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# Identification of photochemical oxidation products of 5,10,15,20-tetra(*m*-hydroxyphenyl)chlorin by on-line high-performance liquid chromatography–electrospray ionization tandem mass spectrometry

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## Abstract

An on-line HPLC–electrospray ionization tandem mass spectrometry system has been developed for the separation and characterization of the photochemical oxidation products of 5,10,15,20-tetra(*m*-hydroxyphenyl)chlorin (*m*-THPC), a new photodynamic therapeutic agent. *m*-THPC was photo-oxidized in methanol solution by exposure to white laboratory light for one week. The products were then separated by isocratic elution on an Apex C<sub>18</sub> column with 0.1% trifluoroacetic acid in acetonitrile (23:77, v/v) as mobile phase and characterized on-line by electrospray tandem mass spectrometry. The results showed the formation of 5,10,15,20-tetra(*m*-hydroxyphenyl) porphyrin (*m*-THPP), hydroxy *m*-THPP and three hydroxy *m*-THPC isomers. Hydroxylation on the reduced pyrrole ring of *m*-THPC was the most prominent reaction.

## 1. Introduction

5,10,15,20-Tetra(*m*-hydroxyphenyl)chlorin, *m*-THPC (Fig. 1), is a new photodynamic therapeutic agent [1,2] currently under clinical trial [3,4] for the treatment of malignant tumours. Studying the photochemical reactions of *m*-THPC is important for understanding the photodynamic therapy process, particularly in the identification of photobleaching products, and for assisting the

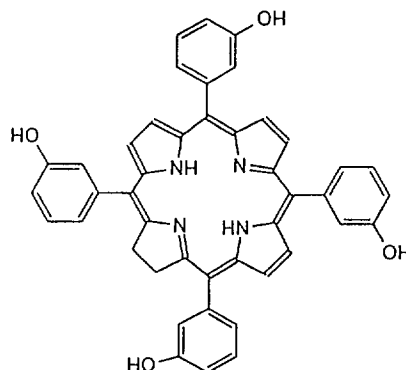


Fig. 1. Chemical structure of 5,10,15,20-tetra(*m*-hydroxyphenyl)chlorin (*m*-THPC).

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characterization of several hydroxylated *m*-THPC “metabolites” detected in the liver of rats and mice treated with the drug [5]. This paper describes the development and application of an

on-line HPLC–electrospray ionization tandem mass spectrometric (ESI-MS–MS) method for the separation and characterization of the photo-oxidation products of *m*-THPC.

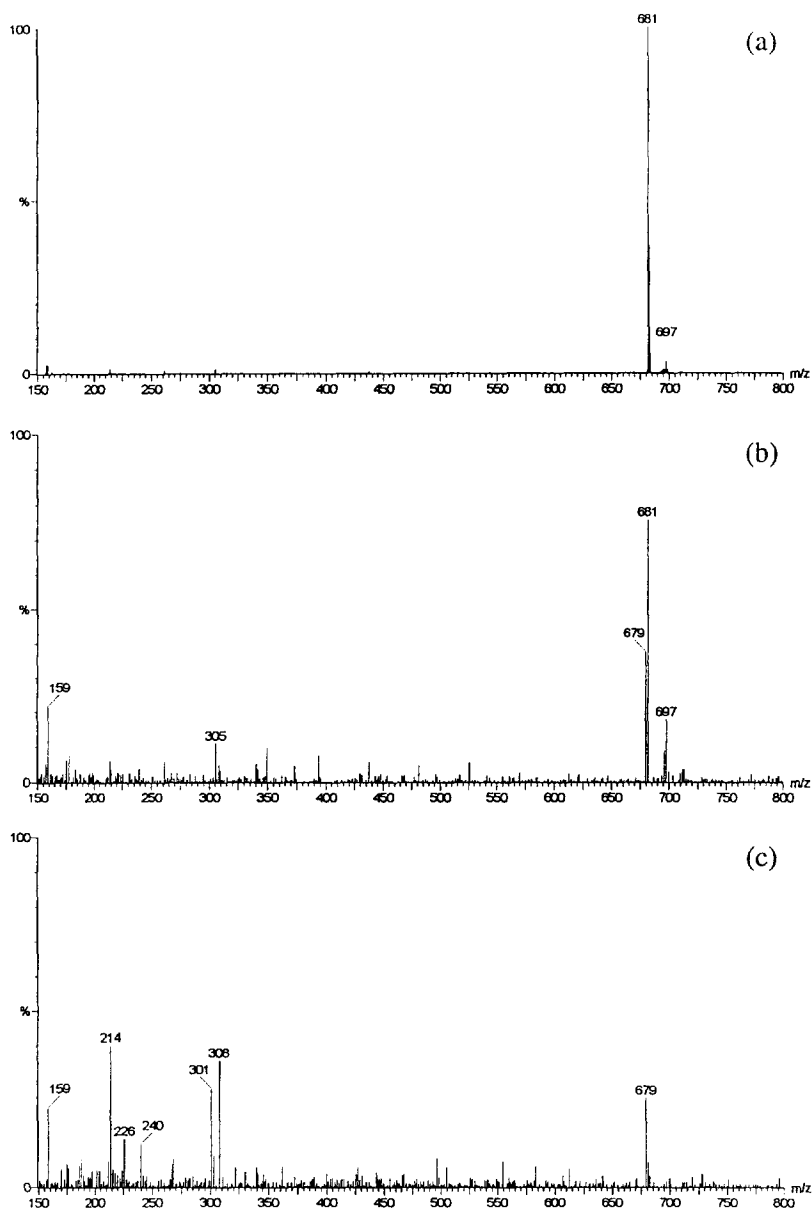


Fig. 2. Mass spectra of *m*-THPC and its photo-oxidation products. *m*-THPC was photo-oxidized by exposure to white laboratory light for a variety of times. Mass spectra were then acquired to identify photo-oxidation products of *m*-THPC: (a) after two weeks in the dark, (b) one week under white laboratory light and (c) two weeks under white laboratory light.

## 2. Experimental

### 2.1. Materials and reagents

5,10,15,20-Tetra(*m*-hydroxyphenyl)chlorin (*m*-THPC) was obtained from Scotia Pharmaceuticals (Guildford, Surrey, UK). 5,10,15,20-Tetra(*m*-hydroxyphenyl) porphyrin was from Queen Mary and Westfield College, London, UK. Acetonitrile and methanol were HPLC grade from

Fisons Scientific (Loughborough, Leicestershire, UK). Trifluoroacetic acid (TFA) was from Pierce and Warriner (Chester, UK). A 0.1% solution was made by dissolving 1 ml (1 ampoule) in 1 l of distilled water.

### 2.2. Photochemical oxidation of *m*-THPC

*m*-THPC was dissolved in methanol (1 mg/ml) and exposed continuously to ambient white lab-

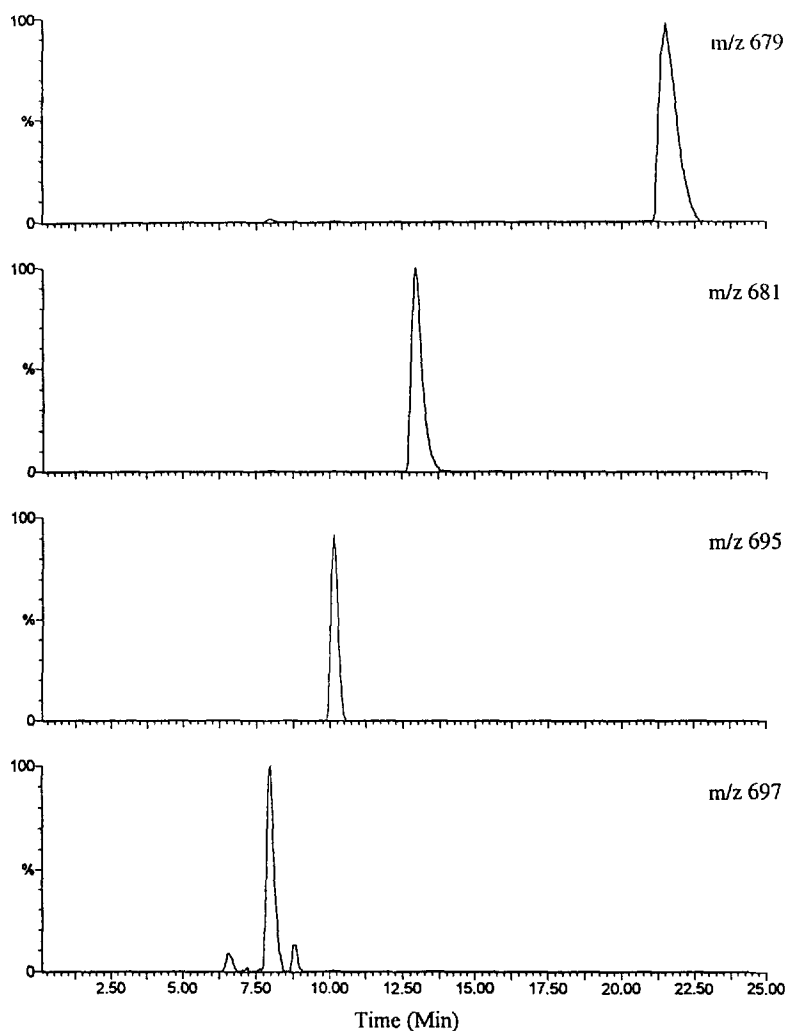


Fig. 3. Selected ion chromatograms following HPLC-ESI-MS separation of the photo-oxidation products of *m*-THPC. *m*-THPC was photo-oxidized by exposure to white laboratory light for one week. *m*-THPC and its photo-oxidation products were separated isocratically on an Apex C<sub>18</sub> column using 0.1% TFA-acetonitrile (23:77, v/v) as mobile phase, at a flow-rate of 1 ml/min.

oratory light from double fluorescent tubes behind a diffuser, plus incidental sunlight, for one and two weeks in the presence of air. The reaction mixture was analyzed first by dynamic ESI-MS without HPLC separation, and then by on-line HPLC–ESI-MS–MS. A control experiment was carried out by keeping a methanolic solution of *m*-THPC in the dark in the presence of air for two weeks and then analyzing it by ESI-MS.

### 2.3. Dynamic electrospray MS conditions

A Varian (Walton-on-Thames, Surrey, UK) 9012 HPLC pump was used for solvent delivery. The solvent was 0.1% TFA–acetonitrile (1:1, v/v) at a flow-rate of 50  $\mu\text{l}/\text{min}$ . The sample was injected via a Rheodyne (Cotati, CA, USA)

7125 injector fitted with a 100- $\mu\text{l}$  loop. A VG Quattro BQ tandem quadrupole mass spectrometer (Fisons Instruments, Manchester, UK) fitted with an API electrospray source was used. The capillary and HV electrode potentials were 0.38 and 3.78 kV, respectively. The source temperature was 90°C. Ion spectra were acquired in the positive-ion mode, over the mass range of 100 to 800 amu, at the rate of 1 scan every 2 s.

### 2.4. On-line HPLC–electrospray ionization MS–MS conditions

The HPLC separation was carried out on an Apex  $\text{C}_{18}$  column (25 cm  $\times$  4.6 mm, 5  $\mu\text{m}$  particle size) from Jones Chromatography, Hengoed, Mid-Glamorgan, UK. The mobile phase was 0.1% TFA–acetonitrile (23:77, v/v). The

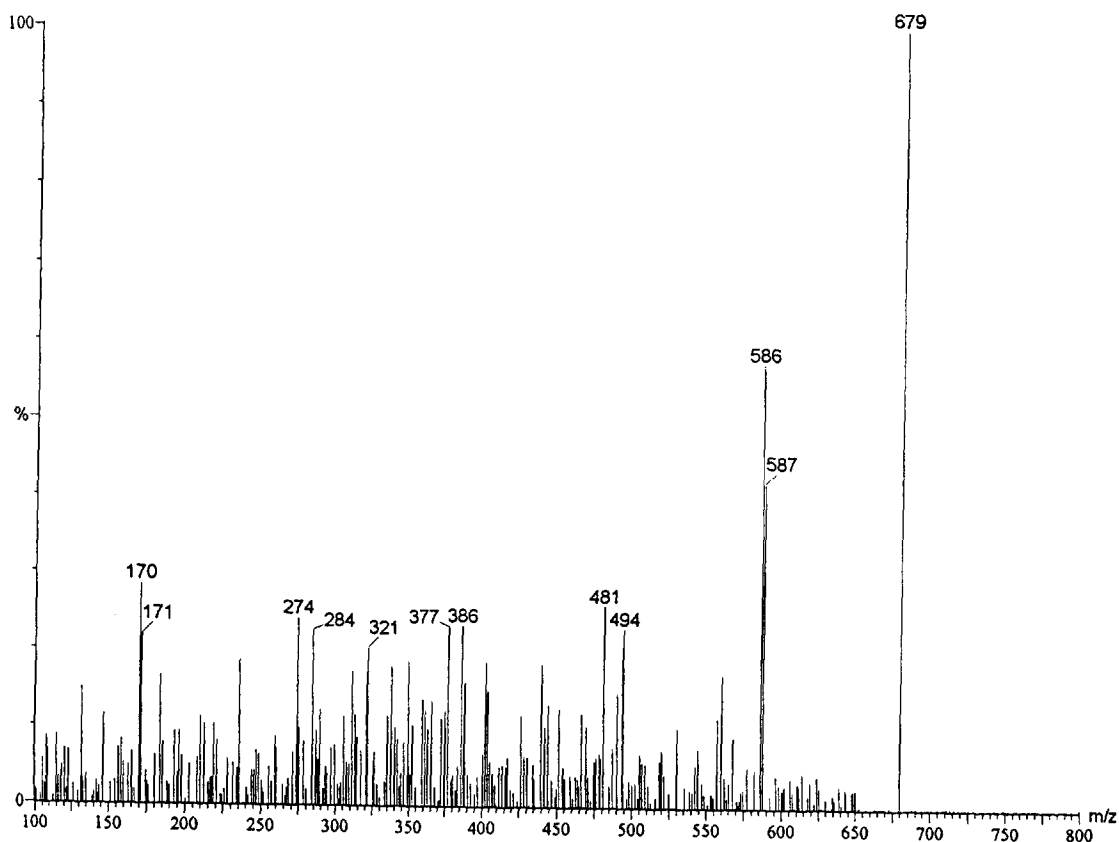


Fig. 4. Product ion spectrum of *m*-THPP. *m*-THPP ( $m/z$  679) was fragmented using a collision energy of 100 eV and argon as collision gas ( $4.3 \times 10^{-4}$  mbar), and the product spectrum was acquired over the mass range 100–800.

flow-rate was 1 ml/min. The eluate leaving the HPLC column was split in the ratio of 1:5 before entering the ESI-MS ion source. The source temperature was set at 150°C. The collision gas was argon. The collision cell pressure and collision energy were  $4.3 \times 10^{-4}$  mbar and 100 eV, respectively. Product ions were scanned between the masses 100 to 800, and the spectra collected in the form of continuum data.

### 3. Results and discussion

Optimization of the photochemical reaction is essential for the production of sufficient quantities of the products for isolation by HPLC and for analysis by HPLC–ESI-MS. Preliminary

study by irradiation of a methanolic solution of *m*-THPC in air with UV light or exposure to strong sunlight led to substantial degradation with little hydroxylated products being detected. This indicated that milder photo-oxidation conditions were needed. *m*-THPC solution was, therefore, exposed to white laboratory light only, and the reaction was monitored by dynamic ESI-MS at regular intervals. The results showed that the optimum conditions for the formation of hydroxylated products was exposure to laboratory light for one week: the products were degraded after two weeks exposure. The ESI spectra are shown in Fig. 2.

Two main groups of products were formed after one week exposure to light. These have the  $[M + H]^+$  signals at  $m/z$  697 and 679, indicating

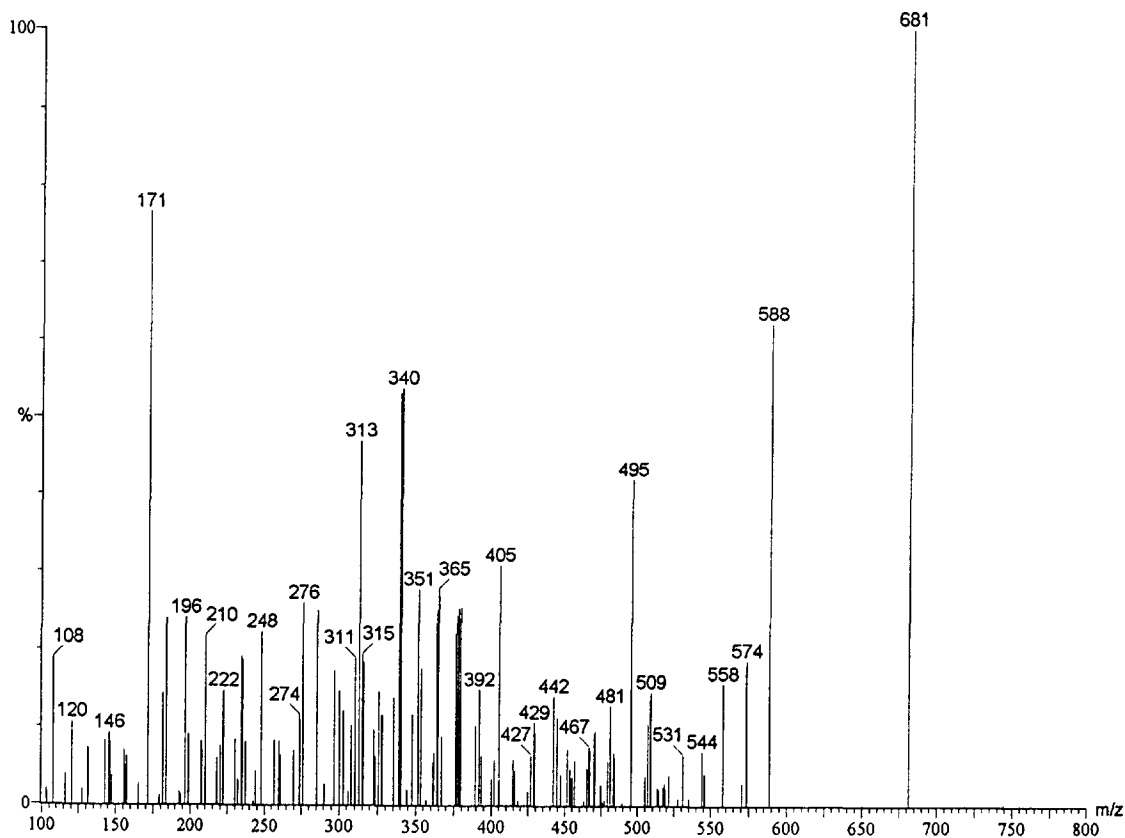


Fig. 5. Product ion spectrum of *m*-THPC. *m*-THPC ( $m/z$  681) was fragmented using a collision energy of 100 eV and argon as collision gas ( $4.3 \times 10^{-4}$  mbar), and the product spectrum was acquired over the mass range 100–800.

hydroxylation and dehydrogenation of *m*-THPC ( $m/z$  681), respectively. A smaller peak at  $m/z$  695 was also detected.

Analysis by on-line HPLC–ESI–MS showed the presence of three compounds with the  $[M + H]^+$  ion at 697, one compound with the  $[M + H]^+$  ion at  $m/z$  695 and one compound with the  $[M + H]^+$  ion at  $m/z$  679 (Fig. 3). The HPLC retention time and MS–MS fragmentation pattern of the peak with the  $[M + H]^+$  ion at  $m/z$  679 were identical to those of authentic *m*-THPP (Fig. 4). The main fragmentation pathway was the sequential elimination of phenolic rings to give ions at  $m/z$  586, 494 and 402, respectively. The fragmentation pathway of *m*-THPP was also shown by the parent compound, *m*-THPC, in which the loss of phenolic groups to give ions at

$m/z$  588, 496 and 404 was clearly observed (Fig. 5).

The three photo-oxidation products with  $[M + H]^+$  ions at  $m/z$  697 correspond to isomers  $C_{44}H_{32}N_4O_5$  in which one oxygen atom has been added to the *m*-THPC molecule. Such an extra oxygen atom could be envisaged as being at a nitrogen to give a chlorin N-oxide, e.g. as at I in Fig. 6; at a double bond to give a  $\beta,\beta'$ -oxiran, e.g. as at II in Fig. 6; or being formally inserted in a C–H bond to give a hydroxyl compound, e.g. as at III, IV and V in Fig. 6. Structures I and II are unusual and do not appear to be known in the tetraphenylchlorin series.

The three compounds  $C_{44}H_{32}N_4O_5$  had a relative abundance of 6, 86 and 8%, respectively (Fig. 3). Their UV–Vis spectra, obtained on-line

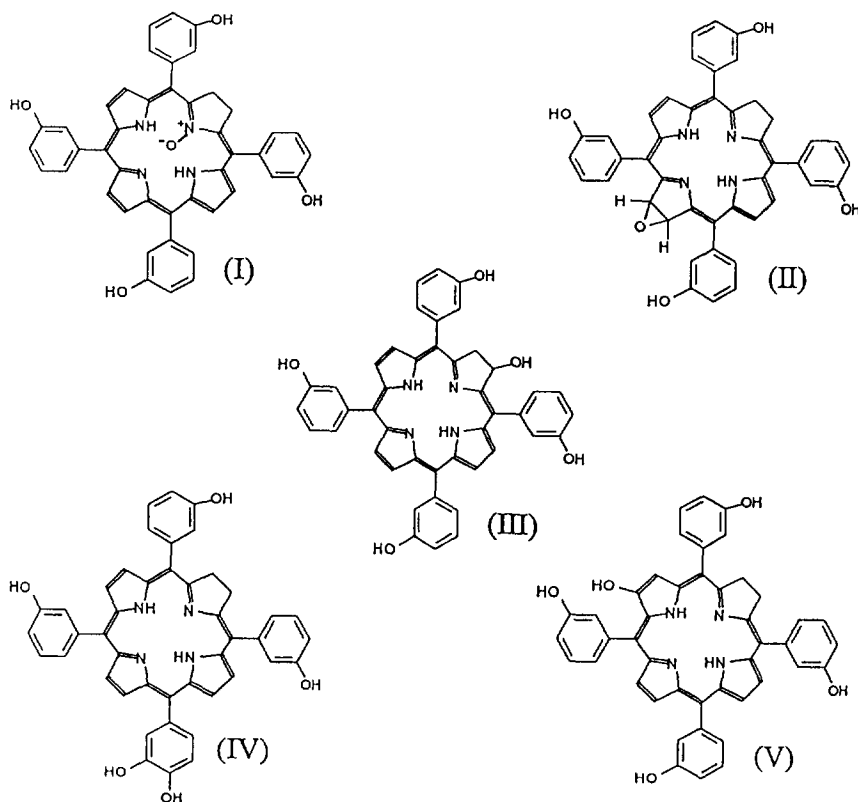


Fig. 6. Possible chemical structures of *m*-THPC derivatives with  $[M + H]^+$  ion of  $m/z$  697. (I) *m*-THPC N-oxide, (II) *m*-THPC- $\beta,\beta'$ -oxiran, (III)  $\beta$ -hydroxy-*m*-THPC (substitution on reduced ring), (IV) hydroxy-*m*-THPC, (V)  $\beta$ -hydroxy-*m*-THPC (substitution on pyrrole ring, isomers possible).

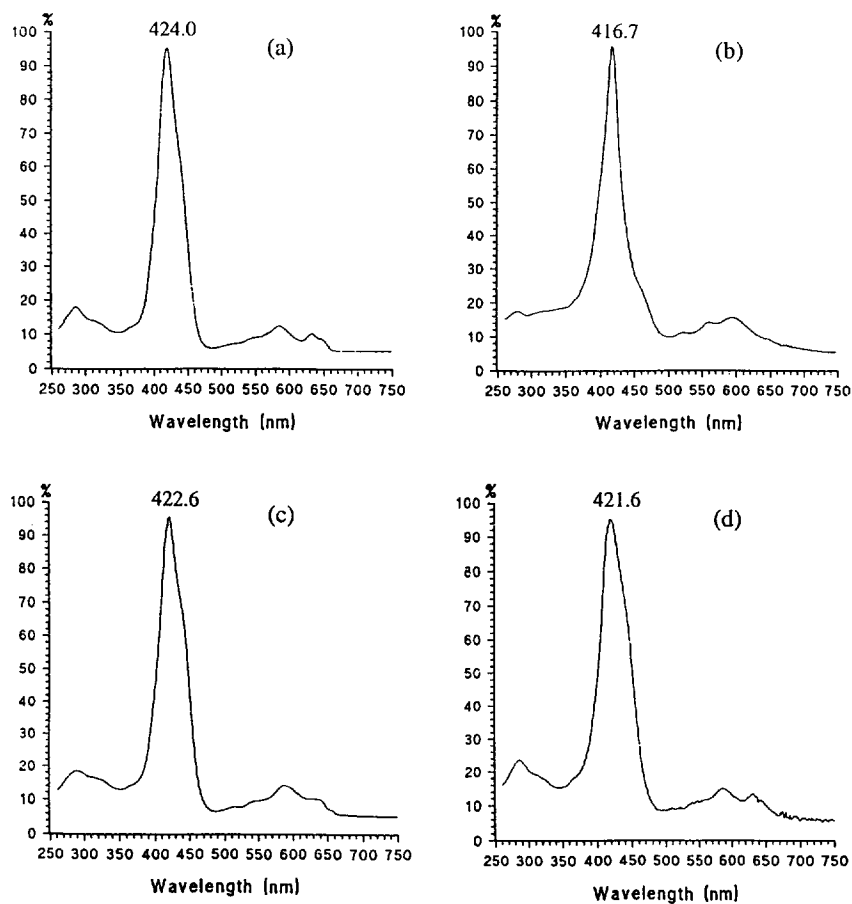


Fig. 7. UV-Vis spectra obtained on-line with a photodiode-array detector. (a) *m*-THPC, (b) hydroxy-*m*-THPC, (c)  $\beta$ -hydroxy-*m*-THPC (III), (d)  $\beta$ -hydroxy-*m*-THPC (V).

with a photodiode-array detector, are shown in Fig. 7. The major compound (retention time 8.0 min) is regarded as having a structure with the hydroxy group attached to the reduced pyrrole ring (as in Fig. 6, III), since it could be dehydrated by heating at 60°C in solution in the mobile phase to give *m*-THPP as the only product. This was confirmed by HPLC-ESI-MS-MS analysis. ESI-MS-MS analysis also showed that the main hydroxychlorin was easily dehydrated to *m*-THPP in the ion source. This was then followed by a fragmentation pattern (Fig. 8) similar to that of *m*-THPP (Fig. 4). The UV-Vis spectrum (Fig. 7c) of compound III was also very similar to that of the parent *m*-THPC (Fig. 7a),

consistent with substitution at the reduced pyrrole ring. Under the acidic conditions of the on-line measurement, the *m*-THPC is in the monoprotonated form [ $\lambda_{\text{max}}$  ( $\epsilon$ ) 424 (183 000), 585 (17 900) and 633 nm (14 200) in methanol:aqueous buffer = 1:1] [6], and the similarity of the UV-Vis spectra suggests that compound III is also monoprotonated under these conditions.

The formation of the  $\beta$ -hydroxychlorin (Fig. 6, III) as the major product detected under mild conditions can be readily rationalized in terms of a photochemically initiated radical chain auto-oxidation at the reactive pseudo-benzylic position. In a control experiment where a methanolic

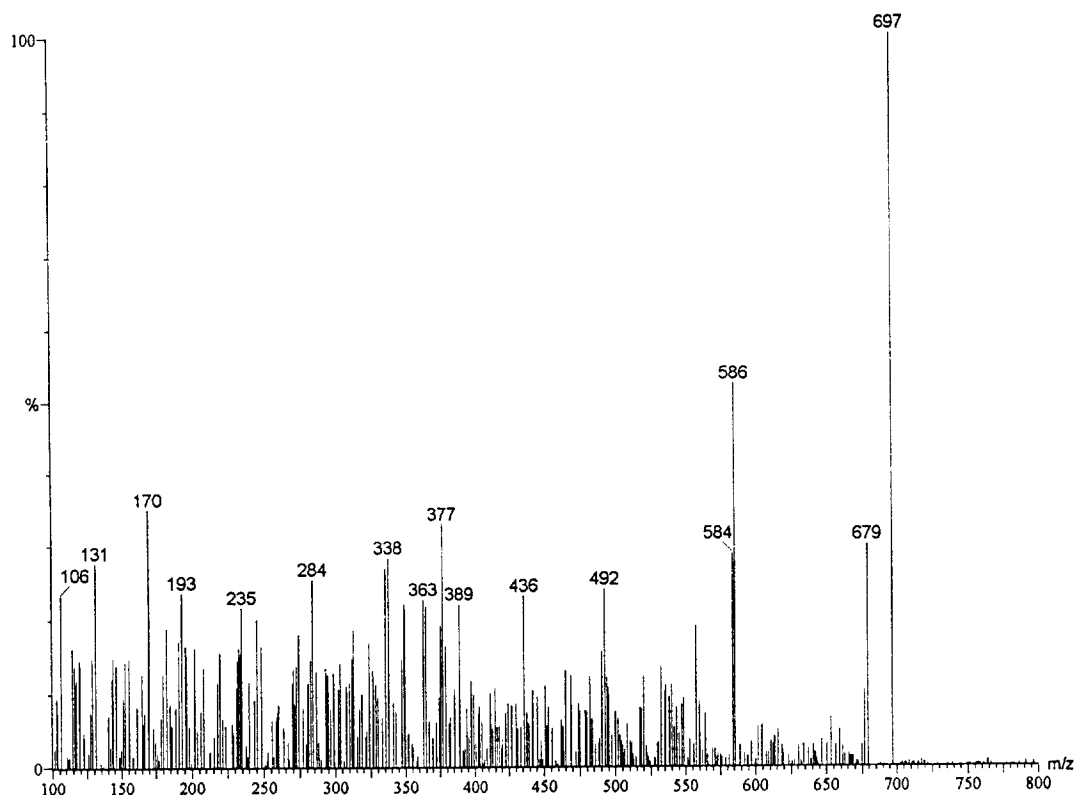


Fig. 8. Product ion spectrum of hydroxy-*m*-THPC ( $t_R = 8.0$  min), formed by photochemical oxidation of *m*-THPC. Hydroxy-*m*-THPC ( $m/z$  697) was fragmented using a collision energy of 100 eV and argon as collision gas ( $4.3 \times 10^{-4}$  mbar), and the product spectrum was acquired over the mass range 100–800.

solution of *m*-THPC was kept in the dark in air for two weeks, a small amount of hydroxychlorin was detected by ESI-MS (Fig. 2a). This would also support the free radical chain auto-oxidation hypothesis for the formation of the  $\beta$ -hydroxychlorin.

The two minor  $C_{44}H_{32}N_4O_5$  isomers were not dehydrated by mild heating in the acidic mobile phase, and are most probably compounds with the extra hydroxy group attached to a hydroxyphenyl ring (as in Fig. 6, IV) or to the  $\beta$ -position of an unsaturated pyrrole ring (as in Fig. 6, V). Isomers are possible in either case. On the basis of the available evidence it is not possible to make definitive structural assignments, and the quantities were insufficient for MS–MS analysis. However, the UV–Vis spectroscopic evidence

does provide some indication. The least mobile (least polar) component ( $t_R = 8.8$  min) possessed the spectrum shown in Fig. 7d. This is very similar to the electronic spectrum of the monocation of *m*-THPC (Fig. 7a), and accords with a structure with further hydroxylation on a phenyl ring (as in Fig. 6, IV). The most mobile (most polar) component ( $t_R = 6.6$  min) has a spectrum (Fig. 7b) which is rather different (e.g. Soret band at  $\lambda_{max}$  416.7 nm), and this would be consistent with substitution on the chlorin chromophore, as in Fig. 6, V. Here, aromatic hydroxylation ( $\beta$ -pyrrole or benzenoid) appears to be occurring, and presumably involves hydroxyl radicals generated from the hydrogen peroxide (or alkylperoxide) formed in the dehydrogenation of *m*-THPC.



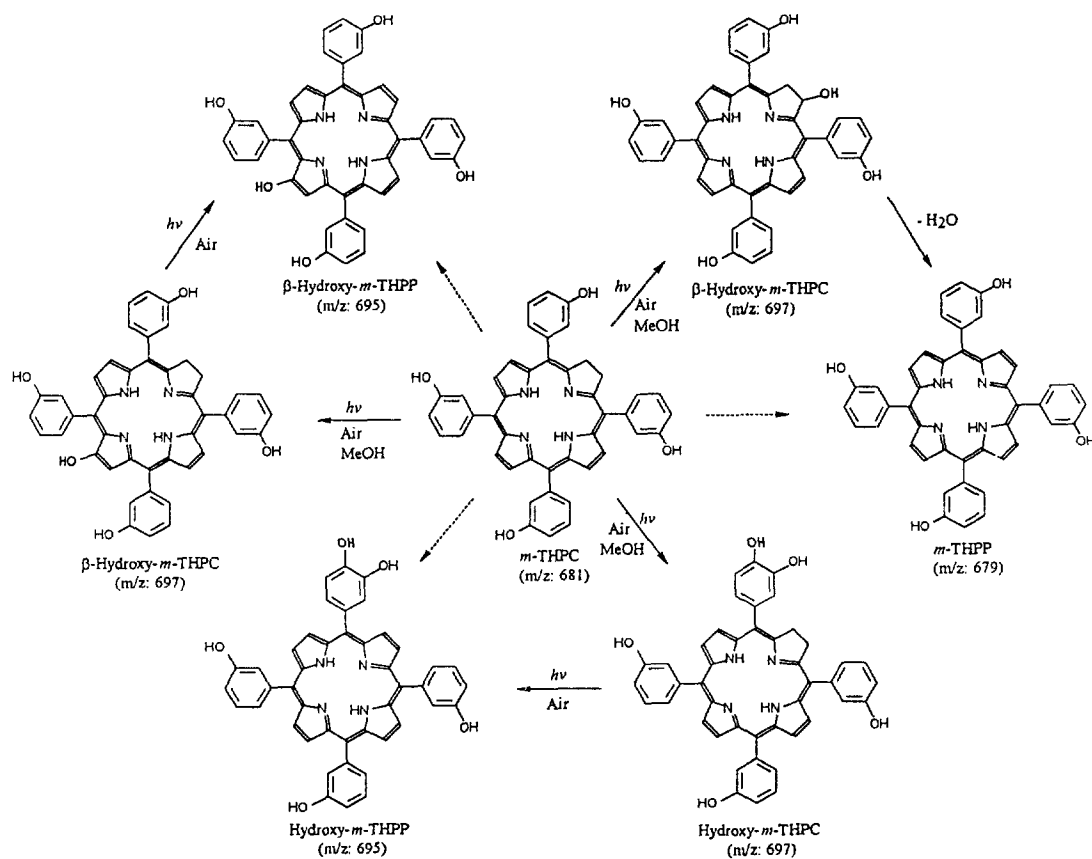


Fig. 9. Proposed scheme for the photo-oxidation of *m*-THPC.

The peak with the  $[M + H]^+$  ion at  $m/z$  695 is consistent with the hydroxy-*m*-THPP structure (Fig. 9) with the OH group attached to a hydroxyphenyl group, or an unsaturated pyrrole ring. This compound might arise by dehydrogenation of the hydroxy-*m*-THPC structures.

The present study has demonstrated that the combination of the high resolution of HPLC with the specificity of MS–MS analysis is a powerful technique for the characterization of *m*-THPC derivatives. Photochemical oxidation of *m*-THPC gave *m*-THPP and a mixture of hydroxylated derivatives. The reaction scheme is outlined in Fig. 9. The technique is currently being applied to the identification of *m*-THPC metabolites and photobleaching products.

## References

- [1] R. Bonnett, R.D. White, U.J. Winfield and M.C. Berenbaum, *Biochem. J.*, 261 (1989) 277.
- [2] R. Bonnett and M. Berenbaum, in G. Bock and S. Harnett (Editors), *Photosensitizing Compounds. Their Chemistry, Biology and Clinical Use*, Wiley, 1989, pp. 41–59.
- [3] H.-B. Ris, H.J. Altermatt, R. Inderbitzi, R. Hess, B. Nachbur, J.C.M. Stewart, Q. Wang, C.K. Lim, R. Bonnett, M.C. Berenbaum and U. Althaus, *Brit. J. Cancer*, 64 (1991) 1116.
- [4] H.-B. Ris, H.J. Altermatt, B. Nachbur, J.C.M., Stewart, Q. Wang, C.K. Lim, R. Bonnett and U. Althaus, *Int. J. Cancer*, 53 (1993) 141.
- [5] Q. Wang, H.J. Altermatt, H.-B. Ris, B.E. Reynolds, J.C.M. Stewart, R. Bonnett and C.K. Lim, *Biomed. Chromatogr.*, 7 (1993) 155.
- [6] B.D. Djelal, Ph.D. Thesis, London, 1994.