**Isolation and Identification of Kahweol Palmitate and Cafestol Palmitate as Active Constituents of Green Coffee Beans That Enhance Glutathione S-Transferase Activity in the Mouse**

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**ABSTRACT**

Glutathione (GSH) S-transferase is a major detoxification enzyme system that catalyzes the binding of a variety of electrophiles, including reactive forms of chemical carcinogens, to GSH. Green coffee beans fed in the diet induce increased GSH S-transferase activity in the mucosa of the small intestine and in the liver of mice. A potent compound that induces increased GSH S-transferase activity was isolated from green coffee beans and identified as kahweol palmitate. The corresponding free alcohol, kahweol, and its synthetic monooacetate are also potent inducers of the activity of GSH S-transferase. A similar diterpene ester, cafestol palmitate, isolated from green coffee beans was active but less so than kahweol palmitate. Likewise, the corresponding alcohol, cafestol, and its monooacetate showed moderatly as inducers of increased GSH S-transferase activity. Kahweol palmitate and cafestol palmitate were extracted from green beans into petroleum ether. The petroleum ether extract was fractioned by preparative normal-phase and reverse-phase liquid chromatographies successively. Final purification with silver nitrate-impregnated thin-layer chromatography yielded the pure palmitates of cafestol and kahweol. The structures were determined by examination of the spectroscopic data of the esters and their parent alcohols and by derivative comparison.

**INTRODUCTION**

GSH3 S-transferase has been studied extensively as a major detoxification enzyme system that catalyzes the binding of a wide variety of electrophiles to GSH (3, 10). Since most reactive ultimate carcinogenic forms of chemical carcinogens are electrophiles, GSH S-transferase may play a significant role in carcinogen detoxification. Enhancement of the activity of this system potentially could increase the capacity of the organism to withstand the neoplastic effects of chemical carcinogens. Experiments have been carried out to determine the correlation between increased GSH S-transferase activity in a target organ of chemical carcinogenesis and its response to the carcinogen. For this purpose, the forestomach of the mouse was used. Members of several classes of inhibitors of BP-induced neoplasia of the mouse forestomach were studied for their effects on the GSH S-transferase activity in that target. Five of the compounds increased the GSH S-transferase activity of the forestomach between 78 and 182%. They were p-methoxyphenol, 2-tert-butyl-4-hydroxyanisole, coumarin, a-angelicalactone, and benzyl isothiocyanate (18). All 5 compounds inhibited BP-induced neoplasia of the forestomach (13, 20, 21). These data suggest that the capacity to enhance GSH S-transferase activity might be used as a method of identifying compounds or natural products likely to inhibit BP or other carcinogens detoxified in a similar manner (18).

In efforts at identifying dietary constituents that might protect against chemical carcinogens, the effects of natural products on GSH S-transferase activity were studied. During this investigation, it was found that consumption of diets containing powdered green coffee beans resulted in a very marked enhancement of GSH S-transferase activity in the liver and mucosa of the small bowel of the mouse. The magnitude of induction was as high as that obtained with any test compound or natural material previously investigated. The coffee beans used in the original study were from Guatemala. In subsequent work, coffee beans from Brazil, Colombia, El Salvador, Mexico, and Peru were all found to have a comparable enhancing effect on GSH S-transferase activity (17). Roasted coffee and instant coffee were found to have a weaker inducing activity than did the green coffee beans studied, i.e., slightly less than 50% as much. Decaffeinated instant coffee showed activity similar to that of instant coffee. Investigations were then begun to identify the constituents of green coffee beans having the capacity to enhance GSH S-transferase activity. In the present study, it was found that the coffee constituent kahweol palmitate is a highly potent inducer of increased GSH S-transferase activity. The palmitate of a closely related diterpene, cafestol, was active as an inducer of GSH S-transferase but less so than was kahweol palmitate.

**MATERIALS AND METHODS**

**Extraction of Green Coffee Beans.** Powdered green coffee beans (Guatemala) were placed in a large modified Soxhlet extractor and were extracted with various solvents of increasing polarity (Chart 1). PE (b.p. 60–70°C) was used first, which was followed by benzene, ethyl acetate, methanol, and water. Each solvent extraction was carried out over a period of 7 days. The solvent of each extraction was removed under reduced pressure. The crude extracts were dried under reduced pressure until constant weights were obtained. The activities of the extracts were monitored by the GSH S-transferase assay.

**Fractionation of the PE Extract.** The active PE extract was separated into 7 fractions by preparative LC using a Waters Associates prep500 liquid chromatograph equipped with prepPak-500 silica columns. The elution solvent was PE:ether (2:1, v/v). The active fraction from preparative LC was further fractionated into 6 subfractions by reverse-phase preparative LC. Two prep PAK-500/C18 columns in series were used with 95% methanol as the eluting solvent. Silver
The amount of each extract added was such that the diets would contain the equivalent of 20% powdered green coffee beans. In each experiment, 20% crude powdered green coffee beans were included as a positive control as well as a control in which there were no additions. The diets were fed for 12 days at which time the mice were killed. The mucosa of the proximal half of the small bowel and the liver were taken for the determination of GSH S-transferase activity. The inductive effects on the small bowel mucosa under these conditions are about one-third of the magnitude as in the feeding procedure. Liver is much less responsive.

GSH S-transferase activity in the cytosol was determined according to previously published procedures (18). All steps were done at 0-4°C. The tissues to be studied were homogenized in 0.1 M sodium phosphate buffer (pH 7.5). The homogenate was centrifuged at 100,000 x g for 1 hr. The supernatant was used for the assay of GSH S-transferase activity. The activity was determined spectrophotometrically at 30°C with 1-chloro-2,4-dinitrobenzene as substrate according to the procedure of Habig et al. (8).

RESULTS

Isolation Procedure

The activity of the enzyme GSH S-transferase in the liver and mucosa of the small bowel of the mouse was enhanced by the addition of 20% green coffee beans to the diet. The enhancement of enzyme activity in the liver was approximately 5 times that of the control (Chart 2B). A slightly smaller enhancement...
was found in the mucosa of the small bowel (Chart 2A). The overall magnitude of enzyme activity in the latter organ, however, was much lower than that in the former. PE extraction for 7 days successfully removed most of the ingredients that had inducing activity. Subsequent extractions by benzene showed activity that affected only the mucosa of the small bowel but had little effect in the liver. The other solvent extracts were inactive. When the extracts were combined with the residue of extraction, a slight increase of activity was observed in comparison with the original green coffee beans (Chart 2).

The PE extract contained a small amount of caffeine which could be removed by dissolving the dried extract in a small volume of PE and filtering. The caffeine-free PE extract was then fractionated by preparative LC to give 7 fractions. Fraction 3 contained materials that gave similar enhancement of activity to that of the PE extract. The weight of Fraction 3 was 20% that of the PE extract. The other fractions were all inactive in the GSH S-transferase assay (Chart 3). All attempts to further fractionate Fraction 3 with normal-phase preparative LC resulted in subfractions of mixtures of similar components. Analysis of Fraction 3 by reverse-phase high-performance liquid chromatography (C<sub>18</sub>μBondapak, eluted with methanol) indicated 6 peaks (Subfractions A to F) that were well separated. Subfractions A to F were separated by preparative LC. All 6 subfractions were active in enhancing GSH S-transferase activity in the mucosa of the small bowel of the mouse (Chart 4). Subfraction C, which constituted approximately 38% of Fraction 3, showed the highest activity in the enzyme assay and was chosen for further purification.

To further separate Subfraction C into its pure components, TLC (Silica Gel G) plates impregnated with silver nitrate were used (Chart 1). These silver nitrate-treated plates were able to separate Subfraction C into 4 components, 3 of which were active (Chart 5A). The activity of Compound 1 was similar to...
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that of the control level. Compound 2 showed the greatest potency in enhancing the activity of GSH S-transferase. Administration of Compound 2 resulted in an increase of GSH S-transferase activity that was 4 times that of the control. Compounds 3 and 4 were able to induce the enzyme activity to twice the control level. Compounds 1 and 2, the components that had the highest $R_f$ values, were isolated on preparative TLC plates impregnated with silver nitrate. Compound 1, which was present in much higher quantity than were the other 3 components, was easily purified and characterized as a pure compound. The behavior of this set of compounds during fractionation and TLC separation indicated that they belonged to the same class of organic compounds with only minor structural differences. The structural determination of any one of these compounds will facilitate the identification of the remaining components. Since Compound 1 was isolated in its pure form in sufficient quantity, structural determination was started with this compound. Spectroscopic evidence and derivative comparison indicated that Compound 1 was the palmitate of cafestol (2). Component 2, which has a molecular weight that is 2 mass units lower than that of Compound 1, was identified to be the palmitate of kahweol (1). The previously known coffee constituents, cafestol and kahweol, could be isolated as the free alcohols from mild alkaline hydrolysis of Compounds 1 and 2, respectively. While cafestol palmitate showed very little activity as an enzyme inducer, cafestol and its monoacetate showed activity that was approximately 1.5 times above control level (Chart 5B) (see "Addendum"). Kahweol and its monoacetate showed activity that was similar to that of kahweol palmitate (Chart 6). Palmitic acid (Compound 1c) was inactive as an inducer of the activity of GSH S-transferase (Chart 5B).

**Structural Identification**

**Compound 1.** Compound 1 has been identified as the palmitate of cafestol (Chart 7). Mass spectrometry (70 eV) yielded: $m/e$ 554 (M⁺, 71.2). The precise mass for $C_{38}H_{58}O_4$ was

Calculated: 554.4335
Found: 554.4353

The loss of 18 mass units ($-\text{H}_2\text{O}$) from 554 ($m/e$ 536) clearly indicated the presence of a hydroxy group. No other significant mass fragments were observed between $m/e$ 554 and $m/e$ 298. The IR spectrum showed the presence of OH stretching frequencies at 3600 and 3450 cm⁻¹. Intense C—H stretching frequencies around 2900 cm⁻¹ indicated the presence of a large number of saturated carbon moieties. The carbonyl stretching frequency at 1740 cm⁻¹ together with the fragmentation pattern of the mass spectrum were indicative of an ester function. The 250-MHz proton NMR spectrum confirmed the presence of large numbers of protons on saturated carbons. A triplet centered at 2.36 ppm was the result of the methylene protons adjacent to the carbonyl function of the ester (Compound 1). A singlet at 4.27 (2H) appeared to be the resonance peak due to the 2 protons at C-17. Two doublets at 7.24 and 6.20 ppm ($J < 0.01$ Hz), respectively, were the resonances from the protons at C-19 and C-18 on the furan ring.

Saponification of Compound 1 yielded an alcohol (Compound 1a) and an acid (Compound 1c). The acid was identified
to be palmitic acid (M, 256) by mass spectroscopy and by melting point.

**Compound 1a.** The molecular formula of the alcohol, Compound 1a, was determined by high-resolution mass spectrometry to be $C_{20}H_{36}O_2$.

Calculated: 316.2038
Found: 316.2027

Gas chromatography-mass spectroscopy of its trimethylsilyl
derivatives revealed mono- and ditrimethylsilane derivatives having molecular ions at m/e 398 and m/e 460, respectively. The proton NMR spectrum at 250 MHz showed the absence of aliphatic protons of the palmitic acid moiety. The resonances at 7.24 and 6.20 ppm were unchanged. The singlet at 4.27, however, has changed into an AB quartet with J = 13.5 Hz. This quartet is the result of hydrogen bonding of the hydroxy groups on C-16 and C-17 which makes the protons on C-17 magnetically nonequivalent. These spectral data and the IR spectrum of Compound 1a are consistent with the structure of cafestol (4, 6, 15).

The monoacetate of Compound 1a, which was synthesized by the treatment with acetyl chloride in pyridine, had an IR spectrum identical to that of cafestol monoacetate, Compound 1b, reported by Djerassi et al. (5).

**Compound 2.** Compound 2 was found to be very similar in structure to Compound 1 and was identified as the palmitate of kahweol (Chart 7). The molecular weight was determined to be 552 by mass spectroscopy. The precise mass for C_{36}H_{56}O_{4} was

Calculated: 552.4178  
Found: 552.4203

The IR spectrum of Compound 2 was almost identical to that of Compound 1 except for a small peak at 3050 cm\(^{-1}\) which is the stretching frequency of C—H bond on a carbon-carbon double bond. The proton spectrum had 2 sets of doublets at 7.26 and 6.25, respectively. These resonances are due to the 2 vinylic protons of the double bond at C-1 and C-2 of Compound 2. Saponification of Compound 2 yielded palmitic acid and an alcohol Compound 2a.

**Compound 2a.** Compound 2a has been identified as kahweol. Mass spectrometry yielded m/e 314 (M\(^+\) 10.4). The molecular formula of Compound 2a was determined by high-resolution mass spectroscopy to be

C_{30}H_{46}O_{3}

Calculated: 314.1882  
Found: 314.1872

Compound 2a is easily oxidized in the presence of air. Its monoacetate, Compound 2b, melts at 133.5—136\(^{\circ}\), which is similar to the melting point of the monoacetate of kahweol reported by Kaufman and Sen Gupta (11, 12).

**DISCUSSION**

In the present investigation, a potent inducer of increased GSH S-transferase activity has been isolated from green coffee beans. This compound is kahweol palmitate. A related compound, cafestol palmitate, was also active as an inducer of GSH S-transferase activity but less so than kahweol palmitate.

The active ingredients from green coffee beans were almost totally extracted into PE when the extraction was carried out for 7 days or more. The PE extract was able to induce increased GSH S-transferase activity in both the mucosa of the small intestine and the liver of the mouse. On an equivalent weight basis, the activity of the PE extract was approximately 40\% higher than that of the green coffee beans. This increase in activity was probably due to the greater availability of the inducing compounds resulting from their extraction. Benzene, the second solvent used in the extraction scheme, was able to extract substances that had inducing activity for the mucosa of the small bowel but were inactive in the liver of the mouse.

The first fractionation of the PE extract by normal-phase preparative LC was able to eliminate 80\% of the inactive substances. Attempts to manipulate the solvent composition to further separate the components in the active Fraction 3 were not successful. Reverse-phase high-performance liquid chromatography, however, was able to resolve this fraction into 6 distinct peaks. Peak C, which constitutes approximately 38\% of Fraction 3, was isolated in the preparative scale using prep PAK-500/C_{18} columns. Preliminary UV spectral data on Subfraction C indicated the presence of conjugated double bonds in the molecules. Since silver nitrate-impregnated TLC had been used successfully to separate olefins, it was anticipated that the technique might be applied to further separate components in Subfraction C (19). At least 4 well-separated components were found by this method which led to the final identification of the active ingredients.

The diterpenes, cafestol and kahweol, were isolated from the unsaponifiable portion of the PE extract of green coffee beans in 1938 and 1932, respectively (1, 16). The structure of cafestol was elucidated in the reports of Djerassi et al. (4, 6) and others (15). In the present investigation, Compound 1a obtained by the saponification of Compound 1 was identical to cafestol by spectroscopic and derivative comparison. The structure of kahweol was elucidated by Kaufmann and Sen Gupta (11, 12). Compound 2a obtained by the saponification of Compound 2 was shown to have the same structure as that of kahweol. The exact location of the double bond was questioned recently by Gershbein and Baburao (7). Our NMR data favor the double bond at C-1 and C-2 which is consistent with the structure proposed by Kaufmann and Sen Gupta. Although the existence of these 2 diterpenes has been known since 1932, their palmitate esters have not been well characterized. Previous work has not established any biological effects of cafestol or kahweol (2, 7, 9, 22).

The concentration of kahweol palmitate in the active Fraction 3 is estimated at 10 to 15\%. The remaining active constituents in Subfractions A, B, and D to F are most probably due to fatty acid esters of kahweol. Preliminary TLC data indicated the presence of cafestol and kahweol in all the active fractions in the isolation scheme.

Several compounds that are potent inducers of increased GSH S-transferase activity inhibit carcogen-induced neoplasia (18). 3-tert-Butyl-4-hydroxyanisole is among the most potent of these compounds and inhibits a wide variety of chemical carcinogens. When compared under the same experimental conditions, i.e., a single p.o. administration 24 hr prior to sacrifice, kahweol palmitate is more than 3 times as potent in enhancing GSH S-transferase activity of the mucosa of the small intestine as 3-tert-butyl-4-hydroxyanisole. It remains to be determined whether kahweol palmitate and related diterpene esters will have carcinogen-inhibitory capacities. The constituents of green coffee are complex mixtures of a very large number of chemicals. The composite biological effects of coffee on occurrence of neoplasia may not be the result of one or 2 discrete components alone. Thus, it is not possible at the present time to assume a dominant biological significance of...
the diterpene esters in the complex mixture of coffee constituents.

ADDENDUM

During the isolation procedure, the activity of cafestol palmitate as an inducer of GSH S-transferase activity was determined using a single p.o. administration of 2.5 mg/mouse. Under these conditions, cafestol palmitate showed only marginal activity (Chart 5). A subsequent experiment using 4 administrations of 2.5 mg cafestol palmitate (once a day for 4 days with mice killed 24 hr after the last administration) resulted in a GSH S-transferase activity of the small bowel mucosa 41% greater than that of the control. Further experiments were carried out using cafestol and kahweol palmitates obtained from the esterification of the alcohols with palmitoyl chloride. The semisynthetic esters used in these experiments were identical to the naturally occurring compounds by TLC, mass spectroscopy, and NMR. With the use of 4 daily administrations and a dose level of 5.0 mg/mouse, the GSH S-transferase activity of the small bowel mucosa of mice receiving cafestol palmitate was 3.9 times that of the controls, and the corresponding GSH S-transferase activity of mice receiving kahweol palmitate was 5.6 times that of the controls. These data indicate that cafestol palmitate enhances GSH S-transferase activity but is less potent than kahweol palmitate.

ACKNOWLEDGMENTS

We thank Gerald Bratt for the NMR and Thomas Krick for the mass spectra determinations. The technical assistance of Chester Yee is gratefully acknowledged.

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