# Sesquiterpenes from Clove (Eugenia caryophyllata) as Potential Anticarcinogenic Agents

Guo-Qiang Zheng, Patrick M. Kenney, and Luke K. T. Lam

*J. Nat. Prod.*, **1992**, 55 (7), 999-1003• DOI:
10.1021/np50085a029 • Publication Date (Web): 01 July 2004

*Journal of Natural Products*
*Vol. 55, No. 7, pp. 999-1003, July 1992*



# SESQUITERPENES FROM CLOVE (*EUGENIA CARYOPHYLLATA*) AS POTENTIAL ANTICARCINOGENIC AGENTS

GUO-QIANG ZHENG, PATRICK M. KENNEY, and LUKE K.T. LAM*

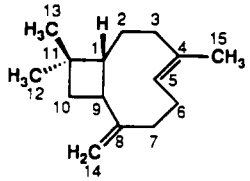
*LKT Laboratories Inc., 2010 East Hennepin Avenue, Minneapolis, Minnesota 55413*

ABSTRACT.—Bioassay-directed fractionation of clove terpenes from the plant *Eugenia caryophyllata* has led to the isolation of the following five active known compounds: β-caryophyllene [**1**], β-caryophyllene oxide [**2**], α-humulene [**3**], α-humulene epoxide I [**4**], and eugenol [**5**]. Their structures were determined on the basis of spectral analysis (hreims, $^{1}H$ and $^{13}C$ nmr). These compounds showed significant activity as inducers of the detoxifying enzyme glutathione *S*-transferase in the mouse liver and small intestine. The ability of natural anticarcinogens to induce detoxifying enzymes has been found to correlate with their activity in the inhibition of chemical carcinogenesis. Thus, these sesquiterpenes show promise as potential anticarcinogenic agents.

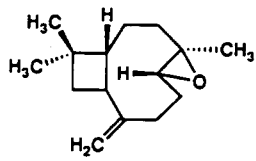
A number of chemopreventive agents are naturally occurring secondary metabolites isolated from plants, which may be useful in reducing the incidence of cancer in humans (1). A short-term enzyme assay has been used successfully for the screening of potential inhibitors of chemical carcinogenesis. This rapid screening assay is based on the induction of the detoxifying enzyme glutathione *S*-transferase (GST) in laboratory animals (2,3). GST is one of the major enzyme systems that are responsible for the detoxification of toxic xenobiotics including carcinogens (4–6). GST catalyzes the reaction of glutathione with electrophiles to form less toxic conjugates which are readily eliminated by excretion. Since most of the reactive ultimate carcinogenic forms of chemical carcinogens are electrophiles, induction of GST activity by anticarcinogenic compounds is believed to be a major mechanism for carcinogen detoxification (1,7,8). Thus, compounds that induce an increase in the activity of this detoxifying enzyme system may be potential inhibitors of carcinogenesis. Positive correlation has been established between the inhibitory activity of natural and synthetic anticarcinogens and their ability to induce increased GST activity. Several classes of compounds that inhibit chemical carcinogenesis have been found to elicit GST enzyme activity significantly in the same mouse tissues (9–13). In the course of our continuing search for chemopreventive agents from natural sources, clove terpenes exhibited high GST-inducing activity (14). To discover the components responsible for this activity observed in the preliminary screening, we have employed the GST assay to guide the subsequent fractionation and isolated five active compounds from clove terpenes. In this paper, we report the isolation, identification and biological evaluation of these compounds.

Clove terpenes are present in the higher boiling fraction of the clove oil, which can be obtained by $H_2O$ distillation from the dried flower buds of *Eugenia caryophyllata* Thunberg (Myrtaceae), a tropical tree originating in the Moluccas (15). The tree is cultivated in many tropical countries. The most important clove-exporting countries are Tanzania, Indonesia, Sri Lanka, and the Malagasy Republic. For oil production the clove buds are brought to European and American distilleries. Clove oil is frequently used in perfumery and medicine, but the largest part by far is used in flavorings.

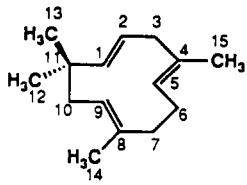
The bioassay-directed separation of clove terpenes involved initial chromatography on a Si gel column to yield 7 fractions (Table 1). Further separation of the active fractions using GST enzyme assay as a guide resulted in the isolation of five



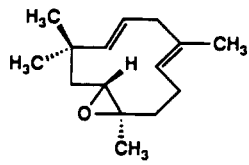
1



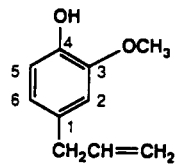
2



3



4



5

active compounds **1–5**. The determination of the structures of **1–5** was based on spectral analysis (hreims, $^{1}H$ and $^{13}C$ nmr). The $^{1}H$- and $^{13}C$-nmr chemical shifts of sesquiterpenes **1** and **2** were consistent with the data for β-caryophyllene (17,18) and β-caryophyllene oxide (16,18) published in the literature. Compound **3** was identified as α-humulene by comparison of its spectral data

TABLE 1. The Effects of Clove Terpene Fractions on the Activity of Glutathione *S*-Transferase in Target Tissues of A/J Mice.

| Fraction[a] | Liver | | Small Bowel Mucosa | | Forestomach | |
|---|---|---|---|---|---|---|
| | GST Sp. Act.[b] | Ratio[c] | GST Sp. Act.[b] | Ratio[c] | GST Sp. Act.[b] | Ratio[c] |
| Control | 1.15 ± 0.39 | | 0.43 ± 0.03 | | 0.47 ± 0.06 | |
| Mixture | 2.14 ± 0.51 | 1.86[e] | 1.25 ± 0.53 | 2.91[e] | 0.64 ± 0.08 | 1.36[e] |
| A | 1.77 ± 0.33 | 1.54 | 0.91 ± 0.50 | 2.12[e] | 0.53 ± 0.05 | 1.13 |
| B | 2.04 ± 0.24 | 1.77[d] | 1.30 ± 0.25 | 3.02[d] | 0.64 ± 0.10 | 1.36[e] |
| C | 2.24 ± 0.58 | 1.95[e] | 1.70 ± 0.77 | 3.95[e] | 0.59 ± 0.01 | 1.26[e] |
| D | 1.29 ± 0.18 | 1.12 | 1.09 ± 0.06 | 2.53[d] | 0.58 ± 0.08 | 1.23[e] |
| E | 1.46 ± 0.29 | 1.27 | 1.26 ± 0.50 | 2.93[e] | 0.63 ± 0.09 | 1.34[e] |
| F | 2.24 ± 0.29 | 1.95[d] | 1.90 ± 0.56 | 4.42[d] | 0.58 ± 0.03 | 1.23[e] |
| G | 1.70 ± 0.37 | 1.48 | 1.57 ± 0.66 | 3.65[e] | 0.59 ± 0.08 | 1.26[e] |

[a]The dosage for all fractions was 20 mg in 0.3 ml of cottonseed oil given once every 2 days for a total of three doses. The control mice were given 0.3 ml of cottonseed oil only.

[b]μmol/min/mg of protein.

[c]Test/Control.

[d]$p<0.005$. All $p$ values were obtained by Student's $t$-test (n = 4).

[e]$p<0.05$.

with those of α-humulene derivatives such as 15-$CH_2OAc$ or 15-$CO_2H$ (19,20). The location of the epoxide group in **4** was determined by the analysis of its proton spectrum. Comparison of $^1$H-nmr spectra of **3** and **4** indicated that the 8,9 double bond of **3** was oxidized because the signal of H-9 at δ 4.85 (t, 1H, $J = 7.5$) disappeared in the spectrum of **4**, while the signals responsible for the doubly allylic methylene ($CH_2$-3) at δ 2.49 and the trans-coupled CH=CH at δ 5.13 and 5.57 remained intact. Finally, the structural assignment of **4** was confirmed by comparison with the standard spectra of α-humulene epoxide I published in the literature (21). Eugenol [**5**] was identified by comparison of its $^1$H- and $^{13}$C-nmr spectra with the standard spectra (22). These compounds have been found in clove oil (15,23). Biosynthetically, β-caryophyllene and α-humulene can be derived from the same precursor (24).

The GST-inducing activity of compounds **1–5** was determined in the liver, forestomach, and small bowel mucosa of A/J mice (Table 2). At the 20 mg dose, sesquiterpenes **1–4** increased GST activity at least 2 times more than the control in the liver and 2.5–4.8 times over the control in the small intestinal mucosa. No significant increase of GST activity in the forestomach was observed with these compounds. Eugenol [**5**] induced GST enzyme activity in the liver by 26% higher than the control. It also increased GST activity significantly in the small bowel.

The present results indicated that essential oils can be a potential source for the discovery of natural anticarcinogenic agents and that these agents may be detected and evaluated by using the GST enzyme assay. For instance, eugenol has been reported to inhibit mutagenicity induced by benzo[*a*]pyrene and 2-acetylaminofluorene (25). Commercial uses of essential oils include food flavorings, cosmetics, and detergents; many are being used by humans on a daily basis (26,27). Thus, pure anticarcinogenic compounds isolated from the same sources may be readily acceptable as diet supplements if the toxicity and side effects are minimal.

## EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Low and high resolution mass spectra were obtained on Kratos MS-25 and VG 7070E-HF mass spectrometers, respectively. $^1$H- and $^{13}$C-nmr spectra were recorded on a Nicolet NT-300 nmr spectrometer. TMS was used as the internal reference (δ 0.00) for $^1$H-nmr spectra measured in

TABLE 2. The Effects of Clove Terpene Compounds on the Activity of Glutathione *S*-Transferase in Target Tissues of A/J Mice.

| Compound[a] | Liver | | Small Bowel Mucosa | | Forestomach | |
|---|---|---|---|---|---|---|
| | GST Sp. Act.[b] | Ratio[c] | GST Sp. Act.[b] | Ratio[c] | GST Sp. Act.[b] | Ratio[c] |
| Control | 0.74 ± 0.18 | | 0.25 ± 0.12 | | 0.62 ± 0.12 | |
| **1** | 1.92 ± 0.47 | 2.59[d] | 0.75 ± 0.18 | 3.00[d] | 0.77 ± 0.11 | 1.24 |
| **2** | 2.13 ± 0.58 | 2.88[d] | 1.20 ± 0.70 | 4.80[e] | 0.59 ± 0.10 | 0.95 |
| **3** | 1.47 ± 0.70 | 1.99 | 0.63 ± 0.13 | 2.52[d] | 0.63 ± 0.04 | 1.02 |
| **4** | 1.54 ± 0.27 | 2.08[d] | 0.81 ± 0.14 | 3.24[d] | 0.77 ± 0.10 | 1.24 |
| **5** | 0.93 ± 0.02 | 1.26 | 0.59 ± 0.18 | 2.36 | 0.75 ± 0.09 | 1.21 |

[a]The dosage for all compounds was 20 mg in 0.3 ml of cottonseed oil given once every 2 days for a total of three doses. The control mice were given 0.3 ml of cottonseed oil only.

[b]μmol/min/mg of protein.

[c]Test/Control.

[d]$p<0.025$. All $p$ values were obtained by Student's $t$-test (n = 3 for compound **1** and **4**, n = 4 for compounds **2**, **3**, and **5**.

[e]$p<0.05$.

$CDCl_3$. The solvent $CDCl_3$ was used as the internal reference (δ 77.00) for $^{13}C$-nmr spectra. Preparative liquid chromatography was performed on the Waters PrepLC/system 500A instrument equipped with two connected PrepPak-500/Silica cartridges purchased from Millipore Waters Associates (Milford, MA). Clove terpenes from *E. caryophyllata* were obtained from Berje Chemical Co. (Bloomfield, NJ). Glutathione was purchased from Sigma Chemical Co. (St. Louis, MO). 1-Chloro-2,4-dinitrobenzene (CDNB) was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of reagent grade or purer and purchased from Aldrich unless noted otherwise.

SEPARATION AND ISOLATION.—A 10-g portion of clove terpenes was subjected to liquid chromatographic separation on two connected Si gel cartridges using 1% (8 liters) and 5% (5 liters) EtOAc in hexane as the solvent system. Fractions were collected and analyzed by Si gel tlc. Fractions with the same $R_f$ values were combined to yield 7 major fractions (A–G). Fractions B and C, which showed higher GST-inducing activity (Table 1), were combined and purified by repeated chromatography on Si gel using 1% and 3% EtOAc in hexane as the solvent system to afford compounds **1** (4.95 g, 49.5% yield), **3** (0.30 g, 3.0%), **2** (0.45 g, 4.5%), and **4** (0.26 g, 2.6%), which exhibited decreased $R_f$ values in the Si gel tlc analysis [hexane-EtOAc (98:2)]. Fractions E, F, and G also exhibited high GST-inducing activity (Table 1) and were combined based on tlc analysis. The combined fraction was chromatographed on a Si gel column eluted with 2%, 3%, and 5% EtOAc in hexane. The first fraction afforded compound **5** (0.61 g, 6.1%) upon removal of the solvent.

β-*Caryophyllene* [**1**].—Colorless oil: hreims *m/z* $[M]^+$ 204.1873 (calcd for $C_{15}H_{24}$ 204.1878). Eims, $^1H$-nmr, and $^{13}C$-nmr data compared with literature values (17,18).

β-*Caryophyllene oxide* [**2**].—Colorless oil; hreims *m/z* $[M]^+$ 220.1815 (calcd for $C_{15}H_{24}O$ 220.1827). Eims, $^1H$-nmr, and $^{13}C$-nmr data compared with literature values (16,18).

α-*Humulene* [**3**].—Colorless oil; hreims *m/z* $[M]^+$ 204.1877 (calcd for $C_{15}H_{24}$ 204.1878). Eims, $^1H$-nmr, and $^{13}C$-nmr data compared with literature values (19,20).

α-*Humulene epoxide I* [**4**].—Colorless oil; hreims *m/z* $[M]^+$ 220.1812 (calcd for $C_{15}H_{24}O$ 220.1827). Eims, $^1H$-nmr, and $^{13}C$-nmr data compared with literature values (20,21).

*Eugenol* [**5**].—Colorless oil; hreims *m/z* $[M]^+$ 164.0844 (calcd for $C_{10}H_{12}O_2$ 164.0844). Eims, $^1H$-nmr, and $^{13}C$-nmr data compared with literature values (22).

GLUTATHIONE *S*-TRANSFERASE ASSAY.—Female A/J mice, six weeks of age, were obtained from the NIH colony at Harlan Sprague Dawley Co. (Indianapolis, IN). Animals were housed in termperature-controlled animal quarters with a 12/12 h light/dark cycle. $H_2O$ was given ad libitum. They were acclimated for 1 week after arrival before they were fed semipurified diet (ICN Nutritional Biochemicals, Cleveland, OH) until the end of the experiment. One week after the start of the semipurified diet they were divided into control and experimental groups, with four mice per group. The experimental groups were given by gavage 20 mg per dose of the test compounds, suspended in 0.3 ml of cottonseed oil, once every 2 days for a total of three doses. The control groups were given cottonseed oil alone. Twenty-four hours after the last administration, the mice were killed by cervical dislocation. The liver, forestomach, and mucosa of small intestine were removed for enzyme preparation. The tissues were homogenized in cold 1.15% KCl solution (pH 7.4) by means of a Brinkman homogenizer. The homogenates were centrifuged at 9000 *g* for 20 min, and the supernatants were centrifuged at 100,000 *g* for 1 h. The cytosolic fractions were kept frozen at −80° until use. Each sample represented one tissue from each individual animal.

The activity of cytosolic GST was determined according to the method of Habig *et al.* (2) using 1-chloro-2,4-dinitrobenzene as the substrate. Assays were performed at 30° in 0.1 M phosphate buffer (pH 6.5), in the presence of 5 mM GSH, 1 mM CDNG, and 20 μl of the cytosol. The reaction was monitored at 340 nm on a Beckman DU-65 spectrophotometer equipped with a temperature-controlled cell compartment. Complete assay mixture without the cytosolic enzyme was used as the control. The protein concentrations of these samples were determined by the method of Lowry *et al.* (28). Data were analyzed by Student's *t*-test, and *P* values were obtained in comparison to the control.

## ACKNOWLEDGMENTS

We thank Thomas P. Krick, College of Biological Science, University of Minnesota, for his assistance in obtaining spectra. This research was supported by the National Cancer Institute (Grant USPHS CA 47720).

## LITERATURE CITED

1. L.W. Wattenberg, *Cancer Res.*, **45**, 1 (1985).
2. W.H. Habig, M.J. Pabst, and W.B. Jakoby, *J. Biol. Chem.*, **249**, 7130 (1974).
3. W.B. Jakoby and W.H. Habig, in: "Enzymatic Basis of Detoxification." Ed. by

W.B. Jakoby, Academic Press, New York, 1980, Vol. 2, pp. 63–94.
4. L.F. Chasseaud, *Adv. Cancer Res.*, **29**, 175 (1979).
5. D.M. Shankel, P.E. Hartman, T. Koda, and A. Hellaender, "Antimutagenesis and Anticarcinogenesis Mechanisms," Plenum Press, New York, 1986.
6. W.B. Jackoby, *Adv. Enzymol.*, **46**, 381 (1977).
7. V.L. Sparnins, P.L. Venegas, and L.W. Wattenberg, *J. Natl. Cancer Inst.*, **68**, 493 (1982).
8. A.M. Benson and P.B. Barretto, *Cancer Res.*, **45**, 4219 (1985).
9. L.K.T. Lam and S. Hasegawa, *J. Agric. Food Chem.*, **37**, 878 (1989).
10. L.K.T. Lam and S. Hasegawa, *Nutr. Cancer*, **12**, 43 (1989).
11. V.L. Sparnins and L.W. Wattenberg, *J. Natl. Cancer Inst.*, **66**, 769 (1981).
12. L.W. Wattenberg, V.L. Sparnins, and G. Barany, *Cancer Res.*, **49**, 2689 (1989).
13. E.S. Fiala, B.S. Reddy, and J.H. Weisburger, *Ann. Rev. Nutr.*, **5**, 295 (1985).
14. L.K.T. Lam and B.-L. Zheng, *J. Agric. Food Chem.*, **39**, 660 (1991).
15. V. Formacek and K.-H. Kubeczka, "Essential Oils Analysis by Capillary Gas Chromatography and Carbon-13 NMR Spectroscopy," John Wiley & Sons, New York, 1982, pp. 61–63.
16. A. Groweiss and Y. Kashman, *Tetrahedron*, **39**, 3385 (1983).
17. B. Maurer and A. Hauser, *Helv. Chim. Acta.*, **66**, 2223 (1983).
18. Y. Kashman and A. Groweiss, *J. Org. Chem.*, **45**, 3814 (1980).
19. A.G. Gonzalez, J. Bermejo, J.G. Ciaz, L. Arancibia, and P.P. DePaz, *J. Nat. Prod.*, **51**, 1140 (1988).
20. F. Bohlmann, C. Zdero, H. Robinson, and R.M. King, *Phytochemistry*, **21**, 685 (1982).
21. N.P. Damodaran and S. Dev, *Tetrahedron*, **24**, 4123 (1968).
22. Sadtler Research Laboratories, "Standard Spectra Collection, 1980 Cumulative Alphabetical Index," Philadelphia, 1980; p. 632, Eugenol: $^{1}$H nmr, #10918; $^{13}$C nmr, #584.
23. T. Deyama and T. Horiguchi, *Yakugaku Zasshi*, **91**, 1383 (1971).
24. K.B.G. Torssel, "Natural Product Chemistry, A Mechanistic and Biosynthetic Approach to Secondary Metabolism," John Wiley & Sons, New York, 1983, pp. 186–188.
25. H. Yokota, J. Hoshino, T. Fujita, N. Ogiya, and A. Yuyasa, *Kachiku Seikagaku Kenkyukaiho*, **17**, 1 (1985); *Chem. Abstr.*, **104**, 28544v (1986).
26. U.-J. Salzer, *CRC Crit. Rev. Food Sci. Nutr.*, **9**, 345 (1977).
27. P.E. Shaw, *J. Agric. Food Chem.*, **27**, 246 (1979).
28. O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

*Received 30 December 1991*