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THE IDENTIFICATION OF A CARBON LINKED OLIGOMER IN HEMATOPORPHYRIN DERIVATIVE AND PHOTOFRIN II

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Summary. The presence of carbon linked oligomeric porphyrins in hematoporphyrin derivative and Photofrin II has been established by FAB mass spectrometry and hydrolysis studies. A dimer derived from this material has been isolated and characterized by nmr spectroscopy and mass spectrometry.

The structure of the oligomeric and active component of the anticancer material hematoporphyrin derivative (HPD) and its partially purified form known as Photofrin II has been the subject of considerable debate. Evidence for both ester and ether linkages between the porphyrin units has been provided by acid and base hydrolysis[1,2,3] and reduction[4] studies. It has been assumed that no porphyrin oligomers remained in either material after acid hydrolysis. We now report the presence of non-acid (or base) hydrolysable oligomers in HPD and Photofrin II and the isolation and characterization of a derived dimer from this material.

This oligomeric component was observed as follows. The acid hydrolysis product from either HPD or Photofrin II was treated with methylating conditions that etherify the benzylic hydroxyl groups as well as esterify the carboxylic acid groups of monomeric porphyrins.[5] The FAB mass spectrum[6] of the product showed, besides the expected porphyrin monomer molecular ions, peaks at 1199, 1213, 1245 and 1277 mass units and peaks in the trimer and tetramer porphyrin regions,[7] (no attempt was made to measure peaks of higher molecular weight). Fully methylated dihematoporphyrin ether or ester dimers would show molecular ions at 1263 and any monovinyl analogues would have their molecular ions at 1231. Although peaks with these values are present in the FAB mass spectrum of fully methylated HPD or Photofrin II before hydrolysis, they are not present in the spectrum of the fully methylated acid hydrolysis product. These data suggest that the acidic hydrolysis product of HPD or Photofrin II contains dimer material with a molecular weight of 1277 and that the porphyrins in this dimer are not linked by either an ether or an ester functional group. Any ester or ether linkage between two hematoporphyrin units would form a dimer with either four carboxylic acid groups and two alcohol

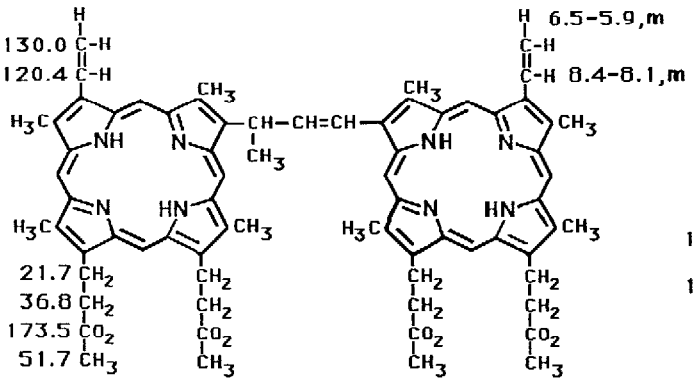
groups (for an ether linked dimer) or three carboxylic acid groups and three alcohol groups (for an ester linked dimer), i.e. both would have a total of six methylatable groups and a molecular weight of 1263. The ion at 1277 corresponds to material with seven methylatable groups, most likely four methyl ester groups and three methoxyl groups. The peaks at 1245 and 1213 could then represent the 1277 material with one or two vinyl groups instead of methoxyethyl groups. The peak at 1199 corresponds to that expected for a dideuteroporphyrin system containing four methyl esters, one hydroxyl group and two vinyl groups, which in turn suggests that one hydroxyl group is more difficult to methylate than the others in these dimer structures.

Attempts to separate the components of the acidic hydrolysis product of Photofrin II by column chromatography were unsuccessful, using either the fully methylated material or material in which only the carboxylic acid groups had been esterified.[5] Treatment of the acidic hydrolysis product with an excess of benzoyl chloride in dimethylformamide,[9] (conditions which convert porphyrins with 1-hydroxyethyl[9] or 1-methoxyethyl[8] side chains to vinyl sidechains but does not affect the methyl esters), gave a product[10] whose FAB mass spectrum showed only one major ion in each of the porphyrin monomer, dimer and trimer regions. These peaks correspond to protoporphyrin dimethyl ester (590), and adducts that formally correspond to two (1181) and three (1772) protoporphyrin dimethyl ester units. This derivatized material can be separated by chromatography on silica into four fractions which differ in their molecular weights.

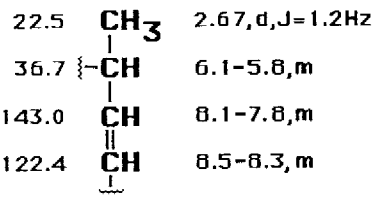
The dimer fraction showed only one strong peak in the dimer region at 1181 and the trimer had a strong molecular ion at 1772 with only a weak ion in the dimer region at 1181. Both fractions also showed some fragmentation to monomers. The oligomeric fraction showed a more complex set of peaks in each region with peaks being observed as high as the pentamer region.

The dimer and trimer samples gave good ^{13}C nmr spectra but only the dimer gave a good ^{1}H nmr spectrum, with very broad signals being obtained for the trimer.[11] Both the ^{13}C and ^{1}H nmr spectra showed the expected signals due to the porphyrin nuclei, the ring methyl, vinyl and propionic ester side chains. The dimer would be expected to have eight ring methyl, four propionic methyl esters and two vinyl side chains, leaving C_4H_6 to be assigned, given the molecular weight of 1181. Analysis of the ^{13}C and ^{1}H nmr data with the aid of a carbon off resonance spectrum, a $^{1}H/^{13}C$ chemical shift correlation and proton homonuclear decoupling showed the structure[12] of the dimer was (1) (relevant ^{13}C and ^{1}H nmr chemical shift data shown). In particular both the methine carbons of the linking double bond change to doublets in the off resonance spectra, (the multiplicities of the other methine and the methyl carbons of the linkage could not be determined because of the overlapping signals from the methylene carbons of the propionate groups). A $^{1}H/^{13}C$

chemical shift correlation established the correlation between the bridge carbon signals to the proton signals in the complex ^{1}H spectra as shown in (2). Decoupling at 5.97ppm in the proton spectrum collapsed the doublet at 2.67ppm (to a singlet) and the multiplet at 8.1-7.8ppm, thus showing the connectivity of the bridging unit. The trimer fraction showed a nearly identical ^{13}C spectrum to that of the dimer, confirming the same linkage.

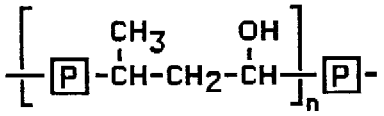


(1)



(2)

The dimer (and oligomer) component in HPD or Photofrin II contains a hydroxyl group (by FAB mass spectrometry), which is removed with the dehydrating conditions to form the double bond in the linking group. Although in principle this hydroxyl could be attached to either of the carbons of the double bond, we believe it is more likely to be on the benzylic carbon (adjacent to the porphyrin nuclei). The structure of this component is represented by (3). This question and the mechanism of formation of this material in the preparation of HPD are the subject of further investigation.



(3)

Where P represents the porphyrin system

The oligomeric material (3) is presumably inactive *in vivo* as an anticancer agent since it is present in substantial amounts in aged, inactive Photofrin II or HPD. However the dehydrated dimer (1) and the corresponding trimer show significant anticancer *in vivo* activity.[13]

Acknowledgments

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References and Notes

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2. Kessel, D., Thompson, P., Musselman, B. and Chang, C. K., *Photochem. Photobiol.*, **46**, 563, 1987.
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4. Kessel, D., Thompson, P., Musselman, B. and Chang, C. K., *Cancer Res.*, **47**, 4642, 1987.
5. Byrne, C. J. and Ward, A. D., *Tetrahedron Lett.*, **29**, 1421, 1988.
6. Fast atom bombardment (FAB) spectrometry was carried out on a VG ZAB-2HF mass spectrometer. Argon atoms accelerated to 8kV were used as the bombarding particles and 3-nitrobenzyl alcohol was used as the matrix.
7. We have synthesized[8] a wide range of porphyrin dimers related to hematoporphyrin, with ether, ester and other linkages; in our experience all show strong molecular ions and relatively weak fragmentation to other peaks in the dimer region. Hence we consider that the peaks at 1199, 1213, 1245 and 1277 are likely to be those of the molecular ions of various dimers present in the mixture.

8(a). Morris, I. K. and Ward, A. D., *Tetrahedron Lett.*, **29**, 2501, 1988.
8(b). Byrne, C. J., Marshallsay, L. V., Morris, I. K., Sek, S. Y. and Ward, A. D., unpublished data.

9. Clezy, P. S., Fookes, C. J. R. and Hai, T. T., *Aust. J. Chem.*, **31**, 365, 1978.
10. A solution of Photofrin II (batch 86056, 150ml, 375mg of porphyrin material), 2M HCl (150ml) and tetrahydrofuran (300ml) was refluxed for 3hr. The cooled solution was diluted with water, the pH was adjusted to 4-5 and the solution was extracted with dichloromethane-tetrahydrofuran (2:1). The extract was washed with water and the solvent was removed under reduced pressure. The residue was esterified[5] using trimethylorthoformate (10ml), methanol (10ml), water (2ml) and concentrated sulphuric acid (2ml) for 45 minutes. This material was then dehydrated[9] using benzoyl chloride (2.5ml) in dimethylformamide (100ml) for 30 minutes and the product was separated into four components by radial chromotography on silica. Protoporphyrin dimethyl ester was eluted first (124mg), followed by the dimer (56mg) then the trimer (36mg) components. The last fraction (145mg) contains unidentified oligomeric material.
11. NMR spectra were recorded on a Bruker CXP300 spectrometer operating at 300MHz for ^{1}H and 75.47MHz for ^{13}C spectra; all spectra were recorded in deuterated chloroform with tetramethylsilane as the internal standard.
12. The structure shown in (1) is only one of the several regio- and stereo-(cis,trans)isomers possible for the dimer.
13. The testing procedure has been described previously, (Cowled, P. A. and Forbes, I. J., *Cancer Lett.*, **28**, 111, 1985).

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