Identification and Characterization of Chlorin e_4 Ethyl Ester in Sera of Individuals Participating in the Chlorophyllin Chemoprevention Trial

Patricia A. Egner,*,† Kevin H. Stansbury,† Edward P. Snyder,† Mark E. Rogers,‡ Paul A. Hintz,‡ and Thomas W. Kensler†

Department of Environmental Health Sciences, Johns Hopkins School of Hygiene and Public Health, Baltimore, Maryland 21205, and M Scan, Inc., West Chester, Pennsylvania 19380

Received March 24, 2000

Chlorophyllin (CHL), a mixture of water soluble derivatives of chlorophyll, has been shown to be an effective inhibitor of aflatoxin B_1 (AFB_1) carcinogenesis and AFB_1−DNA adduct formation in rainbow trout and rats [Breinholt, V., Hendricks, J., Pereira, C., Arbogast, D., and Bailey, G. (1995) *Cancer Res.* **55**, 57−62; Kensler, T. W., Groopman, J. D., and Roebuck, B. D. (1998) *Mutat. Res.* **402**, 165−172]. The chemopreventive action of CHL has been previously attributed to molecular complexing. In 1997, a randomized, double-blind clinical trial of CHL was conducted in Qidong, Jiangsu Province, People's Republic of China. At the completion of the study, when serum samples were regrouped by subject identification number, it was noted that many of the participant samples were green in color. Using HPLC, ESI/MS, and MS/MS techniques, serum samples from individuals receiving CHL were found to contain previously unreported copper chlorin e_4 ethyl ester ($CuCle_4$ ethyl ester) as well as copper chlorin e_4 ($CuCle_4$). Both chlorins originated in the study tablet, were absorbed into the bloodstream, and conferred a green color to the sera. This initial finding of in vivo absorption and bioavailability of two chlorin derivatives suggests that the mechanism of CHL chemoprevention may lie in the actions of these two components in vivo in addition to preventing carcinogen absorption from the gut.

Introduction

Certain naturally occurring food constituents in the human diet exhibit protective effects against a wide range of carcinogens (*1*, *2*). Although most of these agents are relatively insoluble and occur at low concentrations in a well-balanced diet, exceptions may be found in the abundant, photosynthetic porphyrin chlorophyll or chlorophyllin (CHL),[1] a synthetic mixture of water soluble derivatives of chlorophyll. CHL currently has a wide range of uses, dietary and medicinal, including being a food coloring agent, a health food additive (*3*), an accelerant of wound healing and controlling urinary and fecal odors in geriatric patients (*4*). In a wide array of in vitro and in vivo assays, CHL has also been shown to exhibit antimutagenic (*5*), antigenotoxic (*6*), and anticarcinogenic (*7*) activities against numerous carcinogens.

Mechanistic studies suggest that CHL acts as an "interceptor molecule" forming noncovalent molecular complexes especially with partially planar, aromatic carcinogens, including the potent hepatocarcinogen aflatoxin B_1 (AFB_1). The molecular complexing of CHL and AFB_1 is believed to block absorption of AFB_1, thereby reducing the bioavailability of the carcinogen in target tissues (*8*, *9*). The chemoprotective properties of CHL may also be due to antioxidative activities (*10*) or the nonspecific inhibition of cytochrome P450 enzymes involved in the bioactivation of carcinogens (*11*, *12*). The magnitude of the actions of CHL in vivo can be illustrated by the powerful inhibition of AFB_1 tumorigenesis in rainbow trout (*13*) and the dramatic modulation of AFB_1 disposition in rats (*14*). In addition, although pharmacokinetic studies utilizing CHL have not been published, it has no reported toxicities and is believed to be poorly absorbed (*15*). Thus, the chemoprotective properties of CHL in several animal models coupled with the lack of reported toxicities provided a solid justification for the evaluation of the efficacy of CHL in individuals exposed to AFB_1.

Beginning in August 1997, a 4 month, randomized, double-blind clinical trial of CHL was conducted in Qidong, Jiangsu Province, People's Republic of China. AFB_1 exposure from food contamination and a high incidence of liver cancer have been well documented in this region (*16*). During the entire 4 month regimen, 90 participants in each group received three pills daily containing placebo or 100 mg of CHL. Serum samples were taken at biweekly intervals throughout the trial to measure the effect of daily CHL administration on the levels of AFB_1−albumin adducts. It was noted at the completion of the trial when samples were regrouped serially by subject identification number that many participant serum samples were increasingly green in color. Studies were subsequently undertaken to ascertain the source of the tint.

* To whom all correspondence should be addressed: Department of Environmental Health Sciences, Johns Hopkins School of Hygiene and Public Health, Baltimore, MD 21205. Phone: (410) 955-2435. Fax: (410) 955-0116. E-mail: pegner@jhsph.edu.

† Johns Hopkins School of Hygiene and Public Health.

‡ M Scan, Inc.

[1] Abbreviations: CHL, chlorophyllin; AFB_1, aflatoxin B_1; $CuCle_4$, copper chlorin e_4; $CuCle_6$, copper chlorin e_6; Cle_4, chlorin e_4; Cle_6, chlorin e_6; $CuCle_4$ dimethyl ester, dimethyl ester of copper chlorin e_4; $CuCle_4$ ethyl ester, ethyl ester of copper chlorin e_4; ESI/MS, electrospray ionization/mass spectrometry; TFA, trifluoroacetic acid.

10.1021/tx000069k CCC: $19.00
Published on Web 08/17/2000

Experimental Procedures

Overall Design and Structure of the CHL Intervention. The Chlorophyllin Clinical Trial was a randomized, double-blind, placebo-controlled intervention designed to determine whether administration of CHL could modulate levels of urinary and serum biomarkers of aflatoxin and to ascertain the feasibility of long-term administration of CHL three times daily. Study participants were recruited from Daxin Township, Qidong, Jiangsu Province, People's Republic of China. Daxin is a rural farming community of approximately 40 000 residents located at the mouth of the Yangtze River, 15 km southeast of Qidong. A signed, informed consent was obtained from all participants according to institutional and federal guidelines established by both the People's Republic of China and the United States. Initially, 513 individuals (25−65 years of age) received complete physical exams and provided blood and urine samples at the Daxin Medical Clinic. A medical history, physical examination, liver ultrasound, and routine hematological, hepatic, and renal function tests were used to screen the volunteers at the first baseline visit. Individuals were excluded as possible study participants on the basis of criteria previously outlined by Jacobson et al. (*17*). In addition, levels of serum aflatoxin−albumin adducts were measured to assess aflatoxin exposure (*18*). All screening participants in good general health with a positive aflatoxin−albumin adduct level were invited to participate in the CHL trial. A total of 180 agreed to participate; the main reason for declining was the inability to commit to remaining in the area for the duration of the study.

On August 3, 1997, the study participants were randomly assigned into two equal arms and given their first dose of study drug at the clinic (administration three times daily of 100 mg of CHL or placebo). Thereafter and for the duration of the trial, local doctors were assigned the daily task of distributing study tablets and collecting any unused tablets from the participants residing within their village. Doctors received a weekly supply of study tablets for each individual which were packaged in sealed, desiccated, brown bottles. Three tablets were subsequently placed in a plastic pill holders for daily distribution. Both the weekly bottles and the plastic holders bore the participants' unique identification number to ensure proper distribution. Written and verbal directions were also given requesting that individuals take each tablet immediately prior to their three daily meals. Study drug was replenished each Sunday, and any drug not taken the previous week was recorded. As adjudged by the pill counts, overall participant compliance exceeded 97%. Only one participant withdrew from the study before its scheduled completion. Blood and urine samples collected at 2 week intervals throughout the trial provided the basis for monitoring toxicities and measuring the modulation of the aflatoxin biomarkers.

Chemicals. Bulk CHL (Bush, Boake and Allen, Sudbury, Suffolk, England) and the formulated tablets containing 100 mg of CHL or placebo were graciously supplied by Rystan, Inc. (Little Falls, NJ). Authentic disodium copper chlorin e_4 ($CuCle_4$), trisodium copper chlorin e_6 ($CuCle_6$), chlorin e_4 (Cle_4), and chlorin e_6 (Cle_6) were purchased from Porphyrin Products, Inc. (Logan, UT). Diazomethane was prepared using Diazald (Aldrich Chemical Co., Milwaukee, WI). All other chemicals and solvents used in analyses were analytical/reagent grade or higher.

Sample Extraction. To identify the chlorin components in the serum samples obtained from study participants, 100 μL of serum was acidified with 100 μL of 0.1 M phosphate buffer (pH 5.0) and extracted with 600 μL of an ice-cold methanol/chloroform mixture (1:1, v/v). Following centrifugation, the organic layer was removed and dried under nitrogen. Samples were stored at −20 °C until they were chromatographed.

Instrumentation. Bulk CHL and the authentic chlorin standards were fractionated and purified according to the method of Sato et al. (*10*) using silica gel G-60 thin-layer chromatography plates (Whatman, Inc., Clifton, NJ). CHL components and serum extracts were also separated by high-pressure liquid chromatography (HPLC) using a 250 mm × 4.6 mm, 5 μm C_{18} Prodigy column (Phenomenex, Torrance, CA) and quantitated with a Waters 996 photodiode array detector at 626 nm. The best separation of the CHL components was achieved using a 1 mL/min flow rate of the following mobile phase and gradient: solvent A, methanol/water (80:20, v/v) containing 1% (v/v) acetic acid (*19*); solvent B, methanol; initial conditions, 40% B; from 0 to 20 min, linear change to 80% B; from 20 to 30 min, linear change to 90% B; from 30 to 40 min, linear change to 100% B; from 40 to 50 min, 100% B. Using these conditions, the limit of detection for $CuCle_4$ was approximately 10 ng. Fractions following HPLC separation of the parent drug preparation and serum extracts were collected and analyzed further by mass spectrometry.

LC/MS separations were conducted on a Hewlett-Packard UV 1100 system by monitoring 407 nm and utilizing a Vydac C_{18} column (The Nest Group, Inc., Southboro, MA) with a linear gradient of 0.1% aqueous trifluoroacetic acid (TFA) to a 100% acetonitrile/water mixture (9:1, v/v) containing 0.1% TFA. Both ESI/MS and ESI/MS/MS were performed using a Quattro II upgraded BioQ triple-quadrupole instrument. Daughter ion spectra were generated from the appropriate singly charged parent ion using argon as the collision gas. Sample aliquots of 100 μL were directly infused into the instrument source at a flow rate of 10 μL/min using a mixture of 50% aqueous acetonitrile containing 0.1% TFA. Structures and masses of all CHL components as well as the unknown chlorin derivative found in the sera extracts were confirmed by ESI/MS and ESI/MS/MS.

NMR spectra were obtained for the Cle_4 standard and the unknown chlorin e_4 derivative using a Varian Unity plus 400 MHz NMR spectrometer (Varian, Inc., Palo Alto, CA) with a Sun Microsystems Ultra 10 workstation running Solaris 2.6 and V NMR 5.3B software. Prior to the spectra being obtained, chlorin compounds had the copper component removed by extraction with ammonium hydroxide. All samples were dissolved in 1 mL of $CDCl_3$ (100 at. % D). Chemical shifts are reported in parts per million referenced to the residual solvent peaks.

Synthesis of Methyl Esters. The dimethyl ester of copper chlorin e_4 ($CuCle_4$ dimethyl ester) was prepared by isolating $CuCle_4$ from the original bulk CHL by TLC and methylating with diazomethane. Briefly, approximately 2 mg of $CuCle_4$ obtained following TLC separation was eluted from the silica gel with methanol and concentrated under nitrogen. The peak identity was confirmed by HPLC comparison to an authentic standard. To acquire a greater amount of the $CuCle_4$ ethyl ester, a 1.0 mL pool was created from sera samples obtained from a participant receiving CHL throughout the trial and extracted as previously described. The $CuCle_4$ ethyl ester peak was collected following multiple HPLC separations and concentrated. Purified $CuCle_4$ and the isolated $CuCle_4$ ethyl ester were subsequently methylated with diazomethane in ether freshly prepared from Diazald. Upon removal of the residual ether with nitrogen, the resulting $CuCle_4$ dimethyl ester and methylated $CuCle_4$ ethyl ester were analyzed under the identical HPLC and LC/MS conditions outlined above. ESI/MS/MS was used for final structural determinations.

Results

Characterization of Chlorophyllin Components. TLC and HPLC analyses of commercial CHL preparations have indicated that CHL is a mixture of copper chelates of chlorophyll derivatives, including $CuCle_4$, $CuCle_6$, copper pheophorbide *a*, copper rhodin g_7, and their degradation products (*10*, *19*, *20*). The HPLC separation in Figure 1A demonstrates that the particular CHL used in the clinical trial contained approximately 31% $CuCle_6$ (peak I), 33.2% $CuCle_4$ (peak II), and 4.0%

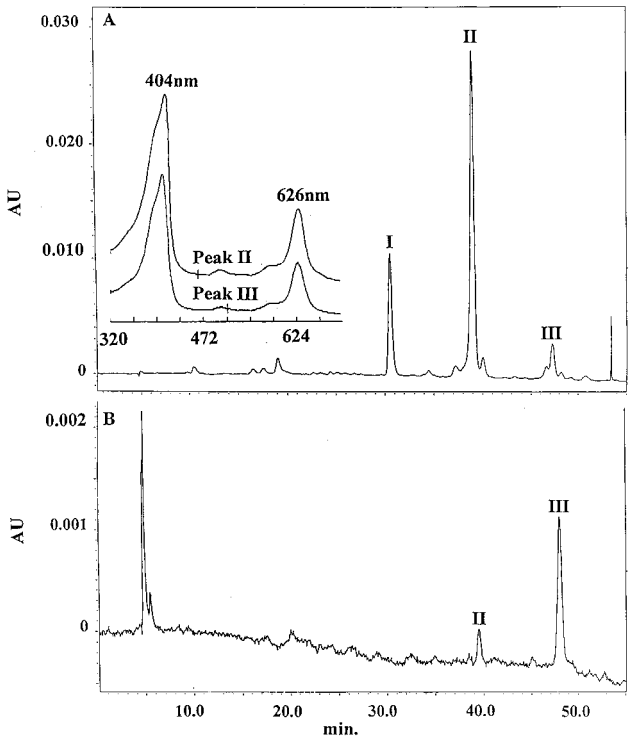

Figure 1. HPLC tracings of bulk CHL constituents (A) and an extracted serum sample obtained from a study participant receiving CHL (B) measured at 626 nm using conditions described in the text. Peaks I−III refer to $CuCle_6$, $CuCle_4$, and $CuCle_4$ ethyl ester, respectively. In the inset are shown full spectrum scans showing characteristic chlorin λ_{max}s at 404 and 626 nm for both $CuCle_4$ and $CuCle_4$ ethyl ester.

copper chlorin analogue (peak III), later identified as $CuCle_4$ ethyl ester. Authentic $CuCle_4$ and $CuCle_6$ standards analyzed under identical HPLC and LC/MS conditions were used to confirm the identity of the components. The balance of the CHL preparation consisted of several additional, copper chlorin derivatives and other chromophores lacking the distinct porphyrin absorption patterns shown in the inset.

ESI/MS spectra of $CuCle_6$, $CuCle_4$, and $CuCle_4$ ethyl ester purified from the CHL tablet possessed molecular ion peaks at *m*/*z* 657, 613, and 641, respectively. In all three cases, isotopic patterns indicated the presence of the copper moiety. A more in-depth examination of these three chlorins using MS/MS of the molecular ion resulted in very similar fragmentation patterns for both $CuCle_4$ (Figure 2A) and $CuCle_4$ ethyl ester (Figure 2B). The $CuCle_6$ daughter ion fragment pattern was distinctly different (spectrum not shown).

Characterization of Serum Components. Figure 1B depicts the HPLC chromatogram from an extracted serum sample originally green in color. Peak III has the same retention time and UV spectral properties as the previously unidentified chlorin, $CuCle_4$ ethyl ester, found in the CHL tablet (Figure 1A). A small amount of $CuCle_4$ (roughly 0.1−0.2 μg/mL) was also detected in this sample. Although $CuCle_4$ ethyl ester was discernible in all of the serum extracts chromatographed from study participants receiving CHL, $CuCle_4$ was detected in only about 10% of these samples. Furthermore, chlorins were not detectable in serum samples assayed from study participants prior to receiving the active drug as well as participants receiving placebo. The early eluting peaks (t_R = 5 min) shown on the chromatogram (Figure 1B) had UV spectral

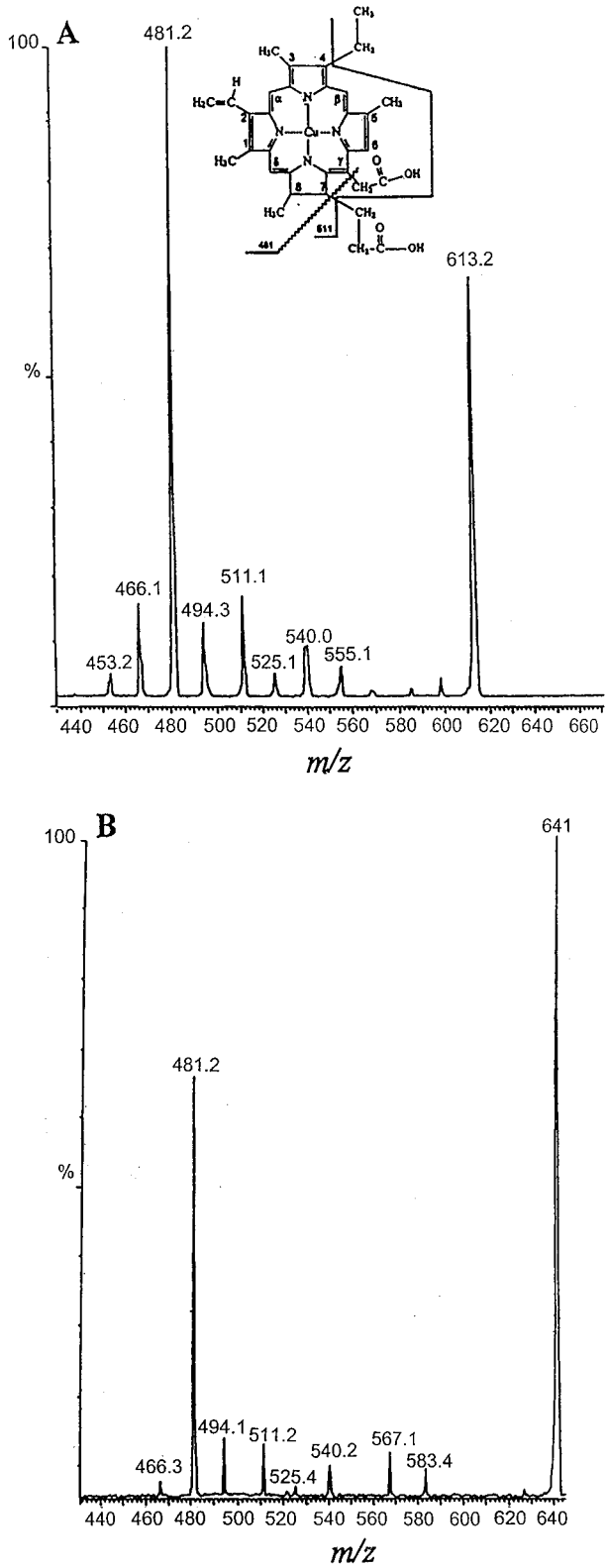

Figure 2. (A) MS/MS spectrum showing daughter ions of $CuCle_4$ isolated from the bulk CHL. In the inset is shown the structure of $CuCle_4$ with fragmentation patterns depicted. (B) MS/MS fragmentation pattern obtained for $CuCle_4$ ethyl ester (peak III) also found in the bulk CHL. Ions at *m*/*z* 540, 525, 511, 494, 481, and 466 are shown for each compound.

characteristics distinctly different from those of the copper chlorins.

Four randomly selected sets of serum samples obtained from study participants receiving CHL were extracted and independently analyzed by LC/MS. ESI/MS spectra

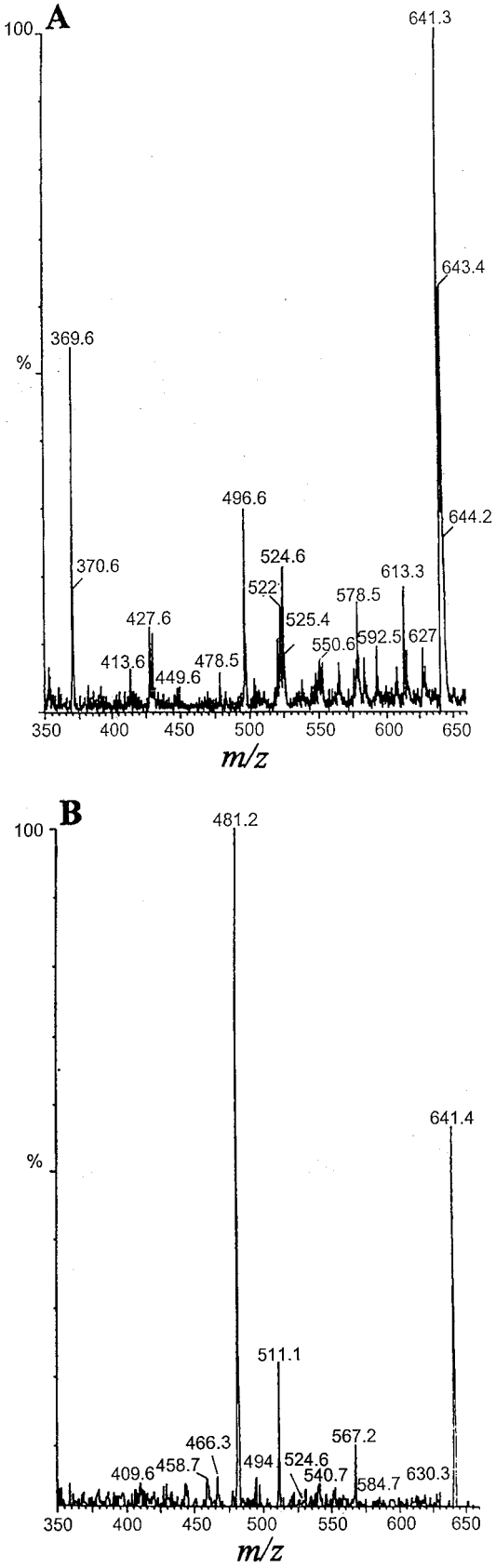

Figure 3. (A) ESI/MS spectrum recorded for the major UV component resulting from an extracted serum sample obtained from a study participant receiving CHL. A major signal is shown at *m*/*z* 641. (B) MS/MS spectrum of the same sample showing fragments at *m*/*z* 540, 525, 511, 494, 481, and 466 found to be the same as the MS/MS of $CuCle_4$ ethyl ester, peak III (Figure 2B).

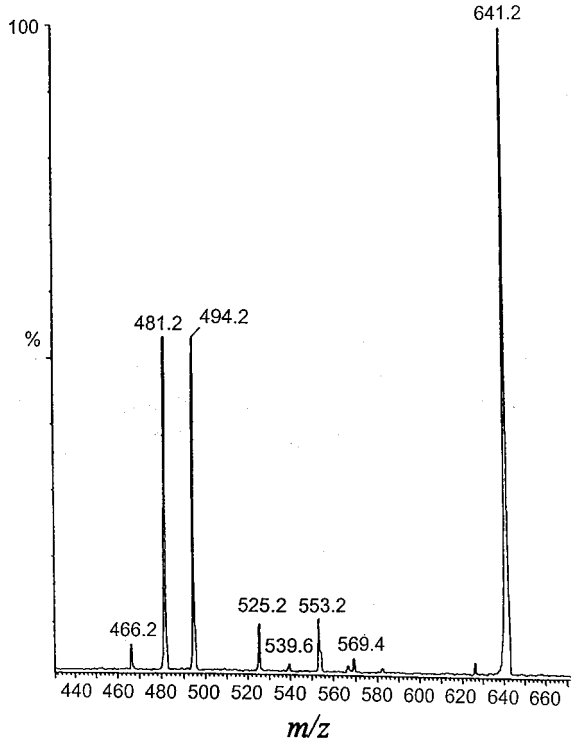

Figure 4. MS/MS daughter ion pattern of the authentic $CuCle_4$ dimethyl ester with ions at *m*/*z* 569, 553, 539, and 525 due to the methylation of both $CuCle_4$ carboxylic acid side chains. Daughter ions at *m*/*z* 555 and 511 previously found in $CuCle_4$ (Figure 2A) are not present.

were recorded for the major UV responsive component in each sample as shown in Figure 3A. In all cases, the fragmentation patterns were identical to those obtained from peak III of the CHL tablet. Furthermore, the MS/MS (Figure 3B) spectra of $CuCle_4$ ethyl ester isolated from the sera revealed that the daughter fragmentation patterns were the same as those found in the CHL tablet for the *m*/*z* 641 peak (Figure 2B). The daughter ion *m*/*z* 555 of $CuCle_4$ is missing in both *m*/*z* 641 peaks of the CHL tablet and the serum. However, an ion at *m*/*z* 583, representing the addition of an ethyl group (*m*/*z* 28) to this daughter ion, is present.

Identification of the $CuCle_4$ Ethyl Ester (Peak III). Due to the presence of the two carboxylic acid groups on $CuCle_4$ (inset of Figure 2A) , two possible derivatives of $CuCle_4$ with molecular weights of *m*/*z* 641 were examined, $CuCle_4$ dimethyl ester and $CuCle_4$ ethyl ester. Authentic dimethyl ester was prepared by methylating $CuCle_4$ with diazomethane as previously described. Using the HPLC conditions outlined above, $CuCle_4$ dimethyl ester had an HPLC retention time ($t_R = 53$ min) that was 5 min longer than that for $CuCle_4$ ethyl ester found in both the sera and CHL tablets ($t_R = 48$ min). In addition, ESI/MS/MS revealed that although both esters had identical molecular ions, *m*/*z* 641, the daughter ion pattern of the dimethyl ester was markedly different from that of $CuCle_4$ (Figure 4). The major $CuCle_4$ daughter ions at *m*/*z* 494, 481, and 466 are similarly found in the dimethyl ester spectrum, representing the loss of fragments containing both diazomethane-added methyl groups. However, the $CuCle_4$ dimethyl ester daughter ions at *m*/*z* 569, 553, 539, and 525 represent fragment losses containing only one of these added methyl groups since corresponding daughter ions of $CuCle_4$, minus the mass of the second methyl group (*m*/*z* 14), are found.

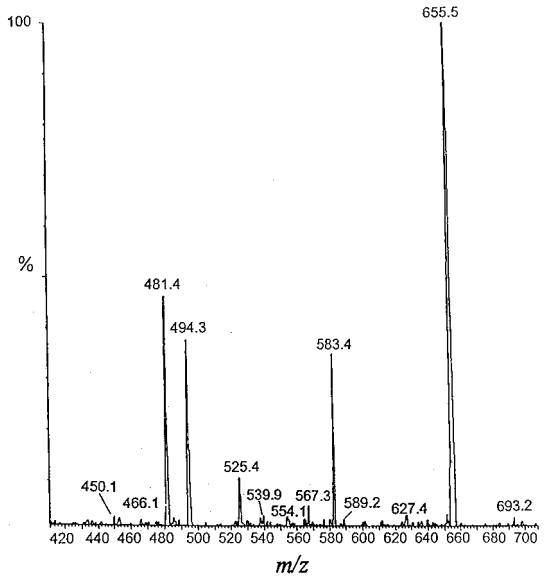

Figure 5. MS/MS fragmentation obtained from the methylation of $CuCle_4$ ethyl ester, with a parent ion at *m/z* 655. Daughter ions found in common with previously depicted chlorins include *m/z* 583, 567, 540, 525, 494, 481, and 466. Ions at *m/z* 597, 571, and 511 are absent.

Treatment of $CuCle_4$ ethyl ester isolated from the serum extract with diazomethane results in a molecular ion at *m/z* 655 (Figure 5), indicating the addition of one methyl group. As with the three chlorin compounds discussed previously, the daughter ions at *m/z* 494, 481, and 466, representing fragment losses containing all modifications to the $CuCle_4$ structure, are present with *m/z* 481 being the major daughter ion. The methylated $CuCle_4$ ethyl ester daughter ions at *m/z* 583 and 567 result from losses which include the methyl modification but retain the ethyl modification on the original $CuCle_4$ structure. The daughter ions at *m/z* 554 and 539 represent fragment losses which do not include the added methyl group and correspond to the $CuCle_4$ ethyl ester daughter ions at *m/z* 540 and 525, respectively. Notably absent in the methylated $CuCle_4$ ethyl ester fragmentation is the ethyl ester daughter ion at *m/z* 511. However, the *m/z* 525 ion peak can represent not only fragment losses from both the 7 and γ positions but also the fragmentation loss similar to that of the $CuCle_4$ ethyl ester *m/z* 511 ion with the methyl modification still intact on the chlorin structure.

Using an independent method of structural identification, the ^1H NMR spectrum for peak III exhibited a quartet signal splitting pattern in the 3.72 ppm ($J = 7.02$ Hz) region and a triplet signal pattern at 0.92 ppm ($J = 7.3$ Hz) which was not observed in the $CuCle_4$ spectrum. The observed chemical shifts are slightly upfield from the values normally exhibited by ethoxycarbonyl groups (*21*). These data are consistent with the existence of an ethyl ester on one of the $CuCle_4$ side chains previously containing a carboxylic acid group (position 7 or γ).

To determine the position of the ethyl ester moiety, the daughter ion at *m/z* 511 (fragment loss, *m/z* 102) on the parent $CuCle_4$ spectrum (inset of Figure 2A) is considered extremely important. This ion results from the loss of the ethyl group (*m/z* 29) at position 4 and the complete loss of the side chain at position 7 (*m/z* 73). While the *m/z* 511 ion is not present in the MS/MS spectrum of either methylated $CuCle_4$ compound (Figures 4 and 5), the *m/z* 525 ion peak is not only present in both methylated $CuCle_4$ derivative spectra but also increased in relative abundance versus its presence in the unmodified parent structures (Figure 2A,B). Therefore, the *m/z* 525 ion peak represents the same fragmentation loss which produces the *m/z* 511 ion peak (inset of Figure 2A), with the addition of the methylation of the carboxylic acid group at the γ position. A structure of the $CuCle_4$ ethyl ester consistent with the data that are obtained is shown in Figure 6.

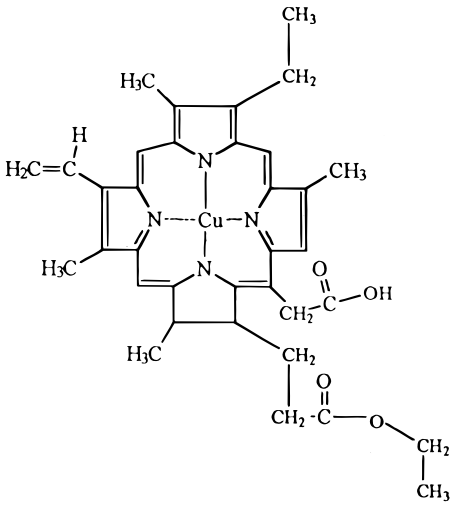

Figure 6. Proposed structure of $CuCle_4$ ethyl ester (peak III) found in both the CHL preparation and serum extracts.

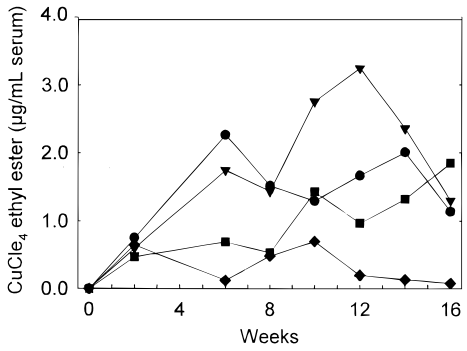

Figure 7. Concentration of $CuCle_4$ ethyl ester in serum samples measured by HPLC in four individuals (▼, ●, ■, ◆) receiving CHL. Samples were obtained biweekly throughout the study.

Absorption and Distribution of the $CuCle_4$ Ethyl Ester. Figure 7 illustrates HPLC measurements of serum levels of the $CuCle_4$ ethyl ester found in four participants receiving CHL throughout the duration of the intervention using an ϵ of 6.6×10^3 μM^{-1} cm^{-1}. Although there are marked differences in individual drug levels (as shown in Figure 7), $CuCle_4$ ethyl ester appears to accumulate throughout the first 8 weeks of treatment and reaches a steady-state level of about 1−2 μg/mL. It should also be noted that the study was not originally designed to be a rigorous pharmacokinetic analysis of CHL and that precise timing of the blood draws relative to pill consumption was not undertaken.

Discussion

$CuCle_4$ ethyl ester can now be identified as a heretofore unreported component found in formulations of CHL. Following daily administration of CHL tablets during a

recent chlorophyllin clinical trial, $CuCle_4$ ethyl ester and $CuCle_4$ were found to be absorbed into the blood stream, thereby lending a peculiar green tint to the sera samples. Using NMR, ESI/MS, and MS/MS techniques, $CuCle_4$ ethyl ester was identified and quantified in both the original CHL preparation and serum extracts from study participants receiving the active drug. The ethyl ester structure (depicted in Figure 6) results from an alkylation of one of the carboxylic acid side chains found on the parent $CuCle_4$ (inset of Figure 2A). We also concluded that the carboxylic acid group at position 7 was ethylated on the basis of the presence of the *m*/*z* 511 daughter ion in the $CuCle_4$ and $CuCle_4$ ethyl ester MS/MS spectra analyses and the existence of an *m*/*z* 525 ion in the methylated $CuCle_4$ ethyl ester.

Although CHL has been widely used for several decades both medicinally and as a dietary supplement, preparations are not routinely examined for composition. Variations in manufacturing techniques as well as starting materials used in producing CHL result in end products with differing compositions (*19*). In addition, the traditional practice of measuring total CHL concentration by spectrophotometry is not only inaccurate but also ineffective in distinguishing between chlorin constituents due to similarities seen in chlorin spectral patterns. However, despite these common practices, several reports using TLC and HPLC techniques (*10, 19, 20*) have documented that commercial CHL preparations are mixtures of various chlorins. This disparity in CHL preparations may modify chemopreventive efficacy and other pharmacological actions based on the variety of biological effects preliminarily attributed to individual chlorins (*10*).

The CHL tablet used in the Chlorophyllin Clinical Trial contained two major chlorin components, $CuCle_4$ and $CuCle_6$, and a previously unreported chlorin, $CuCle_4$ ethyl ester. This less polar chlorin appeared to be readily absorbed as evidenced by the identification and quantitation of this entity in extracted serum samples obtained from the study participants receiving CHL. A much smaller amount of $CuCle_4$ was also discernible in some, but not all, of the serum extracts following administration of CHL. To determine whether the $CuCle_4$ was a result of the action of numerous esterases present in the sera (*22*) or directly absorbed from the tablet, freshly obtained serum was incubated at 37 °C for several hours with isolated $CuCle_4$ ethyl ester. No appreciable $CuCle_4$ was detected (data not shown), suggesting that the esterases did not cleave the ester bond on the $CuCle_4$ ethyl ester. It is not known whether intracellular esterases may hydrolyze the circulating $CuCle_4$ ethyl ester, thereby enhancing its intracellular accumulation. Therefore, it appears that depending on the dose and composition of administered CHL, $CuCle_4$ ethyl ester, $CuCle_4$, or even perhaps additional, less polar chlorins may become bioavailable.

The absorption of $CuCle_4$ ethyl ester and $CuCle_4$ following administration of CHL as demonstrated in this study is significant because previous dogma has held that CHL is poorly absorbed (if at all) and excreted unchanged. Given an estimated daily intake of 12 mg of $CuCle_4$ ethyl ester from the administration of three 100 mg CHL tablets and a steady-state plasma concentration of approximately 2 μg/mL, $CuCle_4$ ethyl ester appears to distribute into total body water. Thus, standing hypotheses concerning the mode of action of CHL and even perhaps its ultimate biological manifestations may have to be reexamined.

To date, the most likely mechanism of action of CHL as a cancer chemopreventive agent has centered around the molecular complexing theory (*8, 9*) in which CHL forms noncovalent molecular complexes with planar aromatic carcinogens, thereby impeding the absorption of these toxins. However, several other realms of CHL action on carcinogen disposition have been examined. Sato and colleagues demonstrated that CHL administered to rats ip was taken into the liver and distributed among the mitochondria, microsomes, and soluble fraction in an active form that not only functioned as an antioxidant (*10*) but also decreased the activities of hepatic microsomal drug-metabolizing enzyme systems (*12*). More recently, CHL was also found to be a potent, nonspecific inhibitor of cytochrome P450 activities in vitro (*11*). A comparison of the steady-state levels of $CuCle_4$ ethyl ester ($\sim$3 μM) with the reported apparent K_i of 4.1 μM for CHL as an inhibitor of cytochrome P450s (*11*) suggests that an inhibitory concentration might have been achieved in this chemoprevention study. Thus, the antigenotoxic, antimutagenetic, and anticarcinogenic effects of CHL may result from the inhibition of cytochrome P450 enzymes in addition to any molecular complexing which may occur. Detailed toxiokinetic analyses of the metabolism and disposition of AFB_1 in blood and urine samples collected during the clinical trial and for other carcinogens in chemoprevention models will be required to rigorously assess the contributions of the ex vivo and in vivo actions of CHL toward inhibition of carcinogenesis.

Acknowledgment. We thank Mr. Herbert Wagner and Dr. Anil Patel of Rystan, Inc., for the generous contribution of placebo and CHL tablets used in the 1997 Chlorophyllin Clinical Trial. This study also would not have been possible without the efforts of our colleagues at the Qidong Liver Cancer Institute and the Daxin Medical Clinic. Insightful discussions with Dr. Jerry C. Bommer (Porphyrin Products, Inc.) were extremely helpful in understanding chlorin properties. We also acknowledge the Johns Hopkins Homewood Campus Kreiger School of Arts and Sciences, Chemistry Department Instrumentation Facility operated by Dr. Charles A. Long, for the NMR analyses. This work was supported by NIH Grants ES 06052 and ES03819.

References

(1) Hayatsu, H. M., Arimoto, S., and Negishi, T. (1988) Dietary inhibitors of mutagenesis and carcinogenesis. *Mutat. Res.* **202**, 429−446.

(2) Dragsted, L. O., Strube, M., and Larsen, J. C. (1993) Cancer-protective factors in fruits and vegetables: biochemical and biological background. *Pharmacol. Toxicol.* **72** (Suppl. 1), 116−135.

(3) Kephart, J. C. (1955) Chlorophyll derivatives: their chemistry, commercial preparation and uses. *Econ. Bot.* **9**, 3−38.

(4) Young, R. W., and Bergei, J. S. (1980) Use of chlorophyllin in the care of geriatric patients. *J. Am. Geriat. Soc.* **24**, 46−47.

(5) Waters, M. D., Stack, H. F., Jackson, H. E., Brockman, D., and DeFlora, S. (1996) Activity profiles of antimutagens: *in vitro* and *in vivo* data. *Mutat. Res.* **350**, 109−129.

(6) Negishi, T., Rai, H., and Hayatsu, H. (1997) Antigenotoxic activity of natural chlorophylls. *Mutat. Res.* **376**, 97−100.

(7) Dashwood, R., Negishi, T., Hayatsu, H., Breinholt, V., Hendricks, J., and Bailey, G. (1998) Chemopreventive properties of chlorophylls towards aflatoxin B_1. A review of the antimutagenicity and anticarcinogenicity data in rainbow trout. *Mutat. Res.* **399**, 245−253.

(8) Negishi, T., Arimoto, S., Nishizaki, C., and Hayatsu, H. (1989) Inhibitory effect of chlorophyll on genotoxicity of 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2). *Carcinogenesis* **10**, 145−149.

(9) Breinholt, V., Schimerlik, M., Dashwood, R., and Bailey, G. (1995) Mechanisms of chlorophyllin anticarcinogenesis against aflatoxin B_1. Complex formation with the carcinogen. *Chem. Res. Toxicol.* **8**, 506−514.

(10) Sato, M., Fujimoto, I., Sakai, T., Aimoto, T., Kimura, R., and Murata, T. (1986) Effect of sodium copper chlorophyllin on lipid peroxidation IX. On the antioxidative components in commercial preparations of sodium copper chlorophyllin. *Chem. Pharm. Bull.* **34**, 2428−2434.

(11) Yun, C.-Y., Hye, G. J., Jhoun, J. W., and Guengerich, F. P. (1995) Non-specific inhibition of cytochrome P450 activities by chlorophyllin in human and rat liver microsomes. *Carcinogenesis* **16**, 1437−1440.

(12) Imai, K., Aimoto, T., Sato, M., Watanabe, K., Kimura, R., and Murata, T. (1986) Effect of sodium metallochlorophyllins on the activity and components of the microsomal drug-metabolizing enzyme system in the rat liver. *Chem. Pharm. Bull.* **34**, 4287−4293.

(13) Brienholt, V., Hendricks, J., Pereira, C., Arbogast, D., and Bailey, G. (1995) Dietary chlorophyllin is a potent inhibitor of aflatoxin B_1 hepatocarcinogenesis in rainbow trout. *Cancer Res.* **55**, 57−62.

(14) Kensler, T. W., Groopman, J. D., and Roebuck, B. D. (1998) Use of aflatoxin adducts as intermediate endpoints to assess the efficacy of chemopreventive interventions in animals and man. *Mutat. Res.* **402**, 165−172.

(15) Dashwood, R. H., and Guo, D. (1992) Inhibition of 2-amino-3-methylimidazo[4,5-*f*] quinoline (IQ)-DNA binding by chlorophyllin: studies of enzyme inhibition and molecular complex formation. *Carcinogenesis* **13**, 1121−1126.

(16) Eaton, D. L., and Groopman, J. D., Eds. (1994) *The Toxicology of Aflatoxins: Human, Health, Veterinary and Agricultural Significance*, Academic Press, San Diego, CA.

(17) Jacobson, L. P., Zhang, B.-C., Zhu, Y.-R., Wang, J.-B., Wu, Y., Zhang, Q.-N., Yu, Y.-L., Qian, G.-S., Kuang, S.-Y., Li, Y.-F., Fang, X., Zarba, A., Chen, B., Enger, C. L., Davidson, N. E., Gordon, G. B., Gorman, M. B., Prochaska, H. J., Egner, P. A., Groopman, J. D., Muñoz, A., Helzlsouer, K. J., and Kensler, T. W. (1996) Oltipraz chemoprevention trial in Qidong, People's Republic of China: study design and clinical outcomes. *Cancer Epidemiol., Biomarkers Prev.* **6**, 257−265.

(18) Kensler, T. W., He, X., Otieno, M., Egner, P. A., Jacobson, L. P., Chen, B., Wang, J.-S., Zhu, Y.-R., Zhang, B. C., Wang, J.-B., Wu, Y., Zhang, Q.-N., Qian, G.-S., Kuang, S.-Y., Fang, S., Li, Y.-F., Yu, L.-Y., Prochaska, H. J., Davidson, N. E., Gordon, G. B., Gorman, M. B., Zarba, A., Enger, C., Muñoz, A., Helzlouer, K. J., and Groopman, J. D. (1998) Oltipraz chemoprevention trial in Qidong, People's Republic of China: modulation of serum aflatoxin albumin adduct biomarkers. *Cancer Epidemiol., Biomarkers Prev.* **7**, 127−134.

(19) Inoue, H., Yamashita, H., Furuya, K., Nonomura, Y., Yashioka, N., and Li, S. (1994) Determination of copper(II) chlorophyllin by reversed-phase high-performance liquid chromatography. *J. Chromatogr.* **679A**, 99−104.

(20) Chernomorsky, S., Rancour, R., Sahai, D., and Poretz, R. (1997) Evaluation of commercial chlorophyllin copper complex preparations by liquid chromatography with photodiode array detection. *J. Am. Org. Anal. Chem. Int.* **80** (2), 433−435.

(21) Sasaki, S.-I., Ed. (1985) *Handbook of Proton-NMR Spectra and Data*, Vol. 5, Academic Press, Tokyo.

(22) Williams, F. M. (1985) Clinical significance of esterases in man. *Clin. Pharmacokinet.* **10**, 392−403.

TX000069K