Microbial Transformation and Butyrylcholinesterase Inhibitory Activity of (−)-Caryophyllene Oxide and Its Derivatives

Muhammad Iqbal Choudhary,* Zafar A. Siddiqui, Sarfraz A. Nawaz, and Atta-ur-Rahman

H. E. J. Research Institute of Chemistry, International Center for Chemical Sciences, University of Karachi, Karachi-75270, Pakistan

Received April 21, 2006

Microbial transformation of the sesquiterpene (−)-caryophyllene oxide (**1**) [(1*R*,4*R*,5*R*,9*S*)-4,5-epoxycaryophyllan-8(13)-ene] by a number of fungi, using a standard two-stage fermentation technique, has afforded as products (1*R*,4*R*,5*R*,9*S*)-4,5-dihydroxycaryophyllan-8(13)-ene (**2**), (1*S*,4*R*,5*R*,8*S*,9*S*)-clovane-5,9-diol (**3**), (1*R*,4*R*,5*R*,9*S*,11*R*)-4,5-epoxycaryophyllan-8(13)-en-15-ol (**4**), (1*R*,4*R*,5*R*,9*S*,11*S*)-4,5-epoxycaryophyllan-8(13)-en-14-ol (**5**), (1*R*,2*S*,4*R*,5*R*,9*S*)-4,5-epoxy-13-norcaryophyllan-8-one (**6**), (1*R*,4*R*,5*R*,8*S*,9*S*)-4,5-epoxycaryophyllan-13-ol (**7**), (1*R*,4*R*,5*R*,8*S*, 9*S*,13*S*)-caryolane-5,8,13-triol (**8**), (1*R*,3*R*,4*R*,5*R*,8*S*,9*S*)-4,5-epoxycaryophyllan-3,13-diol (**9**), and (1*S*,4*R*,5*R*,8*S*,9*S*)-clovane-5,9,12-triol (**10**). Metabolites **6** and **8**−**10** were found to be new compounds, as deduced on the basis of spectroscopic techniques. Compounds **1**−**10** were evaluated for butyrylcholinesterase inhibitory activity, and compound **5** exhibited an IC_{50} value of 10.9 ± 0.2 μM.

Many types of terpenoids are found in fruits and vegetables and are known to possess interesting biological activities. Sesquiterpenoids display analeptic, anthelmintic, anti-inflammatory, antitumor, hypotensive, and sedative properties.[1] A number of transformations of sesquiterpenoids by microorganisms have been reported, including the biotransformation of ginsenol, a sesquiterpene alcohol obtained from the essential oil of *Panax ginseng*, by *Botrytis cinerea*;[2] the fungal transformation of valerianol, a constituent of valerian oil, by *Mucor plumbeus*;[3] the transformation of β-selinene, obtained from the seed oil of celery (*Apium graveolens*), by the plant-pathogenic fungus *Glomerella cingulata*;[4] the biotransformation of (1*R*)-aromadendrene and allo-aromadendrene by the plant-pathogenic fungus *G. cingulata*;[5] and the biotransformation of germacrones, germacrone-4,5-epoxide and (+)-curdione, which were isolated from *Curcuma aromatica*, by *Aspergillus niger*.[6]

In continuation of our studies on microbial transformation of pharmacologically important terpenes and steroids,[7−11] the sesquiterpene (−)-caryophyllene oxide (**1**), has been investigated, which was originally isolated from a medicinal plant, *Sindora sumatrana* Miq. (Leguminosae).[12] (−)-Caryophyllene oxide (**1**) was also obtained from another medicinal plant, *Lavandula latifolia* Vill. (Labiatae), and exhibited potent anti-inflammatory activity.[13] Compound **1** was incubatd with six fungal cultures to obtain nine metabolites, **2**−**10** (Schemes 1 and 2). Metabolites **2**−**10** were evaluated for butyrylcholinesterase (BuChE) inhibitory activities. Butyrylcholinesterase inhibition is considered to be an indicator for possible use in the treatment of Alzheimer's disease and related dementias.[14] BChE (E.C. 3.1.1.8) is produced in the liver and enriched in the circulation. In addition, it is also present in adipose tissue, intestine, smooth muscle cells, white matter of the brain, and many other tissues.[15]

Results and Discussion

Screening-scale experiments showed that six fungal cultures utilized (see Experimental Section) were capable of converting (−)-caryophyllene oxide (**1**) into more polar metabolites. Preparative-scale fermentation was thus carried out to produce sufficient quantities of new and known metabolites for structure elucidation and enzyme inhibition studies.

Incubation of (−)-caryophyllene oxide (**1**) with *Cephalosporium aphidicola* yielded the metabolites **2** and **3**. Metabolites **2** ($C_{15}H_{26}O_2$) and **3** ($C_{15}H_{26}O_2$) were identified as the known compounds (1*R*,4*R*,5*R*,9*S*)-4,5-dihydroxycaryophyllan-8(13)-ene and (1*S*,4*R*,5*R*,-8*S*,9*S*)-clovane-5,9-diol, respectively (Scheme 1). These compounds were earlier isolated as secondary metabolites from the neutral fraction of the dried pods of the medicinal plant *Sindora sumatrana*.[12] Metabolite **2** was formed by the hydrolysis of epoxide by the enzyme epoxide hydrolase, which is present in eukaryotic cells such as mammals and fungi.[16] When **1** was incubated with *Macrophomina phaseolina*, metabolites **4** and **5** were obtained. Metabolites **4** ($C_{15}H_{24}O_2$) and **5** ($C_{15}H_{24}O_2$) were also identified as known compounds, (1*R*,4*R*,5*R*,9*S*,11*R*)-4,5-epoxycaryophyllan-8(13)-en-15-ol and (1*R*,4*R*,5*R*,9*S*,11*S*)-4,5-epoxycaryophyllan-8(13)-en-14-ol, respectively (Scheme 1). Metabolite **4** was earlier obtained from the biotransformation of **1**,[17,18] whereas metabolite **5** was obtained from the biotransformation of **1** by *Botrytis cinerea*.[17] These known metabolites were identified through comparison with their reported data.[12,17−18]

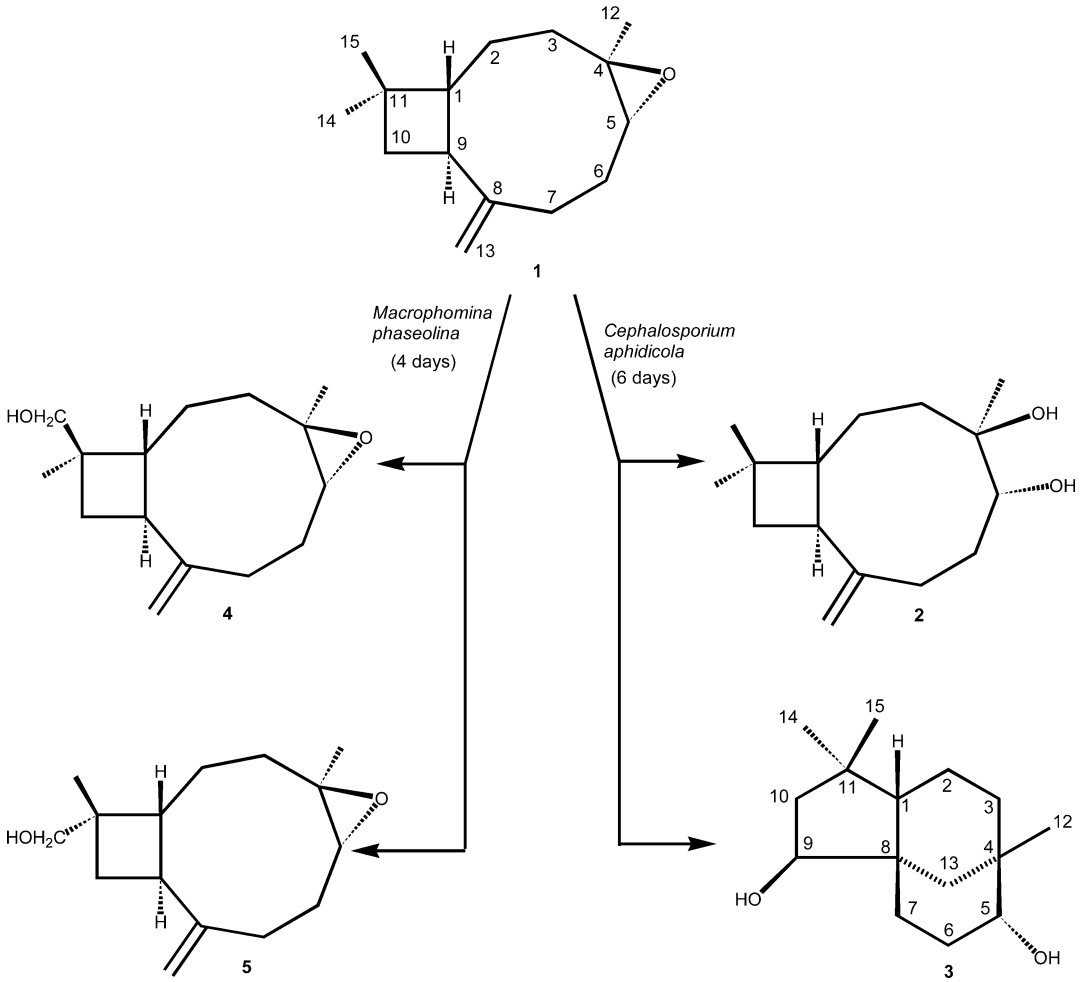
Incubation of (−)-caryophyllene oxide (**1**) with *Rhizopus stolonifer* yielded the new metabolite **6** and the known metabolite **7** (Scheme 2). Metabolite **7** ($C_{15}H_{26}O_2$) was identified as (1*R*,4*R*,5*R*,8*S*,9*S*)-4,5-epoxycaryophyllan-13-ol, which was previously obtained from the biotransformation of caryophyllene oxide (**1**) with *B. cinerea*.[17]

Compound **6** was isolated as a colorless, crystalline solid. The HREIMS exhibited a M^+ peak at *m*/*z* 238.1761, corresponding to the formula $C_{14}H_{22}O_3$ (calcd 238.1776), 18 amu higher than the parent compound, **1**. The IR spectrum showed absorptions at 3408 and 1696 cm^{-1}, indicating the presence of hydroxyl and keto groups, respectively. The absence of any IR absorption band at 1630 cm^{-1} indicated the lack of a double bond. The ^1H NMR spectrum of compound **6** showed an additional downfield signal at δ 3.93 (td, $J_{2\alpha,1\beta;2\alpha,3\beta}$ = 10.9 Hz, $J_{2\alpha,3\alpha}$ = 4.6 Hz), which suggested the presence of a hydroxyl group, as compared to the substrate **1**. The ^{13}C NMR spectrum showed the presence of 14 carbons, including four methines, four methylenes, three methyls, and three quaternary carbons. A new methine carbon appeared at δ 70.1 (C-2). The position of the new hydroxyl group was inferred as C-2 on the basis of COSY 45° interactions between H-2 (δ 3.93) and the C-1 methine proton (δ 2.09) and the C-3 methylene protons (δ 2.47, 1.03), supporting the position of the hydroxyl group at C-2. In the HMBC spectrum, the C-7 methylene protons (δ 2.60, 2.50) and the C-6 methylene protons (δ 1.98) showed interactions with C-8 (δ 213.5). In the NOESY spectrum, H-2α showed interactions with H_3-14α, H_3-12α, and H-9α, indicating the configuration of the C-2 hydroxyl group as β. Similarly, H-1β showed NOESY correlations

* Corresponding author. Tel: 92 21 482 4934. Fax: 92 21 481 9018. E-mail: hej@cyber.net.pk.

10.1021/np0680174 CCC: $33.50
Published on Web 10/03/2006

Scheme 1. Metabolism of (−)-Caryophyllene Oxide (**1**) with *Cephalosporium aphidicola* and *Macrophomina phaseolina*



with H_3-15β and H-5β. This indicated that H-1, H_3-15, and H-5 were all in β configuration (Figure 1). Thus, the structure of the new metabolite **6** was deduced as (1*R*,2*S*,4*R*,5*R*,9*S*)-4,5-epoxy-13-norcaryophyllan-8-one.

Incubation of (−)-caryophyllene oxide (**1**) with *Aspergillus niger*, *Fusarium lini*, and *Gibberella fujikuroi* yielded new metabolites **8**−**10**, respectively (Scheme 2). Compound **8** was isolated as a colorless, crystalline solid. Its HREIMS exhibited a molecular ion at *m*/*z* 254.1631, corresponding to the formula $C_{15}H_{26}O_3$ (calcd 254.1681), 34 amu higher than the parent compound, **1**. The IR spectrum exhibited an absorption at 3377 cm^{-1} for hydroxyl groups. The absence of any IR absorption at 1630 cm^{-1}, as compared to substrate **1**, indicated the absence of a double bond. The ^{1}H NMR spectrum of compound **8** was nearly identical to that of a reported compound, caryolane-1,9α-diol,[12] except in the appearance of an additional downfield signal at δ 3.35 (br s). This indicated the presence of an additional hydroxyl group in compound **8**. The ^{13}C NMR spectrum showed the presence of 15 carbons, including four methines, five methylenes, three methyls, and three quaternary carbons. Resonances for two new methine carbons appeared at δ 76.0 (C-5) and 72.9 (C-13). The position of the new hydroxyl group was inferred as being attached to C-5 on the basis of COSY 45° interactions between H-5 (δ 3.44) and the C-6 methylene protons (δ 1.90). The HMBC spectrum showed interactions of H-5 (δ 3.44) with C-6 (δ 29.3), C-8 (δ 70.7), and C-12 (δ 27.0), further supporting the position of the hydroxyl group at C-5. The position of the second hydroxyl group was placed at C-13 on the basis of HMBC interactions of H-13 (δ 3.35) with C-4 (δ 38.0), C-9 (δ 37.8), and C-3 (δ 29.8). A detailed analysis of the 1D and 2DNMR spectra was used to establish the structure of metabolite **8**. In the NOESY spectrum, H-13α showed correlations with H-9α and H_3-14α, indicating the β-configuration of the C-13 hydroxyl group (axial), whereas H-5β (axial) showed NOE interactions with H_3-12β (equatorial), indicating the configuration of the C-5 hydroxyl group as α (equatorial) (Figure 1). The coupling constants of the C-5 proton signal at δ 3.44 (dd, $J_{5ax,\ 6ax}$ = 11.4 Hz, $J_{5ax,\ 6eq}$ 4.9 Hz) also supported a chair conformation of ring A and equatorial orientations of geminal OH. Thus, the structure of new metabolite **8** was deduced as (1*R*,4*R*,5*R*,8*S*,9*S*,13*S*)-caryolane-5,8,13-triol.

Compound **9** was isolated as a colorless, crystalline solid. The HREIMS of compound **9** showed a M^+ peak at *m*/*z* 254.1321 ($C_{15}H_{26}O_3$, calcd 254.1637). The IR spectrum of **9** did not display any olefinic absorptions, but showed an additional absorption of a hydroxyl functionality at 3743 cm^{-1}, in comparison with substrate **1**. The ^{1}H NMR spectrum of compound **9** showed the disappearance of the C-13 exomethylene olefinic signals, but with the appearance of an additional oxygen-bearing methylene proton signal at δ 3.30 (m). This indicated an *anti*-Markonikov hydration of the C-8/C-13 bond. The ^{13}C NMR spectrum of **9** showed the disappearance of the signals for exocyclic double-bond carbons. Two new carbon signals at δ 49.0 (C-8) and 70.3 (C-13) further indicated the hydration of this double bond. The position of the new hydroxyl group was inferred as C-13 on the basis of COSY 45° interactions between H-13 (δ 3.30) and the C-8 methine proton (δ 1.29). In the HMBC spectrum, H_2-13 showed heteronuclear interactions with C-7 (δ 27.2). The position of the second hydroxyl group was inferred as C-3 on the basis of COSY 45° interactions between H-3 (δ 3.55) and the C-2 methylene protons (δ 1.68). In the NOESY spectrum, H_2-13 showed correlations with H-9α and H_3-12α, suggesting an α-orientation of the C-13 hydroxymethylene group, while H-3 showed NOESY correlations with H-9α and H_3-12α, which indicated a β-orientation of the C-3 OH (Figure 1). Thus,

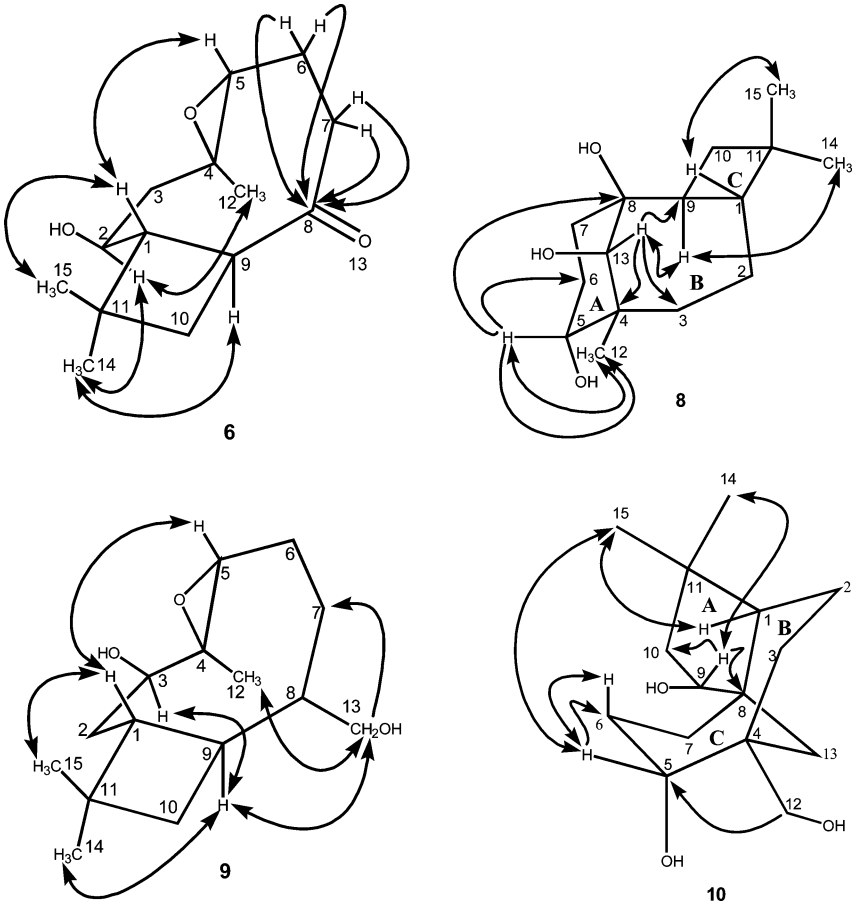
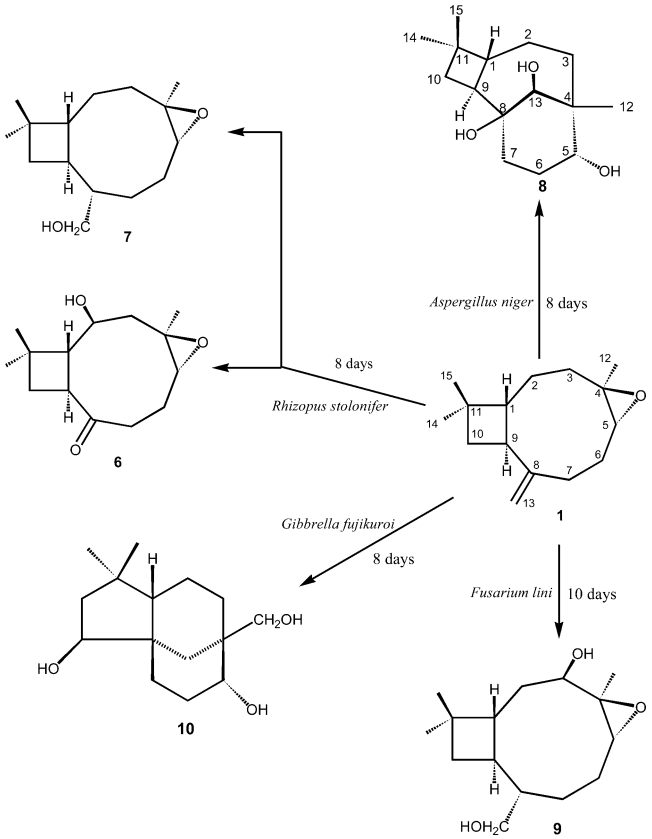


Figure 1. Key NOESY correlations (↔) and HMBC (→) correlations in compounds **6** and **8**−**10**.

Scheme 2. Metabolism of (−)-Caryophyllene Oxide (**1**) with *Rhizopus stolonifer*, *Aspergillus niger*, *Fusarium lini*, and *Gibbrella fujikuroi*



the structure of compound **9** was deduced as (1*R*,3*R*,4*R*,5*R*,8*S*,9*S*)-4,5-epoxycaryophyllan-3,13-diol.

Compound **10** was isolated as a colorless, crystalline solid. The HREIMS exhibited a molecular ion peak at *m*/*z* 254.2530, corresponding to the formula $C_{15}H_{26}O_3$ (calcd 254.2673), 34 amu higher than the parent compound, **1**. The IR spectrum exhibited an absorption at 3633 cm^{-1}, indicating the presence of hydroxyl groups. The ^1H NMR spectrum of compound **10** was nearly identical to that of compound **3**, except in the disappearance of a methyl signal and appearance of additional downfield AB doublets at δ 3.27 (J = 10.0 Hz, 1H) and 3.31 (J = 10.0 Hz, 1H), which indicated that the C-12 methyl group was selectively hydroxylated into a hydroxymethylene group. The disappearance of the H_2-13 signals was consistent with the absence of a double bond, and the appearance of additional downfield signals at δ 3.38 (br s) and 3.62 (t, $J_{5\beta,6\alpha/6\beta}$ = 5.0 Hz) indicated the presence of hydroxyl groups at C-9 and C-5, respectively, as compared to substrate **1**. The ^{13}C NMR spectrum showed the presence of 15 carbons including three methines, seven methylenes, two methyls, and three quaternary carbons. Two new methine carbons appeared at δ 72.0 (C-9) and 80.5 (C-5). One new hydroxymethylene carbon occurred at δ 71.5 (C-12). The position of the new hydroxyl group was inferred as C-5 on the basis of COSY 45° interactions between H-5 (δ 3.62) and the C-6 methylene protons (δ 1.67, 1.88). The HMBC spectrum showed interactions of H-5 (δ 3.62) with C-6 (δ 43.6), supporting the position of a hydroxyl group at C-5. The second hydroxyl group was placed at C-9 on the basis of COSY 45° interactions between H-9 (δ 3.38) and the C-10 methylene protons (δ 1.69, 2.0), as well as HMBC interactions of H-9 (δ 3.38) with C-8 (δ 39.5) and C-10 (δ 45.0). The position of the third hydroxyl group was disclosed as C-12 from the HMBC spectrum, in which H_2-12 showed interactions with C-5 (δ 80.5). In the NOESY spectrum, H-5β showed correlations with H_3-15β and with a methylene proton H-6β

Table 1. ^{1}H NMR Data for Compounds **1**, **6**, and **8**−**10** (400 MHz; $CDCl_3$)

position	1	6	8	9	10
1	1.74, t (10)	2.09, t (9.5)	1.48, m	2.10, m	1.67, m
2	1.42, m; 1.65, m	3.93, td (10.9, 4.6)	1.33, m; 1.51, m	1.68, m; 1.23, m	1.03, m; 0.98, m
3	2.09, m; 0.93, m	2.47, m; 1.03, m	1.82, m; 1.30, m	3.55, dd (10.2, 3.0)	0.80, m; 0.79, m
5	2.85, dd (10.6, 4.2)	2.91, dd (10.9, 3.5)	3.44, dd (11.4, 4.9)	3.01, dd (9.3, 4.8)	3.62, t (5.0)
6	2.23, m 1.32, m	1.98, m; 1.69, m	1.90, m; 1.35, m	2.23, m, 1.65, m	1.66, m, 1.89, m
7	2.32, ddd (12.6, 8.0, 4.3); 2.11, m	2.50, m; 2.60, m	1.61, m; 2.52, m	1.67, m; 1.23, m	0.98, m; 0.92, m
8				1.29, m	
9	2.59, q (7.4)	3.10, q (7.6)	2.24, td (10.5, 4.0)	1.63, m	3.38, brs
10	1.69, m; 1.62, m	1.35, m	1.65, m; 1.60, m	1.70, m; 1.25, m	1.69, m; 2.0, m
12	1.18, s	1.34, s	1.17, s	0.91, s	3.27, d (10.0); 3.31, d (10.0)
13	4.95, brs; 4.83, brs		3.35, brs	3.30, m	1.23 m
14	0.98, s	1.25, s	1.04, s	1.26, s	0.93, s
15	0.96, s	1.16, s	1.02, s	0.92, s	0.95, s

Table 2. ^{13}C NMR Data of Compounds **1**, **6**, and **8**−**10** (100 MHz, $CDCl_3$)

carbon	1	6	8	9	10
1	50.8	54.9	42.6	50.1	43.6
2	27.2	71.0	30.5[a]	40.0[b]	20.8
3	39.2[a]	49.0	29.8[a]	65.5[a]	26.0
4	59.8	56.5	38.0	62.0	63.5
5	63.7	61.9	76.0	65.0[a]	80.5
6	30.3[b]	36.5	29.3	28.5	43.6
7	29.8[b]	38.5	36.5	27.2	22.1
8	151.8	213.5	70.7	49.0	36.5
9	48.7	47.0	37.8	34.0	72.0
10	39.8[a]	31.0	35.0	40.0[b]	45.0
11	34.1	35.5	34.0	34.5	35.5
12	17.0	17.5	27.0	22.0	71.5
13	112.8	−	72.9	70.5	29.8
14	21.7	23.0	16.8	29.0	35.2
15	29.9	31.5	26.0	30.5	28.5

[a,b] Assignments may be interchanged.

Table 3. In Vitro Quantitative Inhibition of BChE by Compounds **1**−**10**

compound	$IC_{50} \pm$ SEM [μM][a]
1	208.4 ± 0.8
2	44.01 ± 0.2
3	455.8 ± 0.1
4	189.5 ± 0.2
5	10.9 ± 0.2
6	458.7 ± 0.5
7	26.37 ± 0.4
8	23.6 ± 0.1
9	154.6 ± 0.3
10	43.63 ± 0.3
Galanthamine HBr[b]	8.5 ± 0.01

[a] Standard error of the mean of five assays. [b] Standard inhibitor of butyrylcholinesterase.

(δ 1.89), suggesting an α-orientation (axial) of the C-5 hydroxyl group, while H-9α showed correlations with H_3-14α, which indicated a β-orientation of the C-9 hydroxyl group (Figure 1). The coupling constant of the C-5 hydroxymethine proton at δ 3.62 (t, J = 5.0 Hz) also supported a chair conformation of ring C and axial orientation of geminal OH. In ring B of the molecule, the boat conformation may be preferred to the chair form in terms of stability. In the chair conformation, the C-2 and C-6 protons may lead to instability, as reported by Aebi et al.[19] Thus, the structure of the new metabolite **10** was deduced as (1*S*,4*R*,5*R*,8*S*,9*S*)-clovane-5,9,12-triol.

Compounds **1**−**10** were screened for butyrylcholinesterase inhibition activity. Their IC_{50} values are shown in Table 3, along with the positive control (galanthamine HBr). Compounds **2**, **5**, and **7**−**10** showed a stronger inhibitory activity against the butyrylcholinesterase enzyme than compound **1**, and compound **5** was found to exhibit potency similar to galanthamine HBr (IC_{50} 10.9 vs 8.5 μM). Structural changes in the transformed products were found to play a role in terms of butyrylcholinesterase enzyme inhibition. Opening of the epoxide ring of compound **1** into a *trans*-diol increased the inhibitory potential (compound **2**; IC_{50} = 44.01 ± 0.2 μM). Hydrogen bonding of the hydroxyl groups with amino acid residues in the active site of BChE is a potentiating factor for the enzyme inhibitor complex and resulted in the higher inhibitory activity of compound **2**. Cyclization of compound **1** into the clovane-type product **3** reduced activity (compound **3**; IC_{50} = 455.8 ± 0.1 μM). Hydroxylation at H_3-15β increased the BChE inhibitory potential (compound **4**; IC_{50} = 189.5 ± 0.2 μM), whereas hydroxylation at H_3-14α increased the activity many-fold because enzymes are stereospecific and hydroxylation at C-14α was assumed to be favorable in the effective accommodation of compound **5** (IC_{50} = 10.9 ± 0.2 μM) at the active site of BChE. Activity was reduced when there was no opening of the epoxide, as in the case of compound **6** (IC_{50} = 458.7 ± 0.5 μM). The presence of an epoxide seems to increase the hindrance in the access of hydroxyl and keto moieties of compounds in the formation of hydrogen bonds.

Addition of a water molecule to a double bond resulted in the formation of compound **7**. In compound **7**, the hydroxyl group at C-13 seems to increase activity (IC_{50} = 26.37 ± 0.4 μM). For the caryolane skeleton represented by compound **8**, hydroxyl groups are more available for hydrogen bonding, resulting in the increase of activity (IC_{50} = 23.6 ± 0.1 μM). Hydroxylation at C-3 in compound **9** further decreased activity, whereas in compound **10**, a hydroxyl group at C-12 is more suitable for hydrogen bonding, resulting in an increase of activity (IC_{50} = 43.63 ± 0.3 μM).

An effort was also made to speculate on the possible pathways by which various bioconversions may have occurred (Supporting Information). Transformation of (−)-caryophyllene oxide (**1**) into **3** may involve a transannular cyclization, which could lead to a tricyclic intermediate **11**, followed by bond migration leading to the formation of the clovane-type compound **3** (Scheme 3 in the Supporting Information).[12,18] Formation of the new clovane derivative **10** may occur through a double-bond formation between C-4/C-12, leading to the intermediate compound **12**, reported earlier by Collado et al.[20] Epoxidation at C-4/C-12 of compound **12** may lead to the metabolite **10** (Scheme 4 in the Supporting Information). In turn, formation of the new metabolite **8** may have occurred by the conversion of **1** into a C-8/C-13 diol via epoxidation, which on rearrangement may yield metabolite **8**. Intermediate **16** may be the precursor of a biotransformed product, as reported by Abraham et al.[18] (Scheme 5 in the Supporting Information). Finally, formation of the norsesquiterpenoid **6** may be through a diol formation at C-8/C-13, followed by oxidative cleavage of the diol, which was also reported by Abraham et al.[18] in the formation of kobusone. This may lead to the formation of metabolite **6** (Scheme 6 in the Supporting Information).

Experimental Section

General Experimental Procedures. (−)-Caryophyllene oxide (**1**) was purchased from Fluka. Mp: Buchi-535 melting-point apparatus. Optical rotations: MeOH solution, JASCO DIP-360 digital polarimeter. IR spectra: $CHCl_3$ solution, FTIR-8900 spectrophotometer, in cm^{-1}. ^1H and ^{13}C NMR spectra: $CDCl_3$ solution, Bruker Avance 400-NMR at 400 and 100 MHz, respectively, 2D experiments with $CDCl_3$ solution, and the same instrument; chemical shifts (δ) in ppm relative to TMS as an internal standard, coupling constants *J* in Hz. Mass spectra were recorded on a JEOL JMS-600H mass spectrometer, in *m*/*z* (rel %). TLC: silica gel precoated plates (Merck, PF_{254}; 20 × 20, 0.25 mm). Column chromatography: silica gel (70−230 mesh, Merck). The compounds were detected on TLC using vanillin spray reagent.

Organisms and Culture Media. Fungal cultures of *Cephalosporium aphidicola* (IMI 68689), *Macrophomina phaseolina* (KUCC 730), *Fusarium lini* (NRRL 68751), *Aspergillus niger* (ATCC 10549), *Rhizopus stolonifer* (ATCC 10404), and *Gibberella fujikuroi* (ATCC 10704) were grown on Sabouraud 4% glucose agar (Merck) at 25 °C and stored at 4 °C. The medium for *C. aphidicola* (IMI 68689) was prepared by mixing the following ingredients into distilled H_2O (1.0 L): glucose (50 g), KH_2PO_4 (1 g), KCl (1 g), $MgSO_4{\cdot}7H_2O$ (2 g), glycine (2 g), and *Gibberella* trace-element solution (2 mL). The latter was prepared by mixing $Co(NO_3)_2{\cdot}6H_2O$ (0.01 g), $FeSO_4{\cdot}7H_2O$ (0.1 g), $CuSO_4{\cdot}5H_2O$ (0.1 g), $ZnSO_4{\cdot}7H_2O$ (0.161 g), $MnSO_4{\cdot}4H_2O$ (0.01 g), and ammonium molybdate (0.01 g) in distilled H_2O (100 mL). The media for *M. phaseolina* and *F. lini* were prepared by adding the following chemicals into distilled H_2O (1.0 L): glucose (10.0 g), glycerol (10.0 g), peptone (5.0 g), yeast extract (5.0 g), KH_2PO_4 (5.0 g), and NaCl (5.0 g). The medium for *A. niger* was prepared by mixing the following ingredients into distilled H_2O (1.0 L): glucose (10.0 g), peptone (5.0 g), KH_2PO_4 (5.0 g), yeast extract (5.0 g), glycerol (5 mL), and NaCl (5.0 g), while the medium for *R. stolonifer* (1.0 L, distilled H_2O) included glucose (20.0 g), glycerol (5 mL), peptone (10.0 g), yeast extract (3.0 g), and KH_2PO_4 (5.0 g); the pH of the solution was adjusted to 5.6 by adding a few drops of 0.04 N NaOH. The medium for *G. fujikuroi* was prepared by mixing the following ingredients into distilled H_2O (1.0 L): glucose (80.0 g), KH_2PO_4 (5.0 g), $MgSO_4{\cdot}2H_2O$ (1.0 g), NH_4NO_3 (0.5 g), and *Gibberella* trace-element solution (2 mL).

General Fermentation and Extraction Conditions. The fermentation medium thus obtained was distributed among 30 flasks of 250 mL capacity (100 mL in each) and autoclaved at 121 °C. The fermentation was carried out according to a standard two-stage protocol.[21] The substrate was dissolved in acetone, and the resulting clear solution was evenly distributed among 30 flasks (20 mg/0.5 mL in each flask), containing 24-h-old stage II cultures, and fermentation was carried out for further days on a rotatory shaker (200 rpm) at 29 °C. During the fermentation period, aliquots from the culture were taken daily and analyzed by TLC in order to determine the degree of transformation of substrate. In all experiments, one control flask without fungus (for checking the substrate stability) and another flask without exogenous substrate (for checking the presence of endogenous metabolites) were used. The culture media and mycelium were separated by filtration. The mycelium was washed with CH_2Cl_2 (1.5 L), and the filtrate was extracted with CH_2Cl_2 (3 × 2 L). The combined organic extract was dried over anhydrous Na_2SO_4, evaporated under reduced pressure, and analyzed by thin-layer chromatography. Control flasks were also harvested and compared with the test by TLC to confirm the presence of biotransformed products.

Fermentation of (−)-Caryophyllene Oxide (1) with *Cephalosporium aphidicola*. (−)-Caryophyllene oxide (**1**) (600 mg), dissolved in 15 mL of acetone, was evenly distributed in 30 flasks containing 24-h-old stage II culture. Fermentation was carried out for 6 days. After filtration, extraction, and evaporation, a brown gum (1.63 gm) was obtained, which on repeated column chromatography (petroleum ether/AcOEt gradient) yielded compounds **2** (16.4 mg; 2.7% yield; with petroleum ether/AcOEt, 72:28) and **3** (8.2 mg; 1.3% yield; with petroleum ether/AcOEt, 68:32).

Fermentation of (−)-Caryophyllene Oxide (1) with *Macrophomina phaseolina*. (−)-Caryophyllene oxide (**1**) (600 mg), dissolved in 15 mL of acetone, was evenly distributed in 30 flasks containing 24-h-old stage II culture. Fermentation was carried out for 4 days. After filtration, extraction, and evaporation, a brown gum (1.8 g) was obtained, which on repeated column chromatography (petroleum ether/AcOEt gradient) yielded compounds **4** (19.1 mg; 3.2% yield; with petroleum ether/AcOEt, 85:15) and **5** (28.0 mg; 4.6% yield; with petroleum ether/AcOEt, 82:18).

Fermentation of (−)-Caryophyllene Oxide (1) with *Rhizopus stolonifer*. (−)-Caryophyllene oxide (**1**) (600 mg), dissolved in 15 mL of acetone, was evenly distributed in 30 flasks containing 24-h-old stage II culture. Fermentation was carried out for 8 days. After filtration, extraction, and evaporation, a brown gum (1.2 g) was obtained, which on repeated column chromatgraphy (petroleum ether/AcOEt gradient) yielded compounds **6** (15.1 mg; 2.5% yield; with petroleum ether/AcOEt, 88:12) and **7** (17.0 mg; 2.8% yield; with petroleum ether/AcOEt, 84:16).

(1*R*,2*S*,4*R*,5*R*,9*S*)-4,5-Epoxy-13-norcaryophyllan-8-one (6): colorless, crystalline solid; mp 62−63 °C; $[\alpha]^{25}_D$ −10 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ∈) 205 (2.1) nm; IR ($CHCl_3$) ν_{max} 3408, 1696, 2922, 2853 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz), Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz), Table 2; EIMS *m*/*z* 238 $[M]^+$ (24), 220 (66), 205 (26), 182 (60), 164 (100); HREIMS *m*/*z* 238.1761 (calcd for $C_{14}H_{22}O_3$, 238.1776).

Fermentation of (−)-Caryophyllene Oxide (1) with *Aspergillus niger*. (−)-Caryophyllene oxide (**1**) (600 mg), dissolved in 15 mL of acetone, was evenly distributed in 30 flasks containing 24-h-old stage II culture. Fermentation was carried out for 8 days. After filtration, extraction, and evaporation, a brown gum (1.4 g) was obtained, which on repeated column chromatography (petroleum ether/AcOEt gradient) yielded compound **8** (14.0 mg; 2.3% yield; with petroleum ether/AcOEt, 55:45).

(1*R*,4*R*,5*R*,8*S*,9*S*,13*S*)-Caryolane-5,8,13-triol (8): colorless, crystalline solid; mp 68−70 °C; $[\alpha]^{25}_D$ −71 (*c* 0.6, MeOH); UV (MeOH) λ_{max} (log ∈) 206 (2.0) nm; IR ($CHCl_3$) ν_{max} 3377, 2965, 2932, 2864 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz), Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz), Table 2; EIMS *m*/*z* 254 $[M^+]$ (12), 236 (14), 220 (26), 186 (60), 160 (100); HREIMS *m*/*z* 254.1631 (calcd for $C_{15}H_{26}O_3$, 254.1681).

Fermentation of (−)-Caryophyllene Oxide (1) with *Fusarium lini*. (−)-Caryophyllene oxide (**1**) (600 mg), dissolved in 15 mL of acetone, was evenly distributed in 30 flasks containing 24-h-old stage II culture. Fermentation was carried out for 10 days. After filtration, extraction, and evaporation, a brown gum (1.8 g) was obtained, which on repeated column chromatography (petroleum ether/ AcOEt gradient) yielded compound **9** (13.0 mg; 2.1% yield; with petroleum ether/AcOEt, 60:40).

(1*R*,3*R*,4*R*,5*R*,8*S*,9*S*)-4,5-Epoxycaryophyllan-3,13-diol (9): colorless, crystalline solid; mp 67−70 °C; $[\alpha]^{25}_D$ −67 (*c* 0.6, MeOH); UV (MeOH) λ_{max} (log ∈) 205 (2.3) nm; IR ($CHCl_3$) ν_{max} 3743, 2926, 2859, 2864 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz), Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz), Table 2; EIMS *m*/*z* 254 [M+] (8), 236 (12), 220 (20), 166 (30), 140 (100); HREIMS *m*/*z* 254.1321 (calcd for $C_{15}H_{26}O_3$, 254.1637).

Fermentation of (−)-Caryophyllene Oxide (1) with *Gibberella fujikuroi* (ATCC 10704). (−)-Caryophyllene oxide (**1**) (600 mg), dissolved in 15 mL of acetone, was evenly distributed in 30 flasks containing 24-h-old stage II culture. Fermentation was carried out for 8 days. After filtration, extraction, and evaporation, a brown gum (1.4 g) was obtained, which on repeated column chromatography (petroleum ether/AcOEt gradient) yielded compound **10** (13.0 mg; 2.1% yield; with petroleum ether/AcOEt, 54:46).

(1*S*,4*R*,5*R*,8*S*,9*S*)-Clovane-5,9,12-triol (10): colorless, crystalline solid; mp 70−73 °C; $[\alpha]^{25}_D$ −54 (*c* 0.6, MeOH); UV (MeOH) λ_{max} (log ∈) 205 (2.1) nm; IR ($CHCl_3$) ν_{max} 3633, 2921, 2854, 2842 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz), Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz), Table 2; EIMS *m*/*z* 254 [M+] (16), 236 (10), 220 (25), 168 (30), 140 (100); HREIMS *m*/*z* 254.2530 (calcd for $C_{15}H_{26}O_3$, 254.2673).

In Vitro Cholinesterase Inhibition Assay. Horse-serum butyrylcholinesterse (E.C 3.1.1.8), butyrylthiocholine chloride, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), and galanthaminehydrobromide were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade. Butyrylcholinesterase-inhibiting activities were measured by the spectrophotometric method, developed by Ellman et al.[15] Butyrylthiocholine chloride was used as substrate to assay butyrylcholinesterase. The reaction mixture containing 150 μL of (100 mM) sodium phosphate buffer (pH 8.0), 10 μL of DTNB, 10 μL of test compound solution, and 20 μL of butyrylcholinesterase solution was mixed and incubated for 15 min (25 °C). The reaction was then initiated by the addition of 10 μL of butyrylthiocholine. The hydrolysis of butyrylthiocholine was monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of butyrylthiocholine

at a wavelength of 412 nm (15 min). Test compounds and the positive control (galanthamine HBr) were dissolved in EtOH. All the reactions were performed in triplicate in 96-well microplates, and absorption was measured on a microtiter plate reader (SpectraMax 340, Molecular Devices, Sunnyvale, CA). The percentage (%) inhibition was calculated as follows: $(E - S)/E \times 100$, where E is the activity of the enzyme without test compound and S is the activity of the enzyme with test compound.

Determination of IC_{50} Values. The concentrations of test compounds that inhibited the hydrolysis of substrates (butyrylthiocholine) by 50% (IC_{50}) were determined by monitoring the effect of various concentrations of these compounds in the assays on the inhibition values. The IC_{50} values were then calculated using the EZ-Fit enzyme kinetics program (Perrella Scientific Inc., Amherst, MA).

Acknowledgment. We gratefully acknowledge the financial support of the Higher Education Commission to various research scholars.

Supporting Information Available: Proposed reaction paths to the rearranged sesquiterpenoids (Schemes 3, 4, 5, and 6) are available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Sticher, O. In *New Natural Products and Plant Drugs with Pharmacological, Biological or Therapeutical Activity*; Wagner, H., Wolff, P., Eds.; Springer: Berlin, 1977; pp 1−22.
(2) Aleu, J.; Hernandez-Galan, R. H.; Hanson, J. R.; Hitchcock, P. B.; Collado, I. G. *J. Chem. Soc., Perkin Trans. 1* **1999**, 727−730.
(3) Arantes, S. F.; Hanson, J. R.; Hitchcock, P. B. *Phytochemistry* **1999**, *52*, 1063−1067.
(4) Miyazawa, M.; Honjo, Y.; Kameoka, H. *Phytochemistry* **1997**, *44*, 433−436.
(5) Miyazawa, M.; Uemura, T.; Kameoka, H. *Phytochemistry* **1995**, *40*, 793−796.
(6) Asakawa, Y.; Takahashi, H.; Toyota, M. *Phytochemistry* **1991**, *30*, 3993−3997. Sakui, N.; Kuroyanagi, M.; Ishitobi, Y.; Sato, M.; Ueno, A. *Phytochemistry* **1992**, *31*, 143−147.
(7) Choudhary, M. I.; Siddiqui, Z. A.; Hussain, S.; Atta-ur-Rahman. *Chem. Biodiversity* **2006**, *3*, 54−61.
(8) Choudhary, M. I.; Siddiqui, Z. A.; Musharraf, S. G.; Nawaz, S. A.; Atta-ur-Rahman. *Nat. Prod. Res.* **2005**, *19*, 311−317.
(9) Choudhary, M. I.; Musharraf, S. G.; Sami, A.; Atta-ur-Rahman. *Helv. Chim. Acta* **2004**, *87*, 2685−2694.
(10) Choudhary, M. I.; Musharraf, S. G.; Khan, M. T. H.; Abdelrahman, D.; Parvaz, M.; Shaheen, F.; Atta-ur-Rahman. *Helv. Chim. Acta* **2003**, *86*, 3450−3460.
(11) Choudhary, M. I.; Musharraf, S. G.; Shaheen, F.; Atta-ur-Rahman. *Nat. Prod. Lett.* **2002**, *16*, 377−382.
(12) Heymann, H.; Tezuka, Y.; Kikuchi, T.; Supriyatna, S. *Chem. Pharm. Bull.* **1994**, *42*, 138−146.
(13) Shimizu, M.; Shogawa, H.; Matsuzawa, T.; Yonezawa, S.; Hayashi, T.; Arisawa, M.; Suzuki, S.; Yoshizaki, M.; Morita, N.; Ferro, E.; Basualdo, I.; Berganza, L. H. *Chem. Pharm. Bull.* **1990**, *38*, 2283−2284.
(14) Yu, S. Q.; Holloway, W. H.; Utsuki, T.; Brossi, A.; Greig, H. N. *J. Med. Chem.* **1999**, *42*, 1855−1861.
(15) Ellman, G. L.; Courtney, K. D.; Andres, V., Jr.; Featherstone, R. M. *Biochem. Pharmacol.* **1961**, *7*, 88−95.
(16) Faber, K. In *Biotransformations in Organic Chemistry*, 5th ed.; Springer-Verlag: Berlin, 2004; pp 135−136.
(17) Duran, R.; Corrales, E.; Hernandez-Galan, R.; Collado, G. I. *J. Nat. Prod.* **1999**, *62*, 41−44.
(18) Abraham, W. R.; Ernst, L.; Arfmann, H. A. *Phytochemistry* **1990**, *29*, 757−763.
(19) Aebi, A.; Barton, D. H. R.; Burgstahler, A. W.; Lindsey, A. S. *J. Chem. Soc.* **1954**, 4659−4665.
(20) Collado, G. I.; Hanson, R. J.; Hitchcock, B. P.; Macías-Sànchez, J. A. *J. Org. Chem.* **1997**, *62*, 1965−1969.
(21) Smith, R. V.; Rosazza, J. *J. Pharm. Sci.* **1975**, *64*, 1737−1759.

NP0680174