from the silica gel as described and crystallized from methylene chloride-hexane.

An overall yield of 25% (4.8 mg) of the mixture of biliverdin methyl esters was achieved. Partial yields when 20 mg of 15-phenyl protohemin IX were oxidized were as follows: for the biliverdin with Rf=0.74, 3 mg (15.6%), mp 189-190ºC; for the isomer with Rf=0.54, 1 mg (5.2%), mp 114-115ºC; and for the one with Rf=0.43, 0.8 mg (4.2%), mp 116-117ºC.

SPECTRAL DETERMINATIONS

UV and VIS spectra were recorded on a JASCO 7850 spectrophotometer. NMR spectra were determined in deuteriochloroform and recorded by means of a Bruker MSL 300 spectrometer. Electronic impact MS were obtained with a VG AutoSpec device (Micromass Inst) at 70 eV.

RESULTS AND DISCUSSION

The overall yield of the chemical oxidation described above proved lower than that for the oxidation of hemin IX to biliverdin IX isomers (42%),[13] but was higher than the one obtained from oxidation of

uroheme III (8.3%),[16] coproheme III (12.1%)[17] or hematohemin IX (11.1%).[14] The biliverdins with Rf=0.74, Rf=0.54 and Rf=0.43 were identified, by ^{1}H NMR and MS spectrometry, as the dimethyl esters of 10-phenyl biliverdin IXα (**1**), 5-phenyl biliverdin IXβ (**2**) and 15-phenyl biliverdin IXδ (**3**) (Figure 2).

In studies carried out with biliverdin dimethyl esters, it has been established that the $\varepsilon_{(vis)}/\varepsilon_{(UV)}$ ratio is a function of verdin molecular extension. An increase in this ratio indicates a change from the porphyrin-like helically coiled form (5Z, 10Z, 15Z) to a more extended form (e.g., 5Z, 10E, 15Z) where the ratio is similar to that of a polyene.[18,19] In our case, the $\varepsilon_{(vis)}/\varepsilon_{(UV)}$ ratio was 0.57 and 0.64 for **2** and **3** respectively, indicating a more stretched conformation than the one present in the helically coiled 10-phenyl biliverdin IXα dimethyl ester (**1**) ($\varepsilon_{(vis)}/\varepsilon_{(UV)}$=0.28) (Table 1).

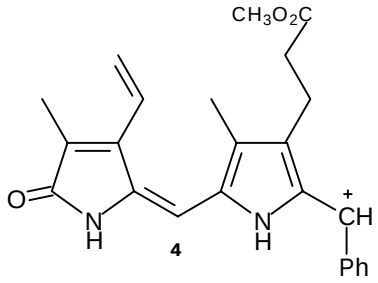
Biliverdin Isomer	UV		VIS		$\varepsilon_{(vis)}/\varepsilon_{(UV)}$
	λmax nm	ε mM^{-1}cm^{-1}	λmax nm	ε mM^{-1}cm^{-1}	
1	395	53.5	675	15.0	0.28
2	385	18.2	632	10.5	0.57
3	382	26.9	611	17.3	0.64

Table 1. Electron absorption spectra of dimethyl esters of phenyl biliverdin IX isomers. Spectra were recorded in methylene chloride; concentrations were 10 μM.

MS of all the three isomers indicated the presence of an *m/z* 686 molecular ion (Table 2).

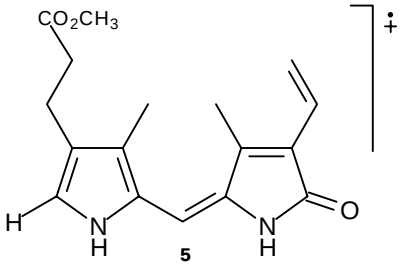
Under electron impact biliverdins are known to fragment across the central methine bridge.[13]

Isomer (**1**) presents ions typical of cleaved propionyl residues, as well as M^{2+} (343, 20) and two fragments from methine 10 cleavage, whose *m/z* are 387 and 300, formulated as **4** and **5**.



4

m/z 389
(and didehydro system *m/z* 387)



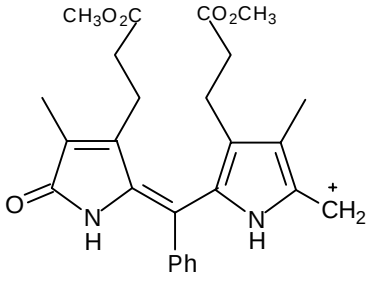
5

m/z 300

Both isomer (**2**) and (**3**) show characteristic fragments but cannot be discriminated from one another. Such fragments are *m/z* 449 (**6**) and *m/z* 240 (**7**) resulting from methine 10 rupture in both isomers, as well as *m/z* 552, resulting from methine 5 rupture in isomer (**2**) and from methine 15 in isomer (**3**).

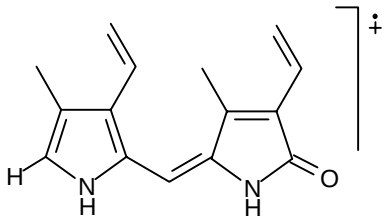
The final identification of the structures of the phenyl biliverdin isomers was assigned by an analysis of their ^{1}H NMR spectra (Table 3). Thus, isomers were distinguished by shifts in methyl signals and

propionate methylene residues. In biliverdins, the exo (α to the amide group) methyl or methylene residue is known to shift to higher fields when compared with similar residues at the endo (the other β-pyrrole) position.[13] Therefore, in isomer **3** there are two exo nuclear methyl residues (1.89 and 2.03 ppm), whereas in **1** and **2** there is only one. In 10-phenyl biliverdin IXα (**1**) both propionate residues are endo and collapse into a single signal (2.07 ppm).



6

m/z 449



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m/z 240

Hence, it may be concluded that, on undergoing chemical oxidation, Iron(III)-complex of 15-phenyl protoporphyrin IX affords three phenyl biliverdin isomers (**1**, **2** and **3**).

Results achieved indicate that oxidation occurs on only three out of the four existing meso-carbon atoms (α, β and δ). The substitution of the γ-meso hydrogen atom by a phenyl group seems to prevent the formation of the γ-meso-hydroxyheme or of any other intermediate oxidized in such position capable of leading to the verdoheme derivative, as shown by the finding that there is no production of the corresponding biliverdin.

Biliverdin Isomer	R_f	M^+ *(m/z)*	Some Fragments *(m/z)*	Molecular Formula	Elemental analysis	
					Calcd	Found
1	0.74	686 (100)	655 (7); 613 (9); 559 (87); 526 (38); 343 (20); 387 (5); 300 (13)	$C_{41}H_{42}N_4O_6$	C, 71.70; H, 6.16; N, 8.16	C, 71.56; H, 6.18; N, 8.32
2	0.54	686 (45)	552 (75); 449 (98); 377 (66); 304 (52); 240 (23)	$C_{41}H_{42}N_4O_6$	C, 71.70; H, 6.16; N, 8.16	C, 71.61; H, 6.20; N, 8.09
3	0.43	686 (100)	671 (10); 599 (18); 552 (20); 449 (13); 436 (11); 416 (6)	$C_{41}H_{42}N_4O_6$	C, 71.70; H, 6.16; N, 8.16	C, 71.75; H, 6.10; N, 8.20

Table 2. MS of the dimethyl esters of phenyl biliverdin IX isomers. Numbers in parenthesis are relative intensities.

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10-phenyl biliverdin IXα (1)	**5-phenyl biliverdin IXβ (2)**	**15-phenyl biliverdin IXγ (3)**
7.49[m, 5, CH(Ph)]	7.43 and 7.34[m, m, 2, 3, CH(Ph)]	7.35[m, 5, CH(Ph)]
6.71[dd, J=17.6 Hz and 11.4 Hz, 1, CH(3^1)]	6.66[dd, J=17.6 Hz and 11.6 Hz, 1, CH(13^1)]	6.76 and 6.63[dd, dd, J=17.0 Hz and 12.0 Hz, J=17.6 Hz
6.56[dd, J=17.6 Hz and 11.4 Hz, 1, CH(18^1)]	6.56[dd, J=17.6 Hz and 11.2 Hz, 1, CH(18^1)]	and 11.4 Hz, 1, 1, CH(3^1,8^1)]
6.14[dd, J=17.6 Hz and 2.2 Hz, 1, CH($18^{2'}$)]	6.27[m, 1, CH($18^{2'}$)]	
5.46[dd, J=11.4 Hz and 2.2 Hz, 1, CH(18^2)]	5.46[m, 3, CH(13^2,$13^{2'}$,18^2)]	5.71, 5.66, 5.50 and 5.46[m, m, s, m, 1, 1, 1, 1,
5.72[m, 1, CH($3^{2'}$)]		CH(3^2,$3^{2'}$,8^2,$8^{2'}$)]
5.67[s, 1, CH(3^2)]		
6.37 and 6.33[s, s, 1, 1, CH(5,15)]	6.88[s, 1, CH(10)]	6.90[s, 1, CH(10)]
	6.15[s, 1, CH(15)]	6.07[s, 1, CH(5)]
3.57[s, 6, CH_3(8^5,12^5)]	3.58 and 3.51[s, s, 3, 3, CH_3(3^5,7^5)]	3.59 and 3.51[s, s, 3, 3, CH_3(13^5,17^5)]
2.07[br, 8, CH_2(8^1,8^2,12^1,12^2)]	2.39 and 2.20[m, m, 2, 6, CH_2(3^1,3^2,7^1,7^2)]	2.47 and 2.23[m, m, 2, 6, CH_2(13^1,13^2,17^1,17^2)]
2.29, 2.14 and 2.11[s, s, s, 3, 3, 3, CH_3(7^1,13^1,17^1)]	2.29, 2.22 and 2.17[s, s, s, 3, 3, 3, CH_3(8^1,12^1,17^1)]	2.20 and 2.13[s, s, 3, 3, CH_3(7^1,12^1)]
1.93[s, 3, CH_3(2^1)]	1.86[s, 3, CH_3(2^1)]	2.03 and 1.89[s, s, 3, 3, CH_3(2^1,18^1)]

Table 3. Proton chemical shifts and assigments of the dimethyl esters of phenyl biliverdins IX isomers. Chemical shifts are quoted in ppm downfield from $(CH_3)_4Si$. Spectra were recorded in $CDCl_3$. The concentrations were **1**: 20 mM; **2**: 6 mM and **3**: 4 mM.

phenyl biliverdins, as well as Romina Falbo and Erika V. Vela for their collaboration in the preparation of some synthetic intermediates.

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