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The susceptibility of trichophyton rubrum to photodynamic treatment

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Chapter II

PHOTODYNAMIC INACTIVATON OF THE DERMATOPHYTE *TRICHOPHYTON RUBRUM*

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Trichophyton rubrum microconidium with a developing germtube

ABSTRACT

The present study shows that *Trichophyton rubrum* in suspension culture is susceptible to PDT, a completely new application in this area. *T. rubrum* could be effectively killed with the use of the light-activated porphyrins DP mme Sylsens B. The photodynamic efficacy was compared to some other photosensitizers, that are well-known in the field of PDT: the porphyrins, deuteroporphyin DP, hematoporphyrin HP, the drug Photofrin and several phthalocyanines. It was demonstrated that with the use of broadband white light the phthalocyanines and Photofrin displayed a fungistatic effect of about one week, whereas all the porphyrins caused photodynamic killing of the dermatophyte. Sylsens B was the most effective sensitizer and showed no dark toxicity; therefore, in an appropriate formulation, it could be a promising candidate for the treatment of various forms of tinea. For Sylsens B and DP mme, displaying the best results, a concentration dependent uptake by *T. rubrum* was established.

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INTRODUCTION

The drugs used today for the treatment of superficial fungal infections are certainly more effective than those available years ago. In the 1960s griseofulvin was introduced (1) as a fungistatic with duration of treatment of 4 weeks up to 18 months (2) while in 1992 a topical treatment with the drug terbinafine (Lamisil) was introduced as a cream that promised to cure a dermatophyte infection after only a few applications (3,4). Recently a sequential pulse therapy with itraconazole (a triazole) and terbinafine (an allylamine) was introduced to treat onychomycosis of fingernails (2,5,6). Allylamines are more effective against dermatophyte infections, but they are more expensive than azoles (2). In general, limitations of the current therapeutic options include: inadequate spectrum of activity, lack of efficacy, multiple drug interactions, inadequate pharmacokinetic profile, excessive costs, recurrence of the infection and duration of treatment (4,7).

The aim of the present study is to determine whether the dermatophyte *Trichophyton rubrum* can be killed with the use of the light-activated porphyrins DP mme and Sylsens B and thus develop a possible clinical application against infections caused by *T. rubrum*. Sylsens B and DP mme are known to be able to kill certain bacteria, Chinese Hamster Ovary cells and the common fruit fly *Drosophila melanogaster* (G.M.T. Smijs, unpublished data). Therefore we selected these compounds to investigate this relatively new application of PDT. Little research has been reported concerning the treatment of fungal infections with the use of light-activated agents. In 1998 the photodynamic effect of different thiophenes on eight strains of dermatophytes with the use of UVA radiation was studied (8). The growth of all tested strains was strongly inhibited by the thiophenes under investigation but a complete inactivation was never detected. Moreover UVA is known to be carcinogenic.

The photodynamic activity of Sylsens B and DP mme towards *T.rubrum* was compared to the photodynamic effect of several photosensitising drugs that have been studied for photodynamic cancer therapy (9), the porphyrins, deuteroporphyin (DP), hematoporphyrin (HP), the drug Photofrin and the phthalocyanines, zinc phthalocyanine (ZnPc), phthalocyanine tetrasulfonate (PcS_4) and aluminium (III) phthalocyanine chloride tetrasulfonate ($AlPcS_4$). For formulae and related names see table 2.1. Photofrin is a commercial product consisting of a complex mixture of monomers, oligomers and aggregates with HP as the starting material.

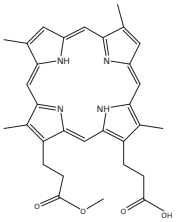
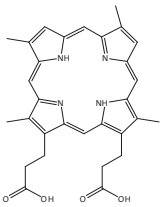
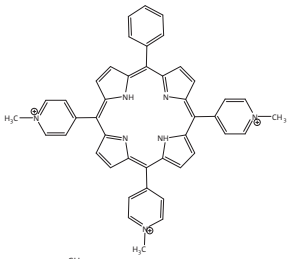
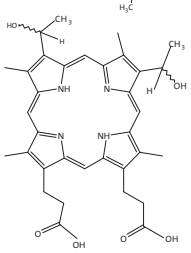
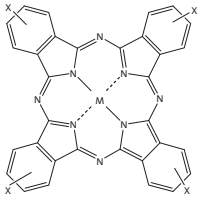
Chemical Structure	*Selected name*	*Abbreviation*
	Deuteroporphyrin monomethylester	DP mme
	Deuteroporphyrin	DP
	5,10,15-tris(4-methylpyridinium)-20-phenyl-[21H,23H]porphine trichloride	Sylsens B
	Hematoporphyrin	HP
	Phthalocyanine	Pc

Table 2.1. Formulae and related names of the porphyrins and phthalocyanines. For ZnPc, M is zinc; for PcS_4, M is hydrogen and X is SO_3H; for $AlPcS_4$, M is aluminium and X is SO_3H.

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MATERIALS AND METHODS

Materials

The fungus *T. rubrum* was purchased from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. Cultures were grown on malt extract agar (MEA; Oxoid, Hampshire, UK). Suspension cultures were made in Dulbecco's modified Eagle medium (GibcoBRL, UK) with 2.5% fetal calf serum (GibcoBRL).

Solid cultures were maintained at 28°C, the suspension cultures at room temperature. HP was purchased from Porphyrin Products Inc. (UT, USA); Sylsens B, DP and DP mme were synthesized and kindly provided by the Department of Bio-Organic Photochemistry, Leiden University, The Netherlands (purity, checked with nuclear magnetic resonance, was more than 99.5%). All the phthalocyanines were purchased from Porphyrin Products Inc., and Photofrin was purchased from Lederle Parenterals Inc. (Carolina, USA). Polyethyleneglycol was obtained from Genfarma B.V. (Maarssen, The Netherlands), and all other chemicals were purchased from J.T. Baker (Deventer, The Netherlands).

The following solvents were used: 50 mM sodium phosphate buffer, pH 7.4, for Sylsens B, HP, Photofrin, ZnPc and $AlPcS_4$; polyethyleneglycol–ethanol–water (3:2:5) for DP; and DP mme and dimethylformamide for PcS_4. Stock solutions of the photosensitizers (2.5 mg/mL solvent) were stored at 4°C for no longer than 1 week.

Photodynamic treatment

Illuminations were performed using a lamp from "MASSIVE" (no. 74900/21), 13 max.500W-230 V-R7s, IP 44. To avoid heating of the samples to be illuminated, the white light produced by the lamp is passed through a 2 cm thick water layer before reaching the samples. Light intensity (30 mW/cm^2) was measured with IL1400A photometer equipped with a SEL033/F/U detector (International Light, Newburyport, MA). Before illumination, the suspension fungal cultures were incubated with the photosensitizer in test tubes for 30 min at a temperature of 28°C. After incubation, the suspension cultures were illuminated in the presence of the sensitizer in 3 cm diameter culture dishes (Greiner, Alphen aan den Rijn, The Netherlands). After 1 hour illumination, the contents of the culture dishes were transferred to 9 cm diameter dishes containing MEA and placed in the incubator at 28°C, and growth was followed during 1 week and quantified by counting the number of inoculates present.

Uptake of photosensitizers

To determine the fungal uptake of the porphyrin photosensitizers Sylsens B and DP mme as a function of time and concentration, suspension cultures (2 mL) were incubated with the photosensitizer at 28°C. Three different concentrations were used with eight different incubation times. After incubation, the suspension culture was centrifuged (Heraeus 3S) for 7 min at 3300 g. The pellet was washed once with medium, three times with Dulbecco's phosphate-buffered saline and finally dissolved in 1 mL of 50% (vol/vol) sulfuric acid. After 24 hours, the concentration of the sensitizer in the sulfuric acid containing dissolved fungus was determined with the use of fluorescence spectroscopy (Perkin–Elmer LS 50B luminescence). The fluorescence emission was measured in the 620–700 nm interval upon excitation at the maximum wavelength of the Soret band of the porphyrin under study. Preliminary experiments for Sylsens B and DP mme showed a linear relationship between fluorescence intensity and porphyrin concentration. A calibration curve was made, and the linear part was used to determine unknown sensitizer concentrations. The values thus obtained were corrected for the recovery, determined separately for Sylsens B and DP mme. To determine the recovery of the sensitizer under study, 2 mL of suspension culture was centrifuged and the pellet was washed as described above. The pellet was dissolved, as described, in sulfuric acid with the addition of predetermined concentrations of the sensitizer, and the fluorescence emission was measured as described above and compared with the fluorescence emission of the actual administered sensitizer concentration in 50% (vol/vol) sulfuric acid. For each sensitizer, five different concentrations were used, and the experiment was repeated six times.

RESULTS

PDT of T. rubrum and dark toxicity

PDT of *T. rubrum* in suspension culture with Sylsens B and DP mme resulted in a complete kill (on the basis of a cutoff at two colonies) of the fungus in almost all experiments. Of the porphyrin derivatives, Sylsens B is by far the most effective. As can be seen in Fig. 2.1A, the photodynamic efficacy of Sylsens B and DP mme above a concentration of 3 µg/mL is analogous to the effect caused by the other porphyrins tested, HP and DP. Below this concentration, Sylsens B displayed a better photodynamic efficacy, whereas the efficacy of DP mme was the same as that of HP; DP gave the

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lowest efficacy in this lower concentration range. On comparing with Fig. 2.1B, it can be seen that at lower sensitizer concentrations, PDT merely results in a delay in growth. Only above a concentration of 20 µg/mL was a true fungicidal effect detected for all the porphyrin sensitizers tested. Sylsens B and DP mme displayed this effect even at a lower concentration, namely, 3 µg/mL. For all the porphyrins, it was established that after successful PDT, even after several weeks, no trace of reoccurrence of the fungus on the MEA dishes could be detected. On examining Fig. 2.1C, it however becomes clear that DP mme as well as DP express a dark toxicity at higher concentrations. However, Sylsens B and HP show no dark toxicity under the given circumstances.

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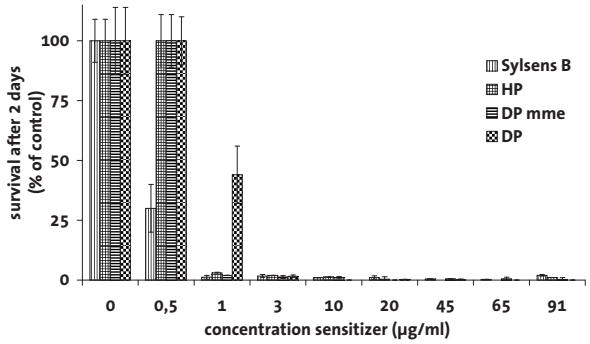

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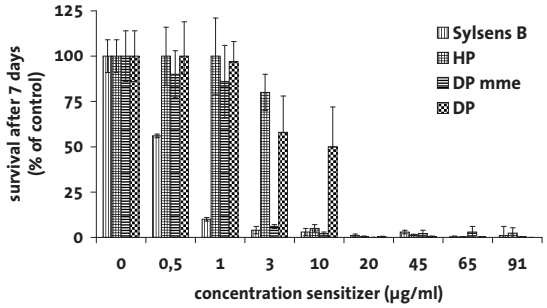

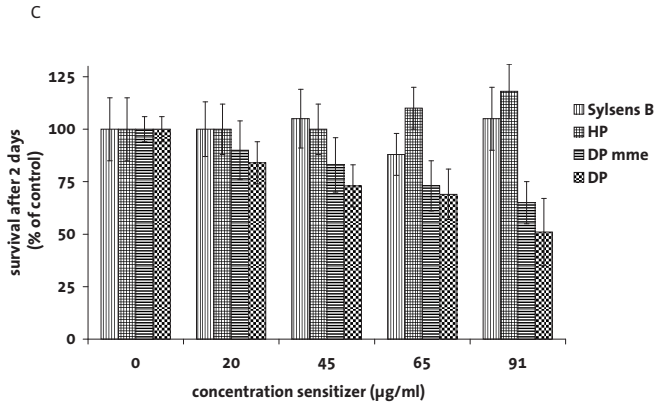

Figure 2.1. Growth of *T. rubrum* on the second (A) and seventh (B) day after light treatment with various porphyrins compared with the growth on the second day after treatment with the porphyrins in the dark (C). Suspension cultures were incubated in the dark for 30 min at 28°C with different concentrations of the porphyrins. After the incubation period, suspension cultures were illuminated with white light (1080 kJ/m^2) or kept in the dark in the presence of the porphyrins. After illumination or dark period, cultures were transferred to MEA dishes and placed in the incubator at 28°C. The survival was measured after 2 (A,C) and 7 (B) days as the number of inoculates present. Untreated cultures containing solvent instead of photosensitizer were considered to have 100% survival. Number of inoculates on control dishes: 175–225 after 2 days and 270–320 after 7 days. The values given are the means of three experiments with the standard deviations.

Figure 2.2A shows the result of PDT of *T. rubrum* using several phthalocyanines and Photofrin. The observed photodynamic efficacy on *T. rubrum* is not as high as that found for the porphyrins (compare with Fig. 2.1A). All the phthalocyanines and Photofrin only induce a growth delay in the first days after the PDT. After 7 days, the fungus grows again as well as it did without PDT, displaying a 100% survival over the whole concentration range used. At day 7, there is no difference between samples subjected to PDT and the blank. So these photosensitizers induce a fungistatic effect that lasts for 7 days. Considering the dark toxicity, only Photofrin shows a positive result when applied at concentrations from 90 µg/mL (see Fig. 2.2B). For the phthalocyanines, no dark toxicity on *T. rubrum* was observed under the conditions used.

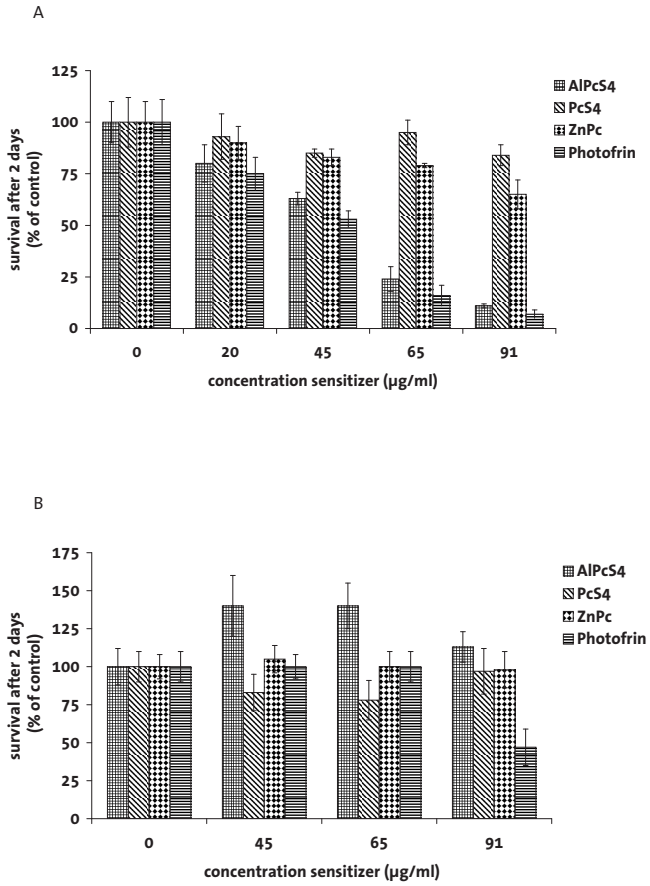

Figure 2.2. Growth of *T. rubrum* on the second day after light (A) or dark (B) treatment with various phthalocyanines and Photofrin. Suspension cultures were incubated in the dark for 30 min at 28°C with different concentrations of the photosensitizers. After the incubation period, suspension cultures were illuminated with white light (1080 kJ/m^2) or kept in the dark in the presence of the photosensitizer. After illumination or dark period, cultures were transferred to MEA dishes and placed in the incubator at 28°C. The survival after 2 days was measured as the number of inoculates present. Untreated cultures containing solvent instead ofphotosensitizer were considered to have 100% survival (number of inoculates: 150–200). The values given are the means of three experiments with the standard deviations.

Uptake of Sylsens B and DP mme

In a first approach to explain the effectiveness of Sylsens B and DP mme, uptake of these two compounds was studied Fig. 2.3 shows the concentration-dependent uptake of the photosensitizers Sylsens B and DP mme by *T. rubrum*, as a function of time. For DP mme, the fluorescence emission was measured at 623 nm (excitation wavelength, 395 nm). For Sylsens B, the emission was measured at 695 nm with an

excitation wavelength of 432 nm. The values shown in the figure are corrected for the recovery (see Materials and Methods), 88% ± 12% for Sylsens B (n = 6) and 84% ± 17% for DP mme (n = 6). Up to an incubation time of 24 h, not much difference is found between the uptake of Sylsens B and DP mme by *T. rubrum*. At longer incubation times, the uptake of the photosensitizer DP mme gives a slightly better result than obtained for Sylsens B. However, significance among the data could only be established for the lowest concentration (16 µg/mL) and highest incubation period and for the highest incubation concentration (55 µg/mL) with 24 h incubation time (Student's *t* -test, *P* = 0.05). Remarkably, no plateau is reached in the uptake of both sensitizers DP mme and Sylsens B. Uptake of the photosensitizer seems to continue.

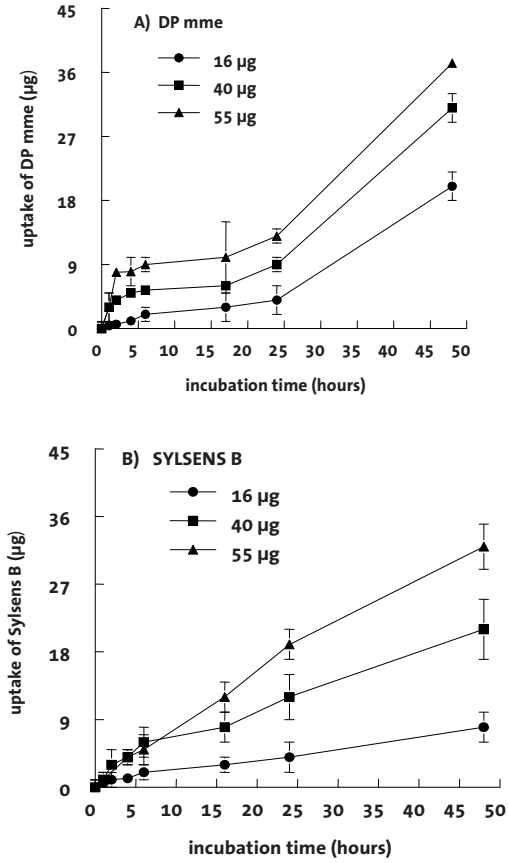

Figure 2.3. Uptake of DP mme (A) and Sylsens B (B) by *T. rubrum*. Incubation temperature: 28°C. For DP mme, the fluorescence emission was read at 623 nm (excitation wavelength, 395 nm). For Sylsens B, the emission was read at 695 nm with an excitation wavelength of 432 nm. Values given are the means of four experiments with the standard deviations. Statistical significance was determined (Student's *t*-test, *P* = 0.05) and was found to be positive for two sets of data points, namely, 55 µg/mL, 24 h and 16 µg/mL, 48 h.

DISCUSSION

Both porphyrins Sylsens B and DP mme were proven to be excellent photosensitizers toward the dermatophyte *T. rubrum*, Sylsens B being the most effective. Both were able to completely prevent the growth of *T. rubrum*, a result that is completely new in the field of PDT. The other tested porphyrins, DP and HP, showed comparable fungicidal effects but at higher concentrations (see Fig.2.1B). For Sylsens B and DP mme, a concentration of 3 µg/mL or higher was sufficient to kill the fungus in most experiments using broadband white light (1080 kJ/m^2). For HP, this concentration appeared to be 10 µg/mL, whereas for DP it was 20 µg/mL. For all the porphyrins, it was established that even several weeks after a successful PDT of the fungus, there was no trace of growth of the fungus on the MEA dishes. This was in contrast with the results obtained with the different phthalocyanines that were tested and Photofrin. They merely displayed a fungistatic effect under the same experimental conditions, whereas all the porphyrins displayed a true fungicidal effect. By 1 week after PDT with the phthalocyanines or Photofrin, the fungus covered the MEA plates completely, a result identical to that obtained with controls, viz. only solvent without sensitizer.

To establish whether the observed photodynamic killing was a true photodynamic effect, we also investigated the dark toxicity of the photosensitizing compounds. We showed that DP and DP mme displayed a dark toxicity under the current experimental conditions at concentrations of 20 µg/mL and higher. Sylsens B and HP showed no dark toxicity in the photodynamic active concentration range. In contrast to these two photosensitizers, with DP mme, dark toxicity most likely contributes to the decrease of the survival as shown in Fig. 2.1. However, the presence of minor dark toxicity does not necessarily have to interfere with a possible future clinical application for PDT of tinea.

On examining Fig. 2.3, it is clear that for Sylsens B and DP mme, there is a similar concentration-dependent uptake into or binding to the fungus. From the current experiments, it cannot be concluded whether the photosensitizers are taken up by the fungus or bound to the outer surface of the fungus. No saturation level could be detected up to 48 h. The difference in uptake kinetics between DP mme and Sylsens B might possibly be explained by the difference in structure of the compounds. Sylsens B is a positively charged, amphiphilic molecule, whereas DP mme is a more hydrophobic molecule.

However, for Sylsens B, the values found for the three different concentrations at the lower incubation times do not significantly differ from each other (Student's *t* -test, $P = 0.05$). This could imply that the uptake curve for Sylsens B has the same shape as that found for DP mme, indicating that there is no difference in uptake kinetics between the two sensitizers. This issue certainly needs to be explored further.

In future research, the photodynamic effect should be examined not only on suspension cultures but also on solid downy colonies grown on agar plates. In a first attempt, we found that when grown as a solid downy colony on an agar plate, *T. rubrum* could not be killed photodynamically in a single treatment with any of the sensitizers described in this article (G. M. T. Smijs, unpublished). At most, a fungistatic effect lasting for no more than 4 days was obtained using short incubation periods. Considering the results of the uptake experiments (see Fig. 2.3), prolonged incubations should be included in further studies. However, it should be taken into account that the growth on an agar plate differs completely from the superficial growth of the fungus on human skin (2). Moreover, PDT *in vitro* could be effective for solid downy colonies using multiple treatments.

The phenomenon of photobleaching (10,11) is a possible source of complications in the clinical application of PDT. However, this is not an issue when using Sylsens B as photosensitizer because this compound does not photobleach (G. M. T. Smijs, unpublished).

Not only the absence of photobleaching and dark toxicity in the photodynamic active concentration range but also the rather low effective concentration supports the idea that Sylsens B is a good candidate for further research into PDT of tinea infections.

Moreover, if the high efficacy that is established *in vitro* can be found again in PDT of tinea infections, this might have certain positive consequences for the costs of the medication.

Because Sylsens B also has absorption peaks in the red part of the spectrum, future research into PDT of the dermatophyte *T. rubrum* with Sylsens B should also include experiments with red light. This can be seen from Fig. 2.4, where the Q-bands of the visible absorption spectrum of the porphyrins used in this study are compared with each other. Because red light has a considerably higher penetration depth in tissue than white light, this property can be particularly useful in treatment of nail infections.

In summary, PDT of tinea infections with Sylsens B as a photosensitizer is a promising entity that deserves further exploration.

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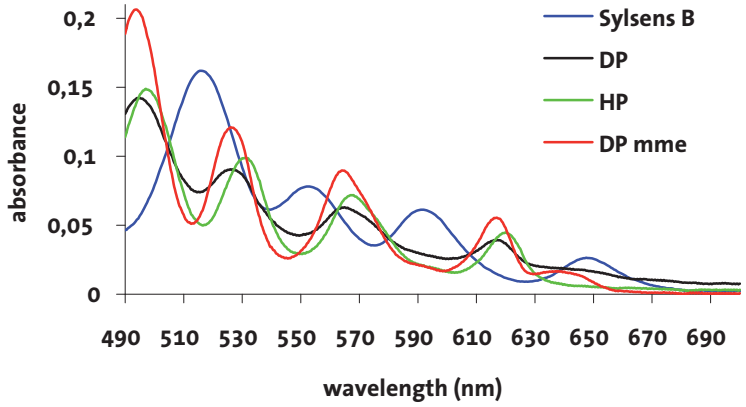

Figure 2.4. Absorption spectra of various porphyrins. The spectra were taken in methanol at a final concentration of 6.5 µg/mL (Shimadzu UV mini 1240). Stock solutions were made in 50 mM sodium phosphate buffer, pH 7.4 (Sylsens B and HP) and polyethyleneglycol–ethanol–water (3:2:5) for DP and DP mme.

ACKNOWLEDGEMENTS

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