Structural Characterization of a Degradation Product of Tin Ethyl Etiopurpurin (SnET2): Comparison of the Long-range Heteronuclear Correlations Detected Using Conventional GHMBC and IMPEACH-MBC In Conjunction with Submicro NMR Probe Technology

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During the terminal heat sterilization of the lipid emulsion final dose formulation of the photodynamic therapeutic (PDT) agent tin ethyl etiopurpurin (SnET2), a new degradant was observed at very low levels. The degradant, which was prone to photo-instability, was isolated by preparative chromatography and subsequently characterized by mass spectrometry and NMR methods. Reproducible parent ion clusters were only observable *via* negative ion APCI methods. Because of the limited isolate sample, NMR characterization was done using 1.7 mm SMIDG (SubMicro Inverse-Detection Gradient) NMR probe technology in conjunction with the accordion-optimized IMPEACH-MBC long-range heteronuclear shift correlation experiment. The "static" 8 Hz optimization of the GHMBC experiment failed to allow the observation of a number of long-range correlations that were of critical importance to the determination of the structure of the impurity. In contrast, all of the correlations required to assemble the structure were obtained from an IMPEACH-MBC experiment optimized for long-range heteronuclear couplings in the range from 2-10 Hz.

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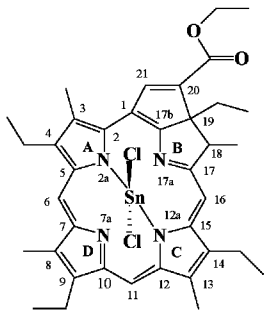
The drug tin ethyl etiopurpurin (SnET2), **1**, is in the final stages of development as a photodynamic ophthalmic therapeutic (PDT) agent. PDT agents are typically compounds activated by the application of laser energy at a specific wavelength afford the actual therapeutic agent. Compounds of this type were first developed as cancer chemotherapeutic agents but there has also been interest in expanding the range of applications to include other tissues to which laser radiation can be directed [1,2]. The compound is formulated in a lipid emulsion suitable for IV administration. It was observed by HPLC analyses that low levels of a previously unidentified degradant were formed during the terminal heat sterilization cycle of the formulation. All impurities/components at levels above 0.1% must be identified to satisfy regulatory requirements. The impurity was subsequently isolated from the lipid emulsion by preparative chromatography for structural characterization. A reproducible determination of the molecular ion cluster was possible only through the use of negative ion APCI methods; mass spectrometry was performed on a PE-Sciex Q-TOF mass spectrometer. NMR data were acquired using 1.7 mm submicro (SMIDG) NMR methods [3-6] at 600 MHz due to the limited quantity of the unstable degradant isolated. Final determination of the structure of the degradant was dependent on long-range heteronuclear shift correlation methods. The conventional GHMBC [7,8] long-range correlation experiment, which was optimized for a single potential long-range coupling value (*i.e.*, static optimization), failed to afford all of the necessary correlations to allow the determination of the structure. In contrast, one of the new accordion-optimized long-range heteronuclear shift correlation methods, IMPEACH-MBC [9], was able to provide sufficient long-range correlation information in the proton-deficient region of the molecule (where structural alteration relative to the parent drug) had occurred, to allow the elucidation of the structure of the degradant.



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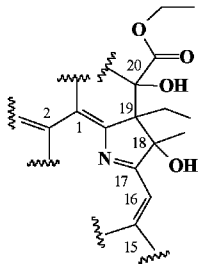
Tin ethyl etiopurpurin, **1**, is a novel therapeutic agent derived from an etioporphyrin I. During the terminal heat sterilization process of the compounds clinical emulsion formulation, a new degradant was observed by HPLC. The degradant was late-eluting relative to the parent drug, with a relative retention time (RRT) of ~2.5 (parent drug RRT ≡ 1.0). The degradant was isolated by normal-phase preparative chromatography using a YMC cyano column with hexane-acetone/*N*,*N*-dimethylformamide (DMF) mobile phase. Pooled fractions containing the green-colored degradant were collected from the column and concentrated under a stream of nitrogen until a lower, green, DMF layer formed. The lower green layer was transferred to a vacuum centrifuge and concentrated to dryness. Care was taken to protect the fractions and isolates from light to prevent further degradation. Degradant isolates were contaminated with small amounts of the bulk drug, which had a tendency to tail on the preparative chromatographic column. Nevertheless, approximately 250 µg of the degradant was isolated, with an HPLC purity of 85%.

Mass spectrometry was attempted in order to determine the molecular weight of the isolated degradant. Reliable parent ion cluster data were only obtained using negative ion APCI performed on a Sciex Q-Star (Q-TOF) mass spectrometer. UV absorbance chromatograms were recorded simultaneously at a wavelength of 412 nm from a serial detector to verify the elution time of the impurity peak. MS interface flow was established at approximately 300 µL/min *via* eluent splitting. With the normal phase chromatography method employed, a 350 °C nebulizer temperature gave stable operation with little thermal degradation of the analyte. A nominal molecular weight for the degradant of 814 daltons was observed, which corresponds to an empirical formula of $C_{37}H_{44}Cl_2N_4O_5Sn$. The calculated and observed isotope profiles for the degradant based on the proposed empirical formula are shown in Figure 1. MS/MS studies did not prove useful in the characterization of the degradant, as only trivial neutral weight losses were observed.

A 100 µg portion of the remaining isolated degradant was dissolved in 30 µL of 99.96% d_7-DMF (Cambridge Isotope Laboratories) and transferred to a 1.7 mm SMIDG NMR tube under argon in a glovebox to provide the sample used for the NMR experiments performed. Only a portion of the isolated degradant was employed for the initial NMR studies due to the photo instability of the isolate; the balance was held in reserve to prepare a second NMR sample if necessary. The usual suite of routine 1D and 2D NMR experiments (homonuclear TOCSY and ROESY, direct heteronuclear correlation GHSQC, and long-range heteronuclear correlation GHMBC) was performed on the isolate. These experiments confirmed that most of the tin ethyl etiopurpurin framework was intact in the structure of the degradant. Specifically, however, the annelated cyclopentadiene ring portion of the molecule (the "northeast quadrant") had been structurally modified in the degradant, as shown by structural fragment **2**.



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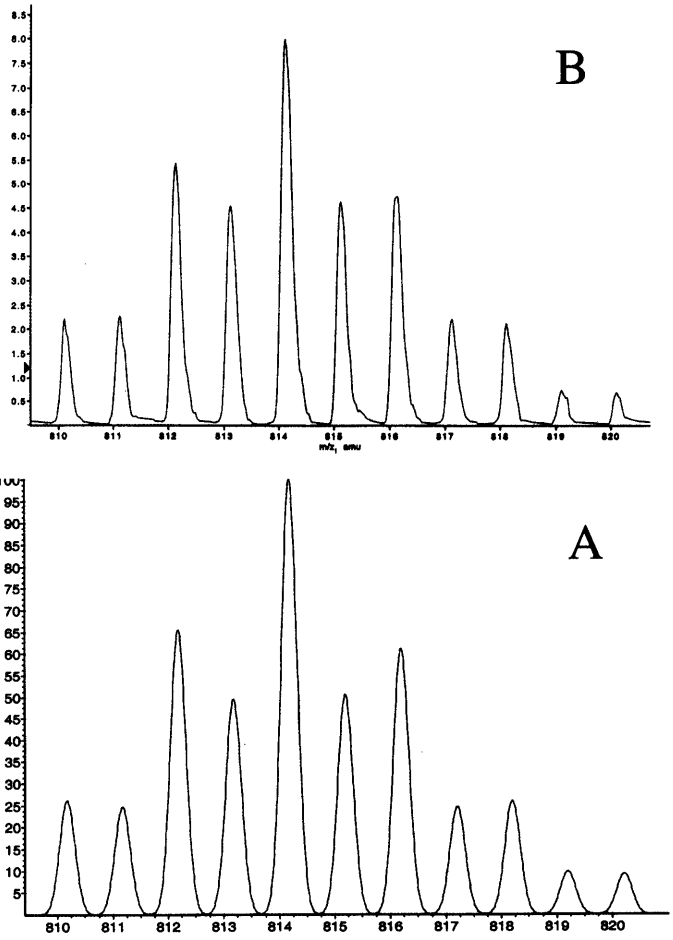


Figure 1. **A**) Calculated and **B**) observed negative ion APCI molecular ion clusters for isolated degradant structure **4**.

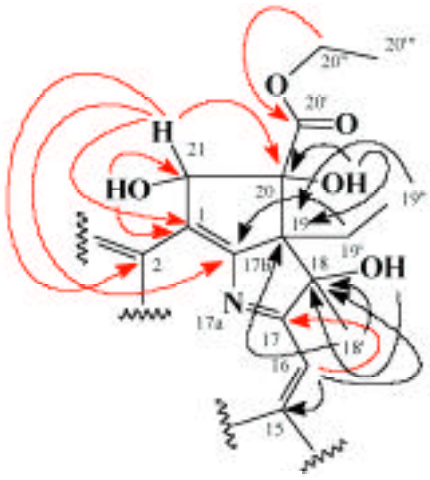
In the proton NMR spectrum of the degradant, the cyclopentadiene vinyl proton (H-21 using the numbering of the bulk drug) was missing. A new NMR resonance occurring at ~6.8 ppm was observed that had the appear-

ance of a “quartet”. A resonance with this type of multiplet structure could not be readily explained. The 18-methyl doublet of the parent molecule was also absent from the spectrum. New resonances attributed to hydroxyl groups were observed at 8.20 and 8.55 ppm. The 19-ethyl group resonances were present in the spectrum.

A larger NMR sample was subsequently prepared (~250 μg) and rather than showing an apparent “quartet” resonating at ~6.8 ppm, instead, showed a separation of this resonance into an AB spin system, with components resonating at 6.82 and 6.92 ppm, probably due to the change in concentration of the analyte. The upfield component of this spin system observed at 6.82 ppm gave a direct carbon correlation response in the GHSQC spectrum at ~88 ppm, which was suggestive of a carbon bearing two oxygen atoms. Alternatively, it could be argued that the carbon resonating at ~88 ppm could be an oxygen-bearing carbon shifted unusually far downfield as a result of the porphyrin ring system. The downfield proton of the pair resonating at 6.92 ppm failed to give a correlation in the GHSQC spectrum, suggesting that this proton doublet was bound to a heteroatom.

Likewise, the18-methine quartet resonating at 4.61 ppm in the proton spectrum of the parent drug was missing in the degradant proton spectrum. In conjunction, the18-methyl doublet had collapsed to a singlet and shifted downfield to 2.96 ppm, consistent with the insertion of an oxygen at the 18-position. The resonances at 8.20 and 8.55 ppm, tentatively ascribed to hydroxyl groups, failed to give responses to carbons in the GHSQC spectrum as would be expected if these protons were attached to heteroatoms.

In the initial effort to elucidate the structure of the degradant, an 8 Hz optimized GHMBC [5,6] long-range heteronuclear shift correlation experiment was performed on the ~100 μg sample (a portion of the 8 Hz optimized GHMBC spectrum is shown in Figure 2). This experiment, unfortunately, failed to provide any long-range correlations from the overlapped resonances at ~6.8 ppm. The GHMBC



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experiment also failed to afford any correlations from the 6.82 and 6.92 ppm resonances when the experiment was repeated on a larger, ~250 μg sample. Black arrows denote long-range heteronuclear correlations observed in the 8 Hz optimized GHMBC experiment from the protons located in the structurally modified region of the SnET2 structure in **3**. Importantly, however, the correlations observed in the 8 Hz GHMBC experiment did allow the location of the two hydroxyl resonances at the 18- and 20-positions of the SnET2 molecular framework (see **3**).

Following the failure of the GHMBC experiment to provide the long-range heteronuclear correlations to assemble the structure of the degradant, we resorted the acquisition of a 2-10 Hz accordion-optimized IMPEACH-MBC experiment [9]. The IMPEACH-MBC pulse sequence utilizes the accordion principle [10] to survey a range of potential long-range heteronuclear couplings in a fashion analogous to the method pioneered by Wagner and Berger [11] with the ACCORD-HMBC experiment. While long-range responses in the ACCORD-HMBC are subject to homonuclear coupling modulation, which has the potential to lead to response overlap [12], these modulations are suppressed in the IMPEACH-MBC experiment [9] through the use of a constant time variable delay pulse sequence element *in lieu* of a simple variable delay as in the ACCORD-HMBC experiment. In terms of absolute sensitivity, the IMPEACH-MBC experiment has been shown to be somewhat less sensitive than the GHMBC experiment in the case of intense long-range responses where the actual coupling constant is close to the optimized value of the long-range delay. In contrast, we have also demonstrated that responses that may be weak or absent in the GHMBC experiment can have appreciable intensity in the IMPEACH-MBC spectrum when the actual coupling constant is within the accordion optimization range of the experiment [13,14]. Usefully, the accordion-optimized long-range experiments require no prior knowledge of any of the long-range heteronuclear couplings for the molecule being studied.

Applying the IMPEACH-MBC experiment to the structural characterization of the SnET2 degradant (see panel in Figure 2 for the region of the spectrum identical to that shown for the GHMBC data), the long-range correlations shown by red arrows on **3** were observed in the 2-10 Hz optimized IMPEACH-MBC spectrum. In addition, most of the correlations denoted by the black arrows from the GHMBC spectrum (see 3) were also observed in the 2-10 Hz optimized IMPEACH-MBC spectrum. The long-range correlations from the doublets resonating at ~6.82 and ~6.92 ppm, in conjunction with chemical shift considerations, allowed the assembly of the 20,21-dihydroxypentene ring fused to the B-pyrrole of the tin ethyl etiopurpurin nucleus of the bulk drug. Correlations observed in the GHMBC and IMPEACH-MBC spectra of the isolated degradant are shown by **4** (black arrows denote correlations from the 8 Hz optimized GHMBC experiment that were also observed in

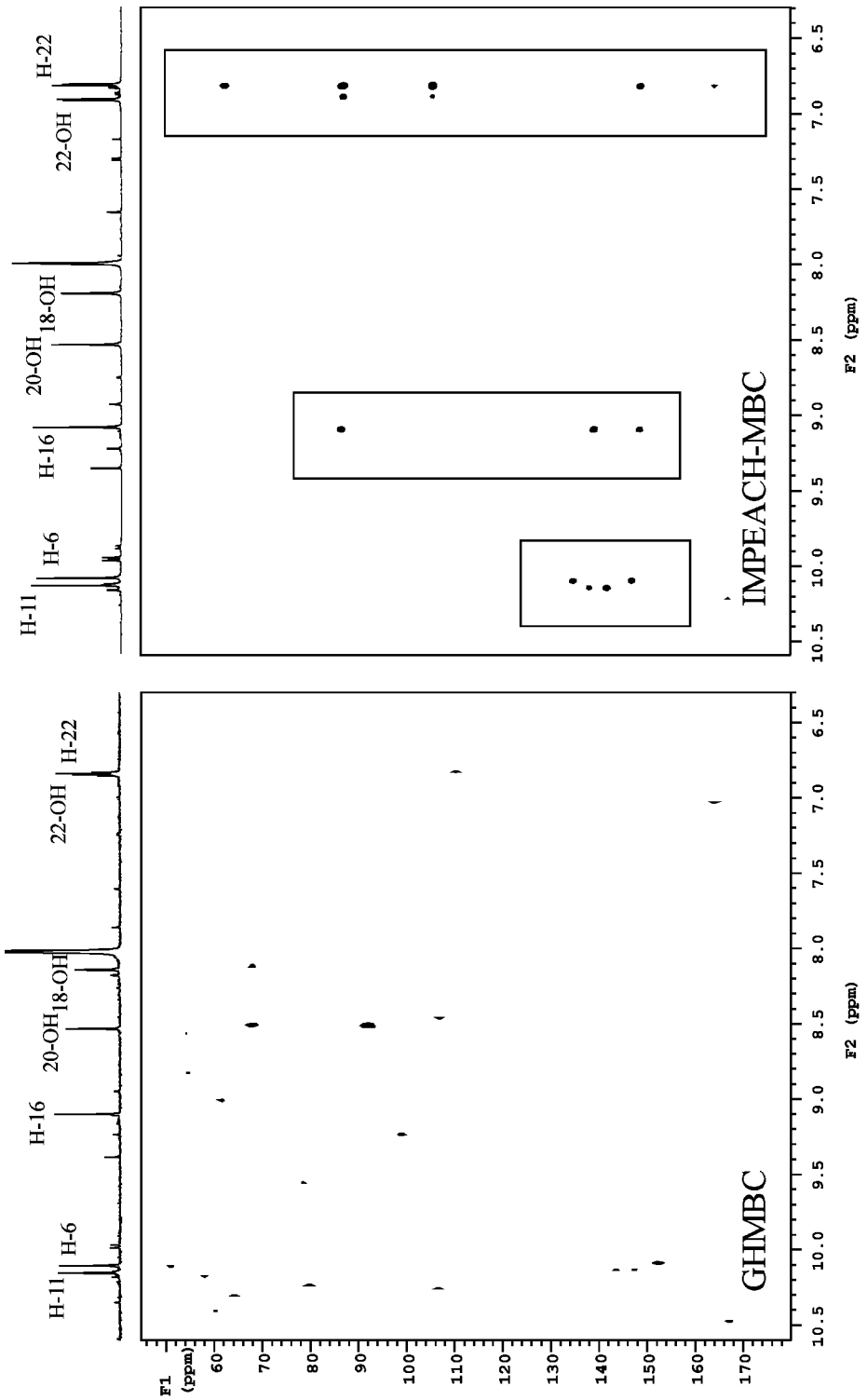
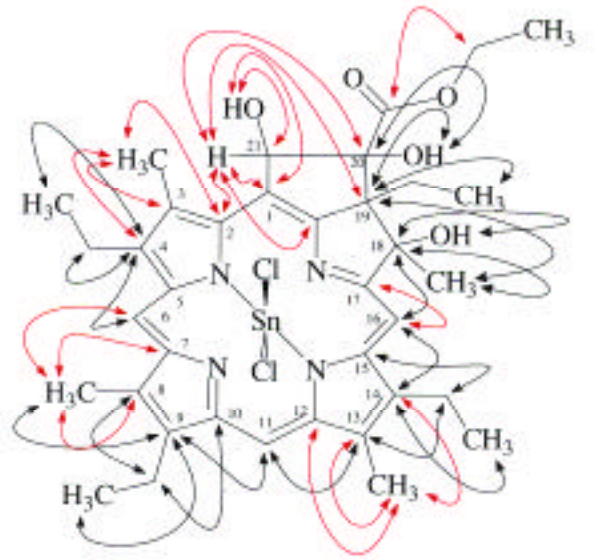


Figure 2. Spectral segments from the 8 Hz optimized GHMBC spectrum (left panel) and the 2-10 Hz accordion-optimized IMPEACH-MBC spectrum (right panel) of an isolated degradant of the drug tin etiopurpurin (**4**). The GHMBC data were acquired using a Varian *INOVA* 600 NMR spectrometer operating at an observation frequency for ^{1}H of 599.75 MHz and equipped with a Nalorac SMIDG™-600-1.7 submicro NMR probe. The samples were prepared in 30 µL d_7-*N*,*N*-dimethylformamide under a dry argon atmosphere and were protected from light during handling and data acquisition. Both data sets were acquired as 4096 x 384 point files. The data were linear predicted to 1536 points in the second frequency domain and were then zero-filled to yield a final data matrix consisting of 2048 x 2048 points. A combination of gaussian and sinebell weighting functions were used prior to the first Fourier transform and cosine multiplication was used prior to the second transform. Acquisition times were comparable for the two experiments. All possible parameters were held constant for the two experiments so as to afford an even comparison.

the 2-10 Hz optimized IMPEACH-MBC experiment; red arrows denote correlations observed *only* in the 2-10 Hz optimized IMPEACH-MBC experiment). Chemical shift assignments for the degradant structure are shown by **5**.



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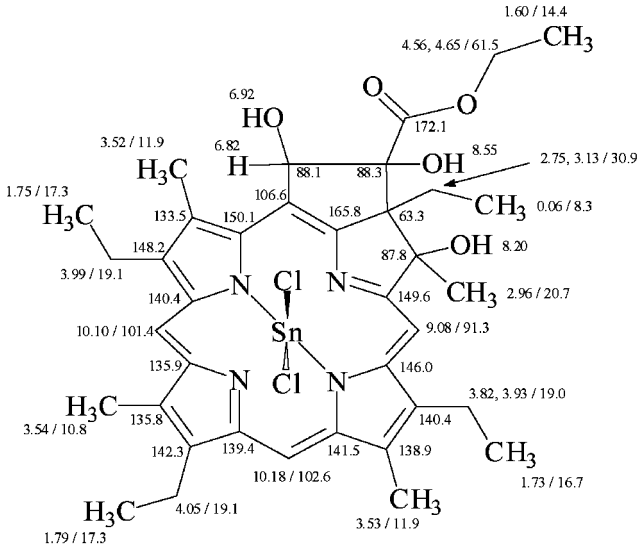
In conclusion, the new family of accordion-optimized long-range heteronuclear shift correlation experiments [9,11,12,15,16], offers considerably enhanced capabilities for the observation of small, and potentially important long-range heteronuclear couplings. These methods were recently reviewed [17]. In addition, as has been shown previously, accordion-optimization also allows the observation of considerable numbers of four-bond ($^4J_{CH}$) long-range couplings [12]. The utilization of accordion-optimized long-range heteronuclear shift correlation techniques, particularly when used in conjunction with high sensitivity submicro (SMIDG) NMR probe technology [3-6] or cryogenic NMR probes [18,19], can provide critical structural information necessary for the characterization of "proton-deficient" impurities and degradants of drugs [20], natural products [21], metabolites, forensic samples, and other scarce samples requiring structural characterization.



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