Photochemistry and Photobiology Vol. 47, No. 5, pp. 713–717, 1988
Printed in Great Britain.

0031-8655/88 $03.00+0.00

BIOLOGICAL ACTIVITIES OF PHTHALOCYANINES—X. SYNTHESES AND ANALYSES OF SULFONATED PHTHALOCYANINES

HASRAT ALI, RÉJEAN LANGLOIS, J. RICHARD WAGNER, NICOLE BRASSEUR, BENOIT PAQUETTE and JOHAN E. VAN LIER*
MRC Group in the Radiation Sciences, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Québec, Canada J1H 5N4

(*Received* 19 *August* 1987; *accepted* 2 *November* 1987)

Abstract—Synthetic methods to obtain selectively sulfonated metallo phthalocyanines are compared. Both condensation and direct sulfonation procedures lead to mixtures of mono- to tetrasulfonated products which are resolved by reverse phase liquid chromatography in buffered aqueous-methanol. The proportion of sulfonated derivatives is examined as a function of the starting reagents in the case of the condensation method, and as a function of the temperature and reaction time in the case of the direct sulfonation procedure. The number of sulfonate groups per phthalocyanine molecule is determined by oxidative degradation of the phthalocyanine ring followed by quantitative chromatographic analysis of the sulfophthalamide and phthalamide fragments.

INTRODUCTION

Phthalocyanines (PC)† and their water soluble sulfonated derivatives (SPC) (Fig. 1) have received increasing attention as sensitizers for photodynamic therapy of cancer [for recent reviews, see Spikes (1986), Ben-Hur (1987), van Lier *et al.* (1988)]. The degree of sulfonation affects the tendency of the SPC to aggregate, and thus changes their ability to generate $^1O_2$ (Wagner *et al.*, 1987), and to be transported through cell membranes which are important factors of their photocytotoxicity (Brasseur *et al.*, 1987a). Accordingly, comparative studies of metallo-SPC demand well characterized dye preparations. We describe here a simple procedure for the analysis of the average number of sulfonate groups per PC molecule in SPC preparations (S/PC ratio) as well as analytical and preparative chromatographic methods for separating SPC. The principal synthetic procedures for metallo-SPC are investigated with respect to the complexity and the degree of sulfonation of the product mixtures.

MATERIALS AND METHODS

Gallium metal, phthalic acid, 4-sulfophthalic acid, phthalamide, 1,2-dicyanobenzene and phthalic anhydride were purchased from Aldrich. Zn-PC and AlCl-PC were purchased from Eastman Kodak. Fuming sulfuric acid (30% free SO_3) and HPLC grade solvents were obtained from Fisher Scientific. Triply distilled water was used throughout this work. The Carbon-13 Nuclear Magnetic Resonance (^{13}C NMR) spectra were recorded on a Bruker WM 250 apparatus with D_2O or D_2SO_4 as the solvent. Chemical shifts are reported in ppm relative to TMS. Ultraviolet-visible absorption spectra were recorded on a Varian 2000 spectrophotometer. Combustion analyses were performed by Guelph Chemical Laboratories, Guelph, Ont. Canada.

Syntheses of metallo sulfophthalocyanines. The syntheses of GaCl-SPC, sulfonated to different degrees, is described in detail as an example. Other metallo-SPC, including AlCl- and Zn-SPC, were prepared in similar manners.

Tetrasodium salt of chlorogallium 4,4′,5″,5‴-tetrasulfophthalocyanine (GaCl-TSPC) by the condensation method. The procedure was adapted from Weber and Bush (1965). It has recently been shown by Gaspard and Maillard (1987) that the substituents of tetrasubstituted metallo-PC, obtained from the 4-substituted phthalo monomer, are oriented on the 4,4′,5″,5‴-positions during the formation of the macrocycle, resulting in the formation of only one out of four possible constitutional isomers. $GaCl_3$ was prepared by reaction of metallic gallium with concentrated HCl. The monosodium salt of 4-sulfophthalic acid (4.3 g, 16.2 mmol), NH_4Cl (470 mg, 90 mmol), urea (5.8 g, 970 mmol), ammonium molybdate (68 mg, 0.06 mmol) and $GaCl_3$ (875 mg, 0.5 mmol) were pulverized to a homogeneous powder. The material was heated to 180°C at which point urea decomposes to yield NH_3. After 30 min the temperature was raised to 200°C for 4 h in order to complete the condensation reaction. The solid cake was dispersed in boiling 1 *N* HCl saturated with NaCl (500 mℓ), cooled to room temperature and the precipitate was collected and redissolved in 0.1 *N* NaOH (200 mℓ). The solution was heated to 80°C, filtered to remove insoluble impurities, neutralized with 1 *N* HCl and evaporated on a vacuum to yield a green solid. Part of the crude material (100 mg) was purified by preparative reverse phase HPLC in water to remove salt, followed by 50% MeOH in water to yield 30 mg of the GaCl-TSPC. ^{13}C NMR data are reported in Table 1. UV-vis (MeOH, monomeric) λ_{max} (ϵ 10^4 cm^{-1} M^{-1}): 674 (22), 609 (2.9), 342 (14.1) nm. Anal. Calc. for $C_{32}H_{12}N_8S_4O_{12}$ ClGa. $9H_2O$ (mol. wt. 1188): C, 32.33; H, 2.54; N, 9.43. Found C, 32.7; H, 2.21; N, 9.75.

Mono- to trisulfonated chlorogallium phthalocyanines (GaCl-SPC) by the condensation method. Mixtures of mono-, to tetrasulfonated GaCl-SPC were obtained in a similar manner as described for the GaCl-TSPC by substituting the sulfophthalic acid with mixtures of various ratios of phthalic acid, or anhydride, and sulfophthalic acid. The homogeneous mono- to trisulfonated complexes were obtained from preparative reverse phase medium pressure

*To whom correspondence should be addressed.

†*Abbreviations*: DSPC, diSPC; MSPC, monoSPC; PC, phthalocyanine; SPC, sulfophthalocyanine; TFA, trifluoroacetic acid.

Table 1. ^{13}C-NMR of phthalocyanines

PC	Chemical shifts in ppm (carbon number)*
Zn-PC	133.76 ($C_{1,8}$), 127.12 ($C_{2,7}$), 124.98 ($C_{3,4,5,6}$)
AlCl-PC	149.12 ($C_{1,8}$), 135.20 ($C_{2,7}$), 126.10 ($C_{3,4,5,6}$)
GaCl-PC	149.67 ($C_{1,8}$), 135.01 ($C_{2,7}$), 126.36 ($C_{3,6}$) and 125.85 ($C_{4,5}$)
GaCl-TSPC	149.4 ($C_{1,8}$), 146.1 (C_4), 132.4 (C_7), 130.9 (C_2), 129.2 (C_6), 125.5 (C_3) and 122.8 (C_5)
GaCl-TSPC†	154.3 ($C_{1,8}$), 148.3 (C_4), 138.3 (C_7), 137.8 (C_2), 131.4 (C_6-H),‡ 127.2 (C_3-H)‡ and 123.2 (C_5-H)‡

*Spectra taken in D_2SO_4, except for b. Carbon numbers refer to Fig. 1 and the structure on Fig. 4 and are tentatively assigned (see discussion in text). †Taken in D_2O. ‡Reversed signals, indicative of methine carbons.

chromatography and HPLC.

Mono- to tetrasulfonated chlorogallium phthalocyanines (GaCl-SPC) by the sulfonation method. The sulfonation procedure was adapted from Linstead and Weiss (1950). Chlorogallium phthalocyanine (GaCl-PC; 308 mg; 0.5 mmol), prepared *via* the condensation method, was dissolved in 15 mℓ oleum (fuming H_2SO_4 containing 30% free SO_3) and heated at 75°C with stirring. The mixture was kept at this temperature for 1–4 h depending on the degree of sulfonation required, whereafter it was poured onto crushed ice. The resulting dark green precipitate was filtered and washed with 1 *N* HCl (10 mℓ), redissolved in 1 *N* NaOH (20 mℓ) and filtered in order to remove water-insoluble impurities including nonsulfonated PC. The filtrate was neutralized with 1 *N* HCl, analyzed by HPLC, concentrated under vacuum and stored at 0°C pending chromatographic purification.

Preparative medium pressure chromatography. Sulfonated metallo-PC were purified on a 30 cm long by 2 cm ID glass column packed with C-18 reverse phase, particle size 25–40 μm (Macherey-Nagel, Düren, Germany) using a linear gradient (140 min) from 0 to 95% MeOH in 10 m*M* sodium phosphate buffer, pH 5, at a flow rate of 2 mℓ min^{-1}. A concentrated dye solution (30 mg) was loaded onto the column with a FMI model RP-SY pump. Eluting SPC were detected at 650 or 680 nm and desalted by rechromatography on the same column in water (100 mℓ). The mixed mono- to tetrasulfonated dyes were eluted with MeOH (25–75%) in water.

Reverse phase HPLC. Metallo-SPC sulfonated to different degrees and their constitutional isomers were separated on a 25 cm long by 0.94 cm ID semi-preparative reverse phase column packed with ODS-2 spherisorb, 5 μm (CSC, Montreal), operated at 2 mℓ min^{-1} with a linear gradient (55 min) from 0 to 95% MeOH in 10 m*M* sodium phosphate buffer, pH 5. SPC were detected by their absorption at 650 or 680 nm. This system was used for both analytical and preparative purposes. Retention times of various metallo-SPC were only slightly affected by the nature of the central metal ion and varied from 20–25 min for the TSPC, 25–35 min for the triSPC, 35–55 for the DSPC, and 55–65 min for the MSPC metallo complexes. The resolution between the various isomers and differently sulfonated SPC in this system depends on the synthetic procedure and the nature of the central metal ion. For example, near-baseline separations between the constitutional isomers of the disulfonated dye were achieved (Fig. 2A) for GaCl-SPC prepared by the condensation procedure.

Degree of sulfonation. The average number of sulfonate groups per SPC molecule (S/PC ratio) of SPC preparations was determined by a simple oxidative degradation procedure followed by HPLC in order to quantify the corresponding sulfophthalimide and phthalimide products. Briefly, 1 mg of the sulfonated dye is dissolved in a minimal volume of concentrated HNO_3 and the solution is heated to 50°C until the characteristic green SPC color disappears (1–5 min). The solution is neutralized with 1 *N* NaOH and analyzed on a reverse phase HPLC column (see above). The solvent program consists of a 5 min isocratic elution with 0.1% trifluoroacetic acid (TFA) in water followed by a 30 min linear gradient from this solvent to 100% MeOH at a flow rate of 2 mℓ min^{-1}. Phthalimide products are well separated in this system and detected by their absorption at 215 nm (Fig. 2). Retention times of the three possible products are, 3-sulfophthalimide 15 min, 4-sulfophthalimide 16 min and phthalimide 28 min. These products were quantified by manual integration of the peak surface.

RESULTS AND DISCUSSION

The synthesis of sulfonated phthalocyanines is easy, however, the resulting mixtures of more or less sulfonated products and constitutional isomers are difficult to separate. We initially examined the feasibility of separating metallo-SPC mixtures by electrophoresis, ion exchange, Sephadex exclusion, silica gel column and thin layer chromatography (Rousseau *et al.*, 1983) and ion-pair reverse phase HPLC (Gloor and Johnson, 1977; Oppenheimer, 1981), but found that conventional reverse phase HPLC, using mixtures of phosphate buffered water and MeOH, resulted in the best resolution. Furthermore, in the case of preparative separations, phosphate buffer is easier to remove from the product than are ion-pairing agents. A comparison of the analysis of GaCl-SPC mixtures obtained either from condensation or from sulfonation methods is presented in Fig. 2. In the case of the condensation method when starting with various mixtures of sulfophthalic-phthalic acid, HPLC analysis gave baseline separation of individual components in each of the four groups of peaks observed. All of these peaks showed the characteristic UV-vis absorption

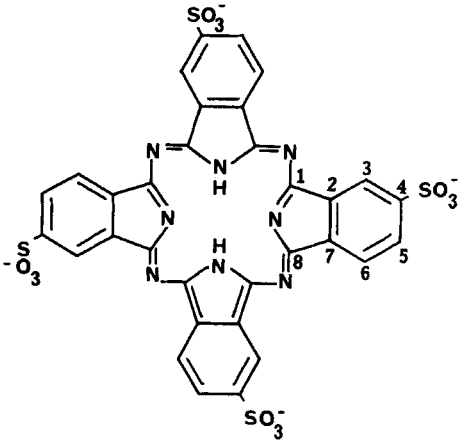

Figure 1. Structure of one of the possible isomers of tetrasulfophthalocyanine (TSPC).

spectrum of the GaCl-PC macrocycle (λ_{max} 674 nm in MeOH), with a constant UV/vis absorbance ratio. The degree of sulfonation (S/PC ratio) of the four major HPLC fractions (Fig. 2) was determined by the degradation assay. In the order of their increasing retention times, fractions were identified as tetra- to monosulfonated GaCl-SPC. The di- and trisulfonated regions are comprised of several peaks representing constitutional isomers. The four disulfonated GaCl-SPC isomers (Fig. 2A) were separately tested for their *in vitro* photosensitizing activities (Brasseur *et al.*, 1987b). Marked differences were observed between the biological activities which correlate well with differences in hydrophobicity and cell penetrating properties of the isomeric dyes. In accordance with the mobilities of isomeric disulfonated tetraphenyl porphyrins (Kessel *et al.*, 1987), we assign the isomer with sulfonate groups in opposite phthalic subunits of the PC molecule as the most hydrophilic (R_t = 40 min) whereas the one with adjacent groups as the most hydrophobic (R_t = 48 min) (Fig. 2). The amphiphilic nature of the latter isomer may well explain its marked cell membrane penetrating properties and high cellular phototoxicity.

Direct sulfonation of GaCl-PC gave a more complex HPLC elution pattern (Fig. 2B) with several peaks at 215 nm lacking the characteristic phthalocyanine UV-vis absorption. These peaks are most likely degradation products formed as a result of the extreme conditions required for sulfonation. Direct sulfonation results in substitution at both the 3- and 4-positions of the phthalic subunits, further contributing to the high complexity of the product mixture. The degradation assay of individual HPLC fractions indicates the presence of equal amounts of 3- and 4-sulfophthalimide (Fig. 3). This contrasts with SPC obtained by way of the condensation method where the phthalic subunits are only substituted at the 4-position (Fig. 3).

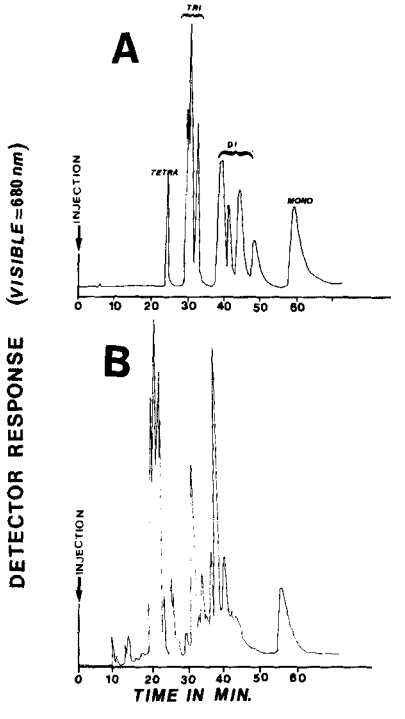

Figure 2. HPLC analysis of GaCl-SPC obtained by the condensation of phthalic and 4-sulfophthalic acid (1 : 1) with $GaCl_3$ (A), and GaCl-SPC obtained by direct sulfonation of GaCl-PC in oleum for 4 h at 75°C (B).

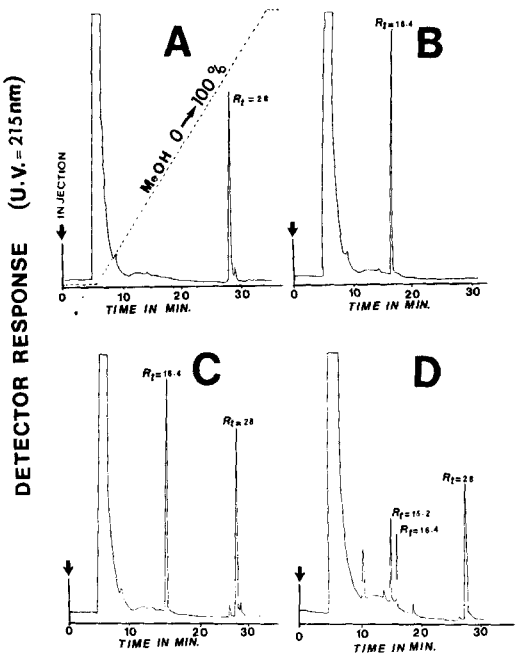

Figure 3. HPLC analysis of phthalimides for the S/PC ratio assay. (A) authentic phthalimide, (B) authentic 4-sulfophthalimide, (C) mixture of phthalimide and 4-sulfophthalimide (1 : 1) obtained from HNO_3 degradation of GaCl-DSPC prepared *via* the condensation method and (D) mixture of phthalimide, 4-sulfophthalimide and 3-sulfophthalimide (2 : 1 : 1) obtained from HNO_3 degradation of GaCl-DSPC prepared *via* the sulfonation method.

The ^{13}C proton decoupled NMR spectra of Zn- and AlCl-PC display three sharp signals in D_2SO_4. As for porphyrins, the carbons closer to the PC center are shifted to higher ppm because of deshielding effects of the macrocycle ring current (Abraham *et al.*, 1982), thereby, these signals can be assigned as the C_1 and C_8 pair furthest downfield followed by C_2 and C_7 and then four equivalent benzyl carbons which are least affected by the PC ring (Fig. 4 and Table 1). Interestingly, the benzyl carbons of GaCl-PC are split into two signals suggesting ring distortion for this metallo complex. This may be caused by an out-of-plane position of the central metal ion, which can be expected more pronounced in GaCl-PC as compared to the analogous complex with the smaller Al ion. The spectra of GaCl-TSPC shows seven carbon signals in D_2SO_4 as well as in D_2O. Concentration effects leading to

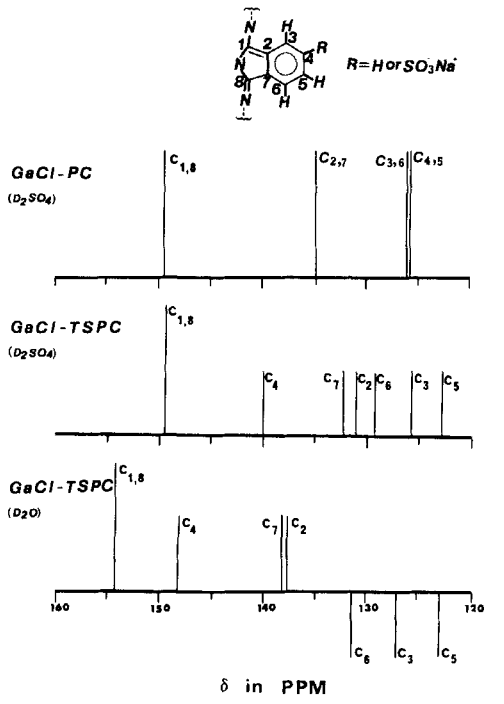

Figure 4. ^{13}C NMR bar plot of signals from GaCl-PC and GaCl-TSPC taken in D_2SO_4 and D_2O. Chemical shifts are in ppm relative to TMS.

intermolecular ring current shielding appear to be responsible for the increased chemical shifts in D_2O, since the GaCl-TSPC solution (40 mg/2 mℓ) is expected to be aggregated. The carbon signals were further characterized by employing J-resolved spin echo which permits separation of the quaternary from methine carbons. Two of these carbons, C_1 and C_2 can be assigned as a single fairly broad multiplet at about 154 ppm (in D_2O). Keeping these results in mind, then, the complete assignment was further aided by comparison with benzene sulfonic acid. The shift of sulfonated substituted aromatic carbons is about 16 ppm which is consistent with a 20 ppm shift of C_4 (Breitmaier *et al.*, 1979). Benzene sulfonic acid also shows the bigger chemical shift at the para position (+ 4.8 ppm) followed by the meta positions (+ 2.3 ppm) while the ortho carbons are shifted slightly upfield (− 1.2 ppm) with respect to benzene. Therefore, we expect C_7 to be at higher ppm than C_2 and for identical meta positions, C_3 would likely appear at higher ppm as compared to C_5 because C_3 is closer to the PC ring.

The effect of the percentage of sulfophthalic *vs* phthalic acid and the nature of other reactants on the composition of the condensation reaction products is summarized in Table 2. It may be seen from this table that the highest percentage of the most biologically active mono- and disulfonated GaCl-SPC (Brasseur *et al.*, 1987) are obtained with 50% 4-sulfophthalic acid. Phthalic acid can be replaced with a number of other reactants, including 1,2-dicyanobenzene, phthalic anhydride and phthalamide. Condensation of these compounds in the presence of sulfophthalic acid always results in similar ratios between the differently sulfonated SPC products. Likewise, the nature of the metal ion has little effect on the product distribution pattern (Table 2). In contrast, with the direct sulfonation of the metallo-PC, the nature of the central metal ion strongly affects the course of the reaction (Table 3). Thus, with Zn-PC as a substrate, good yields of a relative clean mixture of mono- and disulfonated Zn-SPC is obtained after 30 min at 50°C, whereas similar yields of mono- and disulfonated product with the GaCl-

Table 2. Relative yields of mono- to tetrasulfonated metallo SPC obtained *via* the condensation method

Metal	Reactants (%)		Relative yield of metallo SPC				
			TSPC	TriSPC	DSPC	MSPC	PC
$GaCl_3$	Phthalic acid	4-sulfophthalic acid					
$GaCl_3$	(100)	(0)	—	—	—	—	100
$GaCl_3$	(0)	(100)	100	—	—	—	—
$GaCl_3$	(50)	(50)	0	0	46	54	—
$GaCl_3$	(33)	(67)	8	35	41	16	—
$GaCl_3$	(25)	(75)	16	55	25	4	—
$GaCl_3$	1,2-Dicyanobenzene						
$GaCl_3$	(50)	(50)	1	27	49	23	—
$GaCl_3$	Phthalamide						
$GaCl_3$	(50)	(50)	0	13	54	33	—
$GaCl_3$	Phthalic anhydride						
$GaCl_3$	(50)	(50)	9	15	45	35	—
$AlCl_3$	Phthalic anhydride						
$AlCl_3$	(50)	(50)	26	28	21	25	—
$AlCl_3$	Phthalic acid ammonium salt						
$AlCl_3$	(50)	(50)	35	12	33	20	—
$Zn(OAc)_2$	Phthalic anhydride						
	(50)	(50)	6	49	28	17	—

Table 3. Relative yields of mono- to tetrasulfonated metallo SPC obtained *via* the sulfonation method

Substrate	Temp. (°C)	Time (h)	Relative yield of metallo SPC			
			TSPC	TriSPC	DSPC	MSPC
GaCl-PC	50	8	4	8	16	72
GaCl-PC	75	1	2	6	10	82
GaCl-PC	75	2	11	12	27	50
GaCl-PC	75	4	44	16	31	9
GaCl-PC	85	6	>95	—	—	—
Zn-PC	50	0.5	—	2	48	50
Zn-PC	75	1	—	29	71	—
Zn-PC	75	2	—	62	38	—
Zn-PC	75	4	9	77	14	—
Zn-PC	85	6	>90	—	—	—
AlCl-PC	75	1	0	3	7	90
AlCl-PC	75	2	4	9	25	62
AlCl-PC	75	4	14	20	30	36

or AlCl-PC require substantially longer reaction times and higher temperatures (Table 3). Although the condensation method gives less complex mixtures and is more reproducible, comparison of the two methods (Tables 2 and 3) shows that if mono- and disulfonated products are required, direct sulfonation is the preferred procedure.

In conclusion, phthalocyanines and their sulfonated derivatives are readily prepared but often require extensive chromatography to ensure homogeneity. We have presented chromatographic procedures to analyse the composition, including the degree of sulfonation, of phthalocyanine preparations. In view of the extreme differences in biological activities as to the degree of sulfonation and type of isomers, it is important to report such characteristics of dye preparations used in photodynamic protocols.

Acknowledgements—Generous financial support for this work was provided by the Medical Research Council of Canada and the National Cancer Institute of Canada.

REFERENCES

Abraham, R. J., K. M. Smith, D. A. Goff and J.-J. Lai (1982) NMR spectra of porphyrins. 18. A ring-current model for chlorophyll derivatives. *J. Am. Chem. Soc.* **104**, 4332–4337.

Ben-Hur, E. (1987) Photochemistry and photobiology of phthalocyanines: new sensitizers for photodynamic therapy of cancer. In *From Photophysics to Photobiology* (Edited by A. Favre, R. Tyrrell and J. Cadet), pp. 407–420. Elsevier, New York.

Brasseur, N., H. Ali, R. Langlois and J. E. van Lier (1987b) Biological activities of phthalocyanines—VII. Photoinactivation of V-79 Chinese hamster cells by selectively sulfonated gallium phthalocyanines. *Photochem. Photobiol.* **46**, 739–744.

Brasseur, N., H. Ali, R. Langlois, J. R. Wagner, J. Rousseau and J. E. van Lier (1987a) Biological activities of phthalocyanines—V. Photodynamic therapy of EMT-6 mammary tumors in mice with sulfonated phthalocyanines. *Photochem. Photobiol.* **45**, 581–586.

Breitmaier, E., G. Haas and W. Voelter (1979) *Atlas of Carbon-13 NMR data, Vol. 2*, Compound No. 1971. Heyden, New York.

Gaspard, S. and Ph. Maillard (1987) Structure des phthalocyanines tetratertio-butyles: Mécanisme de la synthèse. *Tetrahedron* **43**, 1083–1090.

Gloor, R. and E. L. Johnson (1977) Practical aspects of reverse phase ion-pair chromatography. *J. Chromatogr. Sci.* **15**, 413–423.

Linstead, R. P. and F. T. Weiss (1950) Phthalocyanines and related compounds. Part XX. Further investigations on tetrabenzoporphin and allied substances. *J. Chem. Soc.* 2975–2981.

Oppenheimer, L. E. (1981) Ion-pair chromatography of sulfonated copper phthalocyanine. *J. Chromatogr. Sci.* **19**, 266–269.

Rousseau, J., D. Autenrieth and J. E. van Lier (1983) Synthesis, tissue distribution and tumor uptake of ^{99}Tc-tetrasulfophthalocyanine. *Int. J. Appl. Radiat. Isot.* **34**, 571–579.

Spikes, J. E. (1986) Yearly review: Phthalocyanines as photosensitizers in biological systems and for the photodynamic therapy of tumors. *Photochem. Photobiol.* **43**, 691–699.

van Lier, J. E., N. Brasseur, B. Paquette, J. R. Wagner, H. Ali, R. Langlois and J. Rousseau (1988) Phthalocyanines as sensitizers for photodynamic therapy of cancer, In *Photosensitization: Molecular and Medical Aspects* (Edited by G. Moreno, R. H. Pottier and T. G. Truscott). Springer-Verlag, Berlin. In press.

Wagner, J. R., H. Ali, R. Langlois, N. Brasseur and J. E. van Lier (1987) Biological activities of phthalocyanines—VI. Photooxidation of L-tryptophan by selectively sulfonated gallium phthalocyanines: singlet oxygen yields and effect of aggregation. *Photochem. Photobiol.* **45**, 587–594.

Weber, J. H. and D. H. Busch (1965) Complexes derived from strong field ligands. XIX. Magnetic properties of transition metal derivatives of 4,4′,4″,4‴-tetrasulfophthalocyanines. *Inorg. Chem.* **4**, 469–471.