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# PHOTO-OXIDATION AND PHOTOPROTECTION IN THE IXγ BILE PIGMENT SERIES

## A COMPARISON OF THE PHOTOPROTECTIVE ROLES OF PTEROBILIN, PHORCABILIN AND SARPEDOBILIN *IN VITRO*

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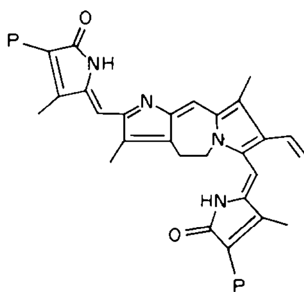
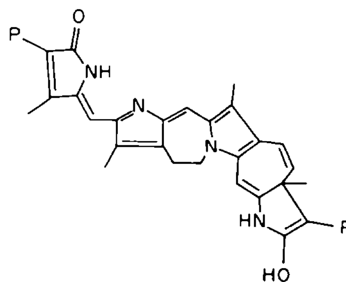
**Abstract**—Pterobilin (**L9**, biliverdin IXγ), a blue green bile pigment from butterflies, is an effective photoprotector of the methylene blue-sensitized photo-oxidation of bilirubin (both as dimethyl esters). The observed effect is of the same order as that observed for the isomeric biliverdins IXα, IXβ, and IXδ (**L4**, **L8**, and **L10**, respectively). However the dimethyl esters of phorcabilin (**1**) and sarpedobilin (**2**), the cyclized derivatives of pterobilin, are unable to provide photoprotection under the same conditions. The possible significance of this result is considered.

AS A result of the systematic search performed in our laboratory for the past 14 years (review[1]) it has been shown that pterobilin (biliverdin IXγ) is widely distributed among butterfly species, larvae and adults. The structure (**L9**) was established in 1968 for this pigment,[2] the biosynthesis of which was shown to proceed through the usual pathway from labelled glycine[3] and various intermediates[4,5] (coproporphyrinogen-III, protoporphyrin-IX). The screening of the Lepidoptera, finally extending to some 100 species, allowed the isolation of two other blue green bile pigments belonging to the same IXγ series. These two new pigments were named phorcabilin and sarpedobilin since they had been isolated first from *Papilio phorcas* and *Papilio sarpedon* respectively.[6–8] Phorcabilin (**1**) and sarpedobilin (**2**) can be formed *in vitro* by the internal cyclization of pterobilin (**L9**) by irradiation with visible light.[9,10] Substances **L9**, **1** and **2**, have not been found in nature elsewhere than in butterflies and the reason for a selective cleavage at the γ position of protoporphyrin IX in these animals is still unknown. Recently, the iron complex of a synthetic γ-hydroxy derivative of protoporphyrin IX was shown[11] to undergo oxidative ring cleavage to give pterobilin and this may provide a model for the study of its biosynthesis in the future. That the formation of phorcabilin and sarpedobilin by the irradiation of pterobilin is related to the presence of the vinyl groups in a central position in the linear tetrapyrrole structure was later emphasized by the demonstration that the IXδ isomer is able to give only one cyclized derivative.[12] Stoll *et al.*[13] have reported the photocyclization of bilirubin to give photobilirubin II, NMR spectroscopy suggesting an internal addition to a vinyl group.

A hypothesis has been proposed according to which pterobilin would have a role in the larval development of *Pieris brassicae*.[14,15] Following this scheme, pterobilin would act as a photoreceptor for the red wavelengths of the visible spectrum, corresponding to a sort of biological clock, connected with the day–night relationships and the determination of diapause. It has been established[16–18] that the red wavelengths of the spectrum are responsible for an increased production of ATP in *Pieris brassicae* integument. The mechanism of such a process, for example by the photo-isomerization of pterobilin at the methene bridges, still awaits demonstration. Edmunds and Adams[19] have proposed a molecular basis for metering time by means of pigments present in membranes, depending on some "chronogen segments". Our observations concerning the pterobilin chromoprotein obtained from *P. brassicae*, which is resistant to a light-induced transformation into phorcabilin *in vitro*, argues against a mechanistic hypothesis involving cyclization, but does not formally rule out photo-




**1** Phorcabilin




**2** Sarpedobilin

($P = -CH_2CH_2COOH$ or $-CH_2CH_2COOCH_3$)

isomerization at the methene bridges. As far as the cyclizations are concerned, the formation of phorcabilin and of sarpedobilin from pterobilin *in vivo* may also be due to enzymic reactions, or to the action of light on free pterobilin, for instance during moulting, when the biosynthesis of tetrapyrroles is more active.

Lightner *et al.*[20,21] have reported the photo-oxidation of biliverdin IXα (**L4**) leading to imides and propentdyopents formed by the action of $^1O_2$. McDonagh[26] has shown that biliverdin IXα inhibits the photo-oxidation of bilirubin. We now report the results of sensitized photo-oxidations performed in the presence of a variety of verdinoid quenchers.

*Photo-oxidation of the verdins*

Irradiation of pterobilin dimethyl ester in methanol with visible light gave after 2 hr a mixture of blue pigments which contained phorcabilin (**1**) and sarpedobilin (**2**) as previously established. If no sensitizer was added, the formation of imides through photo-oxidation did not occur in detectable amount within 10 hr (or within some 10 days under natural light). Compared with bilirubin which catalyzes its own photodegradation, pterobilin (**L9**) was relatively stable. Adding Rose Bengal (1 : 1) to the pterobilin ester solution in methanol led to the rapid photo-oxidation of the pigment with the formation of imides and propentdyopents, as reported for the dimethyl ester of biliverdin IXα (**L4**) by Lightner *et al.*[20,21] The esters of the isomeric biliverdins (**L8**, **L10**) gave similar results.

*Methylene blue sensitized photo-oxidation of bilirubin dimethyl ester in the presence of verdinoid quenchers*

The photo-oxidation of bilirubin dimethyl ester was performed in the presence of the isomeric biliverdins IXα (**L4**), β (**L8**), γ (pterobilin, **L9**) and δ (**L10**), of phorcabilin (**1**) and of sarpedobilin (**2**) (all as dimethyl esters), using methylene blue as a photosensitizer. The oxidation of bilirubin was followed at its absorption maximum at 440 nm, the reference solution being the same as the experimental sample, except that it did not contain bilirubin. The reference solution and the experimental sample were irradiated simultaneously using a (S 3.66) filter ($\lambda > 550$ nm) to avoid the direct excitation of bilirubin. It was shown that the bilin employed for photoprotection was not destroyed during the experiment.

It was found that pterobilin ester and the other biliverdin esters appreciably slowed down the photo-oxidation of the bilirubin ester. After 2 min, 76% of bilirubin ester was oxidized in the absence of a verdin quencher but only 38% in the sample containing pterobilin ester ($A_1$, D, Table 1). Increasing the concentration of pterobilin (B, C, D, Table 1) showed a proportional increase of the photoprotective effect. As the absorption spectra of methylene blue and of the biliverdin esters overlap at 650 nm, the experiment was also repeated using a solution of the pterobilin ester ($1.4 \times 10^{-5}$ M) as an external filter, to check that the observations were not due to a screening effect.

However when the same experiments were performed with the dimethyl esters of phorcabilin (**1**) and sarpedobilin (**2**) at the same concentrations, these two pigments did not protect bilirubin from photo-oxidation (Table 2).

The results obtained are illustrated in Fig. 1 and Fig. 2.

It is concluded that, for the dimethyl esters, pterobilin (**L9**) is an efficient photoprotector in the methylene blue sensitized photo-oxidation of bilirubin (**L6**) by visible light, as are the biliverdins IXα (**L4**), β (**L8**), and δ (**L10**); but phorcabilin (**1**) and sarpedobilin (**2**) are not efficient under the same conditions. The

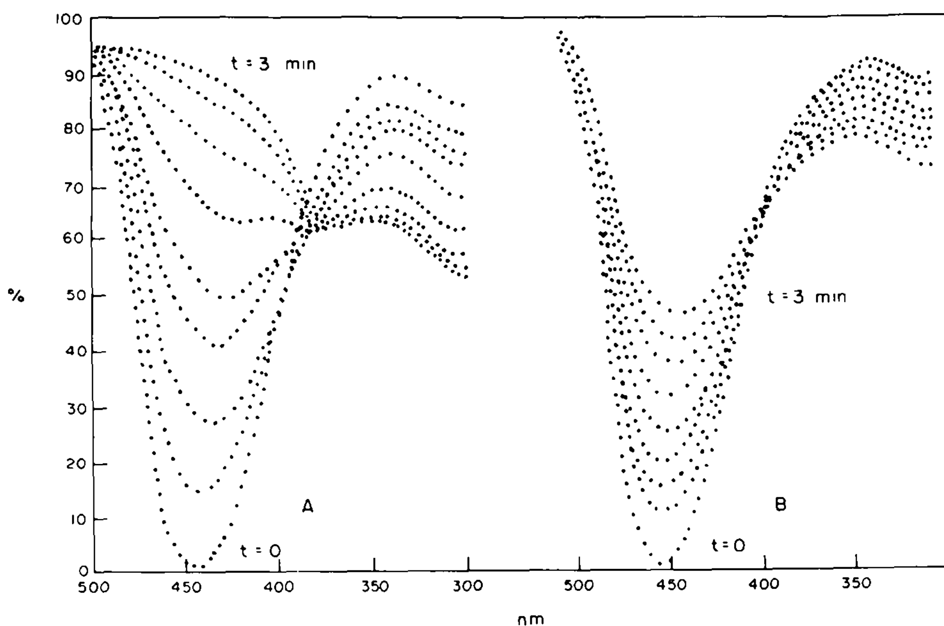



Fig. 1. Methylene blue ($5 \times 10^{-7}$ M) sensitized photo-oxidation of bilirubin ester ($1.5 \times 10^{-5}$ M) in chloroform (0.2% ethanol). A: without pterobilin ester (expt. A, Table 1); B: with pterobilin ester (expt. D, Table 1). The curves have been recorded after 15 sec, 45 sec, 1 min, 2 min, 2 min 30 sec and 3 min irradiation.

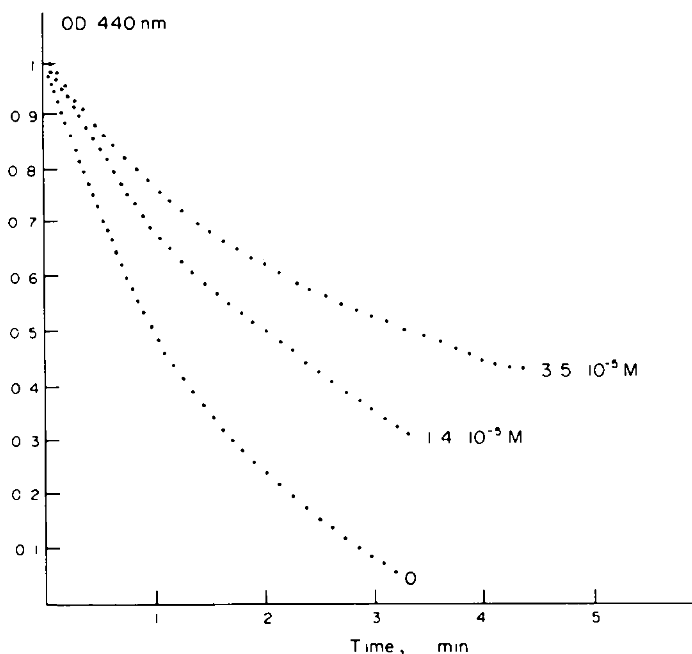



Fig. 2. Inhibition of the methylene blue ($5 \times 10^{-7}$ M) sensitized photo-oxidation of bilirubin ester ($1.5 \times 10^{-5}$ M) by pterobilin dimethyl ester (1.4 and $3.5 \times 10^{-5}$ M) in chloroform solution (0.2% ethanol).

photoprotective effect of pterobilin is probably due to the inhibition of the excited states of the sensitizer or of bilirubin, or to the direct quenching of $^1O_2$.[26]

This study allows the comparison of the photoprotective effect of the four isomeric biliverdins to that of the carotenoid pigments[22-24] for which the inhibitory action on photo-oxidations is well documented. In particular, biliverdin IX$\alpha$ has been shown to be as efficient as $\beta$-carotene in the photoprotection of bilirubin.[25-26] This result and the fact that pterobilin is predominantly accumulated in the cuticle of butterfly larvae, lead to the hypothesis of an *in vivo* photoprotection against the combined action of oxygen, light and internal photosensitizers.

## EXPERIMENTAL

The four biliverdins were prepared according to Bonnett and McDonagh[27] by oxidation of protohaemin in pyridine.

Table 1. Methylene blue sensitized photo-oxidation of bilirubin dimethyl ester in the presence of a biliverdin ester[a]

| Expt. | $A_1$ | $A_2$ | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|---|
| Biliverdin dimethyl ester | — | | IX$\gamma$ | IX$\gamma$ | IX$\gamma$ | IX$\alpha$ | IX$\beta$ | IX$\delta$ |
| Conc./$10^5$ M | -- | Filter* | 0.7 | 1.4 | 3.5 | 1.4 | 1.4 | 1.4 |
| Irradiation time | | | | | | | | |
| 0 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| 15 s | 0.86 | 0.92 | 0.84 | 0.90 | 0.90 | 0.90 | 0.90 | 0.93 |
| 30 s | 0.73 | 0.72 | 0.71 | 0.83 | 0.86 | 0.87 | 0.79 | 0.86 |
| 45 s | 0.58 | 0.62 | 0.62 | 0.76 | 0.81 | 0.80 | 0.73 | 0.79 |
| 1 min | 0.50 | 0.54 | 0.54 | 0.67 | 0.76 | 0.76 | 0.63 | 0.74 |
| 1.5 min | 0.35 | 0.39 | 0.38 | 0.58 | 0.68 | 0.66 | 0.49 | 0.66 |
| 2 min | 0.24 | 0.28 | 0.29 | 0.50 | 0.62 | 0.58 | 0.43 | 0.58 |
| 2.5 min | 0.15 | 0.17 | 0.16 | 0.40 | 0.58 | 0.53 | 0.36 | 0.51 |
| 3 min | 0.10 | 0.13 | 0.12 | 0.34 | 0.53 | 0.43 | 0.29 | 0.44 |
| 3.5 min | | | | | 0.49 | 0.37 | 0.24 | 0.40 |
| 5 min | | | | | 0.41 | | | 0.30 |
| 7 min | | | | | 0.31 | | | |
| 10 min | | | | | 0.22 | | | |

[a]Determinations performed using $\varepsilon$ 62,000 for bilirubin, and 15,000 for the biliverdins, at the $\lambda_{max}$ of bilirubin at 440 nm. In experiments B to G, the reference solution consisted of a cuvette containing a biliverdin ester and methylene blue irradiated simultaneously with the experimental sample. In all assays, a filter CS 3.66 ($\lambda > 550$ nm) was used except in the case *($A_2$) where the filter consisted of an external cuvette containing a $1.5 \times 10^{-5}$ M solution of pterobilin dimethyl ester as an external filter. Concentrations throughout: bilirubin dimethyl ester, $1.5 \times 10^{-5}$ M; methylene blue, $5 \times 10^{-7}$ M. Solvent: chloroform + 0.2% ethanol.

Table 2. Methylene blue sensitized photo-oxidation of bilirubin dimethyl ester in the presence of phorcabilin or sarpedobilin esters[a]

| Verdinoid quencher Conc./$10^5$ M | Phorcabilin dimethyl ester 1.8 | Phorcabilin dimethyl ester 0 | Sarpedobilin dimethyl ester 1.5 | Sarpedobilin dimethyl ester Filter* | Sarpedobilin dimethyl ester 0 |
|---|---|---|---|---|---|
| Irradiation time | | | | | |
| 0 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| 15 s | 0.84 | 0.85 | 0.84 | 0.83 | 0.84 |
| 30 s | 0.70 | 0.67 | 0.69 | 0.70 | 0.66 |
| 45 s | 0.58 | 0.56 | 0.58 | 0.60 | 0.56 |
| 1 min | 0.44 | 0.42 | 0.50 | 0.52 | 0.46 |
| 1.5 min | 0.31 | 0.30 | 0.36 | 0.33 | 0.32 |
| 2 min | 0.22 | 0.20 | 0.28 | 0.29 | 0.20 |
| 2.5 min | 0.15 | 0.13 | 0.19 | 0.20 | 0.14 |
| 3 min | — | — | 0.10 | 0.10 | |

[a]In chloroform + 0.2% ethanol, with a filter CS 3.66, as mentioned Table 1, except in the case* where the filter was an external cuvette containing a $1.5 \times 10^{-5}$ M solution of sarpedobilin ester. The concentrations of bilirubin dimethyl ester and of methylene blue were the same as before (Table 1, legend).

The methyl esters were obtained by the action of MeOH-$H_2SO_4$ (2%, 20 hr at 5°), extraction by $CHCl_3/H_2O$ and preparative tlc on silica with $CHCl_3 : Me_2CO$ (10 : 1); $R_f$ values of the biliverdin esters: IX$\beta$: 0.85; $\alpha+\gamma$: 0.78; $\delta$: 0.65. A second tlc in benzene : hexane : MeOH (9 : 5 : 1) separated the biliverdin esters $\alpha$ and $\gamma$ ($R_f$ 0.45 and 0.55 respectively). The pigment dimethyl esters were crystallized from $CHCl_3$-pentane. Phorcabilin dimethyl ester was prepared by warming a DMSO soln of **L9** dimethyl ester in an evacuated sealed tube for 20 hr at 80°, and sarpedobilin dimethyl ester by irradiation of **L9** in a Pyrex tube for 2 hr with 2 Philips lamps (300 W each) according to the literature.[9,10] The final yields in phorcabilin and sarpedobilin dimethyl esters were 25% and 45% respectively. Phorcabilin dimethyl ester had $R_f$ 0.80 on silica developed with benzene : hexane : methanol (9 : 5 : 3) and while the $R_f$ of sarpedobilin dimethyl ester was 0.20 under these conditions.

The absorption spectra were measured on a Leres S-66 spectrophotometer. Bile pigment concentrations were determined on the basis of the molar extinction coefficients of the dimethyl esters at $\lambda_{max}$ in MeOH which were as follows: pterobilin (**L9**) ($\lambda_{max}$ 650 nm, $\varepsilon$ 15,000); phorcabilin ($\lambda_{max}$ 550 nm, $\varepsilon$ 45,000); sarpedobilin ($\lambda_{max}$ 590 nm, $\varepsilon$ 62,000); and bilirubin ($\lambda_{max}$ 440 nm, $\varepsilon$ 62,000). To simplify the determinations and because this had no significant influence on the results, the concentrations of the biliverdins IX$\alpha$, $\beta$ and $\delta$ were also calculated on the basis of the absorptions of the esters at $\lambda_{max}$ 650 nm ($\varepsilon$ 15,000).

The irradiation experiments were performed on the pigments in a $CHCl_3$ containing 0.2% EtOH soln, using a Philips 300 W lamp with a CS 3.66 filter ($\lambda > 550$ nm). This filter was necessary to avoid the direct photo-oxidation of bilirubin using the experimental conditions reported (Tables 1 and 2). All results were obtained with this filter. References solns (verdin + methylene blue) were simultaneously irradiated. In order to check that the observed effects were not due to the absorption of the effective wavelengths by the bilins, solns of pterobilin ester and sarpedobilin ester were used as external filters in two complementary experiments (Tables 1 and 2).

## REFERENCES


[1]M. Barbier, *Experientia* **37**, 1060 (1981).
[2]W. Rüdiger, W. Klose, M. Vuillaume and M. Barbier, *Ibid.* **24**, 1000 (1968).
[3]W. Rüdiger, W. Klose, M. Vuillaume and M. Barbier, *Ibid.* **25**, 487 (1969).
[4]M. Choussy, M. Vuillaume and M. Barbier, *Biochimie* **57**, 369 (1975).
[5]M. Bois-Choussy, Thesis, Orsay University, France (1977).
[6]M. Vuillaume and M. Barbier, *C. R. Acad. Sci. Paris* **268**, 2286 (1969).
[7]M. Choussy and M. Barbier, *Helv. Chim. Acta* **58**, 2651 (1975).
[8]M. Bois-Choussy and M. Barbier, *Experientia* **33**, 1407 (1977).
[9]M. Choussy and M. Barbier, *C. R. Acad. Sci. Paris* **282** Ser. C, 619 (1976).
[10]M. Bois-Choussy and M. Barbier, *Heterocycles* **9**, 677 (1978).
[11]A. H. Jackson, R. M. Jenkins, D. M. Jones and S. A. Matlin, *J. Chem. Soc.*, Chem. Commun. 763 (1981).
[12]M. Bois-Choussy and M. Barbier, *Helv. Chim. Acta* **63**, 1098 (1980).
[13]M. Stoll, N. Vicker, C. H. Gray and R. Bonnett, *Biochem. J.* **201**, 179 (1982).
[14]M. Barbier, J. Bergerard, B. Hurpin and M. Vuillaume, *C. R. Acad. Sci. Paris* **271**, 342 (1970).
[15]M. Vuillaume, J. Seugé and J. Bergerard, *Intern. J. Chronobiol.* **2**, 181 (1974).
[16]M. Vuillaume and J. Bergerard, *Chronobiologia* **5**, 286 (1978).
[17]M. Vuillaume and M. Best-Belpomme, *Experientia* **36**, 1169 (1980).
[18]M. Vuillaume and Y. Dattée, *Arch. Zool. Exper. Gén.* **121**, 159 (1980).
[19]L. N. Edmunds and K. J. Adams, *Science* **211**, 1002 (1981).
[20]D. A. Lightner and D. C. Crandall, *FEBS Letters* **20**, 53 (1972).
[21]D. A. Lightner and D. C. Crandall, *Tetrahedron Letters* 953 (1973).
[22]C. S. Foote and R. W. Denny, *J. Am. Chem. Soc.* **90**, 6233 (1968).
[23]M. M. Matheus, *Nature, London* **203**, 1092 (1964).
[24]C. S. Foote, Y. C. Chang and R. W. Denny, *J. Am. Chem. Soc.* **92**, 5212 (1970).
[25]A. F. McDonagh, *Biochem. Biophys. Res. Commun.* **44**, 1306 (1971).
[26]A. F. McDonagh, *Ibid.* **48**, 408 (1972).
[27]R. Bonnett and A. F. McDonagh, *J. Chem. Soc.*, Perkin Trans. 1, 881 (1973).