## *Original Research Article*

# Microbial Biotransformation of Male Specific Pheromone α-Copaene with *Cunninghamella elegans* and Antibacterial Activity of Its Transformed Products

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## Abstract

Pharmaceutical industries are increasingly turning to microbial transformation instead of chemical methods. This is because microbial biotransformation is a more effective way to produce pharmacologically active molecules with high specificity and efficient yield. Additionally, it is a step towards eco-friendly synthesis. α-Copaene (**1**), a tricyclic sesquiterpene, is a potent attractant of the male Mediterranean fruit fly *Ceratitis capitata*. This paper presents the utilization of the filamentous fungus *Cunninghamella elegans* TSY 0865 for large-scale biotransformation of 1. α-Copaene (**1**) has been incubated with *Cunninghamella elegans for* 11 days and extracted with $CH_2Cl_2$. Four new hydroxylated metabolites 10β,13-dihydroxycopaene (**2**); 11,13-dihydroxycopaene (**3**); 11-hydroxycopaen-5-one (**4**); and 11-hydroxy-13-copaenic acid (**5**) were afforded. The structures of the new metabolites were elucidated by 1D ($^1$H, $^{13}$C) and 2D NMR (COSY, HMBC, HMQC, and NOESY) techniques and MS analyses. Metabolite **5** exhibited significant antibacterial activity against *Bacillus subtilis* and *Pseudomonas aeruginosa*.

**Keywords:** Microbial Biotransformation, α-copaene, *Cunninghamella elegans*, Pheromone, Antibacterial activity

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## 1.0 Introduction

α-Copaene ((1*R*,2*S*,6*S*,7*S*,8*S*)-8-isopropyl-1,3-dimethyltricyclo[4.4.0.0$^{2,7}$]dec-3-ene, **1**), a tricyclic sesquiterpene exists abundantly in sweet potatoes, lettuce, carrots, ginger root oil and citrus oils. It enhances the mating performance (copulatory success) of male medflies. It is a potent attractant of the male Mediterranean fruit fly *Ceratitis capitata*. It induces in virgin female flies a "Pseudomale" courtship behaviour. α-Copaene (**1**) has also been identified as a minor component of the essential oils of several plants, such as the liverwort *Dumortiera hirsuta* (Swaegr.) Nees. (Saritas, 1998), *Betula pendula* Roth., *Salvia xanthocheila* Boiss. and *Pinus pinea* L.(1-4). Despite its specific pheromone-like property, the biological significance of copaene remains largely unknown. Only a few reports described the anti-microbial activity of the essential oils containing (α)-copaene (5).

Microbial enzymes and those derived from animal and plant cells have been exploited for reactions at chemically inaccessible positions of organic compounds and have significant regio- and stereo-selectivity (6-17). Microbial biotransformation, also known as xenobiotic metabolism, is a highly effective process that involves the modification of an external substance using a complex system of microorganisms. This method produces valuable pharmaceutical products in an affordable and environmentally friendly manner (7,18,19). The microbial biotransformation of terpenoids has been investigated to produce new and useful metabolites as an alternative to the chemical method, and microbial biotransformation is advantageous for preparing new oxygenated steroid derivatives due to its regio- and stereo-selectivity, environment-friendly procedures, and mild reaction conditions compared to chemical synthesis for the preparation of biologically active analogues (20). Microbial-oriented biotransformation has also been used to expand the chemical diversity of terpenoids, the largest group of natural products (21,22). Thus, the microbial transformation technique is increasingly used as one of the most feasible approaches for structurally modifying natural and synthetic compounds. The biotransformation of α-Copaene (**1**) ($C_{15}H_{24}$) by *Cunninghamella elegans* TSY 0865 has been carried out for the first time (Figure 1). Fermentation of **1** with *C. elegans* for 11 days afforded four new hydroxylated metabolites 10β,13-dihydroxycopaene (**2**); 11,13-dihydroxycopaene (**3**); 11-hydroxycopaen-5-one (**4**); and 11-hydroxy-13-copaenic acid (**5**). Functionalized positions of **1** during 11 days of fermentation are mainly C-10, C-11 and C-13.

## 2.0 Materials and Methods

α-Copaene (**1**) was purchased from Sigma-Aldrich (USA). Melting points were determined on a Yanaco MP-S3 apparatus. UV spectra were measured on a Shimadzu UV 240 spectrophotometer. JASCO DIP–360 Digital polarimeter was used to measure the optical rotations in chloroform using a 10 cm cell tube. FTIR-8900 Spectrophotometer was used to record IR spectra in $CHCl_3$. The $^1$H-NMR and 2D NMR spectra were recorded on a Bruker Avance III 500 spectrometer, while $^{13}$C-NMR spectra were recorded on *Bruker* Avance III 500 spectrometer operating at 125 MHz using $CDCl_3$ as solvent. Chemical shifts were reported in $\delta$ (ppm), relative to $SiMe_4$ as the internal standard, and coupling constants (*J*) were measured in Hz. The HREI MS was measured on a Jeol HX 110 mass spectrometer. TLC was performed on Si gel precoated plates ($PF_{254}$, 20 × 20, 0.25 mm, Merck, Germany) using a petroleum ether/EtOA solvent system. Ceric sulphate in 10% $H_2SO_4$ spraying reagent was used to stain compounds on TLC. All reagents used were of analytical grade.

### 2.1 Fungi and Culture Conditions

Microbial cultures of *Cunninghamella elegans* TSY 0865 were grown on potato

dextrose agar (PDA) at 25°C and stored at 4°C. *Cunninghamella elegans* TSY 0865 medium was prepared by adding glucose (10.0 g), peptone (5.0 g), $KH_2PO_4$ (5.0 g), yeast extract (5.0 g), NaCl (5.0 g) and glycerol (10 mL) into distilled water (1 L) and maintained pH at 5.6.

### 2.3 General Stage II Fermentation and Extraction Procedure

The fungal media were transferred into 250 mL conical flasks (100 mL each) and autoclaved at 120ºC. Seed flasks were prepared from a three-day-old slant, and fermentation was allowed for two days on a shaker at 25 ºC. The remaining flasks were inoculated from seed flasks. After one day, α-Copaene (**1**) (0.9 mL) was dissolved in 20 mL of acetone and transferred in each flask, and the clear solution was evenly distributed among the 40 flasks (20 mg/0.5 mL in each flask) containing 24-h-old stage-II cultures. Fermentation was carried out for 11 days on a rotatory shaker (200 rpm) at 25°C. For TLC analysis, the content of one flask each was harvested every second day. 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 $CH_2Cl_2$ (12 L). The combined organic extracts were dried over $Na_2SO_4$ (anhydrous) and evaporated under reduced pressure to obtain brown gum (2.1 g). The crude residue was subjected to column chromatography. Elution was carried out with gradient mixtures of petroleum ether and ethyl acetate, which yielded metabolites **2-5**.

### 2.4 Incubation of α-Copaene (1) with *Cunninghamella elegans* TSY 0865

Compound **1** was fermented for 11 days on a rotatory shaker (200 rpm) at 25°C. For TLC analysis, the contents of one flask each were harvested every second day. The rest of the media flask was filtered after 11 days, extracted with chloroform, and evaporated under reduced pressure to obtain a brown gum finally. The transformed metabolites **2-5** were then isolated by column chromatography.

**10β,13-dihydroxycopaene (2)**: colorless solid; M.p.: 64-66° C; $[\alpha]^{25}_D$ : -182° (*c* = 0.35, MeOH); $R_f$: 0.4 (Petroleum ether/EtOAc 78:22); UV (MeOH) $\lambda_{max}$ nm (log ∈) : 202 (3.1); IR ($CHCl_3$) $\nu_{max}$ $cm^{-1}$ : 3392 (OH), 2964 (C-H), 1651 (C=C); HREI MS *m/z* 236.1617 [$M^+$] calcd for $C_{15}H_{24}O_2$, 236.1620; $^1$H-NMR ($CDCl_3$, 500 MHz) δ: 5.52 (1H, brs, H-4), 3.98 (2H, brs, H-13), 3.81 (1H, t, $J_{(10eq,9ax)}$= 3.9 Hz, H-10), 2.30 (1H, m, H-6), 2.23 (1H, m, $H_b$-5), 2.21 (1H, m, $H_a$-5), 1.88 (1H, m, H-8), 1.71 (1H, m, H-7), 1.65 (1H, s, H-2), 1.25 (1H, m, $H_b$-9), 1.23 (1H, m, $H_a$-9), 0.89 (3H, s, Me-12), 0.85 (3H, d, $J_{(14,11)}$= 6.4 Hz, Me-14), 0.82 (3H, d, *J* (3H, d, $J_{(15,11)}$= 6.3 Hz, Me-15); $^{13}$C NMR ($CDCl_3$, 125 MHz) δ data, see Table 1.

**11,13-dihydroxycopaene (3)**: crystalline compound; M.p.: 71-72° C; $[\alpha]^{25}_D$ : -167° (*c* = 0.2, $CHCl_3$); $R_f$: 0.4 (Petroleum ether /EtOAc 75:25); UV (MeOH) $\lambda_{max}$ nm (log ∈): 202 (1.8); IR ($CHCl_3$) $\nu_{max}$ $cm^{-1}$: 3417 (OH), 3325 (OH), 1657 (C=C); HREI MS *m/z* 236.1611 [$M^+$] calcd for $C_{15}H_{24}O_2$, 236.1627; $^1$H-NMR ($CDCl_3$, 500 MHz): 5.47 (1H, brs, H-4), 3.97 (2H, brs, H-13), 2.32 (1H, m, $H_b$-5), 2.24 (1H, m, $H_a$-5), 2.19 (1H, m, H-6), 1.86 (1H, m, H-7), 1.83 (1H, m, H-8), 1.80 (1H, m, $H_b$-10), 1.67 (1H, m, $H_a$-9), 1.65 (1H, s, H-2), 1.63 (1H, m, $H_a$-10), 1.13 (3H, s, Me-15), 1.10 (3H, s, Me-14), 0.79 (3H, s, Me-12), 0.77 (1H, m, $H_b$-9); $^{13}$C NMR ($CDCl_3$, 125 MHz) δ data, see Table 1.

**11-hydroxycopaen-5-one (4)**: white solid; M.p.: 59-60° C; $[\alpha]^{25}_D$: -201° (*c* = 0.11, $CHCl_3$); $R_f$: 0.4 (Petroleum ether/EtOAc 78:22); UV (MeOH) $\lambda_{max}$ nm (log ∈): 242 (4.1); IR ($CHCl_3$) $\nu_{max}$ $cm^{-1}$ : 3442 (OH), 1736 (C=O), 1655 (C=C); HREI MS *m/z* 234.1528 [$M^+$] calcd for $C_{15}H_{22}O_2$,

234.1536; $^{1}$H-NMR ($CDCl_3$, 500 MHz) δ: 5.74 (1H, s, H-4), 2.87 (1H, m, H-8), 2.72 (1H, s, H-2), 1.99 (3H, s, Me-13), 1.94 (1H, s, H-6), 1.90 (1H, m, H-7), 1.74 (1H, m, $H_a$-10), 1.70 (1H, m, $H_b$-10), 1.24 (1H, m, $H_b$-9), 1.21 (1H, m, $H_a$-9), 1.15 (3H, s, Me-15), 1.12 (3H, s, Me-14), 0.97 (3H, s, Me-12); $^{13}$C NMR ($CDCl_3$, 125 MHz) δ data, see Table 1.

**11-hydroxy-13-copaenic acid (5)**: amorphous material; M.p.: 73-74° C; $[\alpha]^{25}_D$: -222° (*c* = 0.23, $CHCl_3$); $R_f$: 0.5 (Petroleum ether/EtOAc 60:40); UV (MeOH) $\lambda_{max}$ nm (log ∈): 238 (2.2); IR ($CHCl_3$) $\nu_{max}$ cm$^{-1}$ : 3567 (OH), 1712 (C=O, carboxylic acid); HREI MS *m/z* 250.2132 calcd for $C_{15}H_{22}O_3$, 250.2143; $^{1}$H-NMR ($CDCl_3$, 500 MHz) δ: 6.93 (1H, brs, H-4), 2.51 (1H, m, $H_b$-5), 2.40 (1H, s, $H_a$-5), 2.24 (1H, m, H-6), 1.98 (1H, d, $J_{(2,7)}$= 7.1 Hz, H-2), 1.91 (1H, m, H-8), 1.85 (1H, m, H-7), 1.61 (1H, m, $H_b$-10), 1.50 (1H, m, $H_b$-9), 1.13 (3H, s, Me-15), 1.01 (3H, s, Me-14), 0.90 (1H, m, $H_a$-9), 0.75 (3H, s, Me-12); $^{13}$C NMR ($CDCl_3$, 125 MHz) δ data, see Table 1.

## 3.0 Results and Discussion

(α)-Copaene (**1**) is a colorless liquid; its HREI MS provided a molecular ion [M$^+$] at *m/z* 204.1130, suggesting the molecular formula of $C_{15}H_{24}$. The UV spectrum of **1** showed a weak absorption at 202 nm. The $^{1}$H-NMR spectrum of **1** showed signals for four methyl groups at δ 0.83 (d, $J_{(14,11)}$= 6.3 Hz), 0.85 (d, $J_{(15,11)}$= 6.3 Hz), 0.91 (s), and 1.16 (s). Fermentation of (α)-copaene (**1**) with *C. elegans* yielded four new metabolites **2**-**5**. All the metabolites were screened against various gram-positive and gram-negative strains.

10β,13-dihydroxycopaene (**2**) was obtained as a colourless solid. Its molecular formula was determined as $C_{15}H_{24}O_2$ according to the HREI MS data (*m/z* 236.1617: calcd for $C_{15}H_{24}O_2$, 236.1620). It had two more oxygen atoms than substrate **1**. The IR spectrum of **2** showed an additional strong and broad absorption at 3392 cm$^{-1}$, indicating the presence of hydroxyl groups. Compared to substrate **1**, the $^{1}$H NMR spectrum of metabolite **2** exhibited an additional downfield methylene signal at δ 3.98 (brs) and a new methine signal at δ 3.81 (t, $J_{(10eq,9ax)}$= 3.9 Hz) but no singlet for Me-13. Correspondingly, the $^{13}$C NMR spectrum of **2** (Table 1) showed the presence of 15 carbon atoms, including three methyl, three methylene, seven methine, and two quaternary carbons. The appearance of a new methine carbon signal at δ 75.7 and downfield shifts of C-9 and C-1 signals at δ 32.1 and 39.7, respectively, suggests introducing a hydroxyl group in the molecule of **1**. Similarly, the downfield methylene carbon signal at δ 65.9 suggested the presence of an OH group at C-13. The HMBC spectrum of **2** showed long-range interactions between the H-10 (δ 3.81) and C-2 (δ 48.1) and C-6 (δ 33.8), as well as between the $H_2$-13 (δ 3.81) and C-2 (δ 48.1), C-3 (δ 148.3), and C-4 (δ 118.8). This further supported the presence of OH groups at C-10 and C-13. The configuration at the newly introduced stereogenic center at C-10 was deduced to be based on the NOESY correlation between H-10 (δ 3.81) and Me-12 (δ 0.89) (Figure 2). 2D NMR spectra unambiguously assigned all the $^{1}$H and $^{13}$C NMR spectral data.

11,13-dihydroxycopaene (**3**) was obtained as a crystalline compound. The HREI MS of metabolite **3** displayed the M$^+$ at *m/z* 236.1611, supporting the formula $C_{15}H_{24}O_2$, indicating that two oxygen had been incorporated into the molecule, compared to **1** (Figure 1). The IR spectrum showed absorption peaks at 3417 (OH) and 1657 cm$^{-1}$ (C=C). Compared to substrate **1**, the $^{1}$HNMR spectrum of metabolite **3** exhibited a new downfield methylene signal at δ 3.97 (brs) and the disappearance of the Me-13 signal. Correspondingly, the $^{13}$C

NMR spectrum of **3** (Table 1) exhibited 15 carbon signals, including three methyl, four methylene, five methine and three quaternary C-atoms. Compared to substrate **1**, two new carbon signals at δ 65.9 and 74.0 appeared, indicating the presence of two OH groups. Hydroxylation at C-11 was inferred from the downfield shift of C-8 (δ 49.6), the upfield shifts of C-7 (δ 42.3), and C-9 signals (δ 20.5). The other downfield carbon signal at δ 65.9 suggested the presence of the C-13 OH group. The evidence for the hydroxylations at C-11 and C-13 was based on HMBC interactions of H-7 (δ 1.86) and H-8 (δ 1.83) with C-11 (δ 74.0) and between C-13 methylene protons (δ 3.97) and C-2 (δ 51.2), C-3 (δ 147.1), and C-4 (δ 117.9). 2D NMR spectra assigned all the $^{1}$H and $^{13}$C NMR spectral data.

11-hydroxycopaen-5-one (**4**) was obtained as a white solid. The molecular formula of compound **4** was determined to be $C_{15}H_{22}O_2$ by HREI MS ($M^+$ *m/z* measured 234.1528; calcd 234.1536), which indicated the 30 a.m.u. increment in the $M^+$ of the metabolite **4**, compared to substrate **1** (Figure 1). The IR spectrum of **4** displayed hydroxyl and ketonic absorptions at 3442 and 1736 cm$^{-1}$, respectively. The $^{1}$H NMR spectrum of metabolite **4** displayed a downfield shift of H-4 (δ 5.74) compared to substrate **1**. The $^{13}$C NMR spectrum showed **4** (Table 1), indicating the presence of 15 carbons, including four methyl, two methylene, five methine, and four quaternary carbons. Two additional quaternary carbon resonances at δ 73.4 and 203.7, as compared to **1**, were due to C-11 and C-5, respectively. The presence of a C-5 ketonic group was deduced by the downfield shifts of C-4 (δ 121.4), C-3 (δ 169.8), and C-6 (δ 57.1). The HMBC interaction between H-4 (δ 5.74) and C-5 (δ 203.7) further indicated the location of a ketonic carbonyl group at C-5. The location of the OH group at C-11 was inferred from the downfield shift of C-8 (δ 53.5) and the upfield shifts of C-7 (δ 50.2) and C-9 signals (δ 29.7), as well as the HMBC correlations between H-7 (δ 1.90), and H-8 (δ 2.87) with C-11 (δ 73.4). 2D NMR spectra unambiguously assigned all the $^{1}$H and $^{13}$C NMR spectral data.

11-hydroxy-13-copaenic acid (**5**) was obtained as an amorphous material. An HREI-MS analysis indicated that the molecular formula of **5** was $C_{15}H_{22}O_3$ ($M^+$, *m/z* 250.2132, calcd 250.2143) (Figure 1). The infrared (IR) spectrum of **5** displayed intense absorption bands at 3567 and 1712 cm$^{-1}$ for hydroxyl and carbonyl groups, respectively. The $^{1}$H NMR spectrum was recorded in chloroform-d and displayed a downfield shift of C-4 olefinic proton from δ 5.47 to 6.93 and the disappearance of the Me-13 signal, as compared to substrate **1**. The $^{13}$C NMR spectrum (Table 1) revealed 15 carbon signals, including three methyl, three methylene, five methine, and four quaternary C-atoms. A new quaternary carbon signal at δ 73.9 was assigned to C-11, as observed in metabolites **3** and **4**. The disappearance of the Me-13 signal and the appearance of a quaternary signal at δ 177.0, compared to substrate **1**, indicated the carboxylation of the Me-13 group. This was further deduced based on downfield shifts of conjugated H-4 (δ 6.93), C-3 (δ 138.7) and C-4 (δ 169.0) in $^{1}$H and $^{13}$C NMR spectra. The HMBC spectrum showed an interaction of H-4 (δ 6.93) with C-13 (δ 177.0) and C-3 (δ 138.7), further deducing the carboxylation of Me-13. 2D NMR spectra assigned all the $^{1}$H and $^{13}$C NMR spectral data.

α-Copaene (**1**) and its transformed products 2-5 were tested for their antibacterial activity against two Gram-positive bacteria, including *Bacillus subtilis* and *Staphylococcus aureus*, in addition to four Gram-negative bacteria (*Escherichia coli, Shigella flexneri*, *Pseudomonas aeruginosa* and *Salmonella typhi*) using previously described zone of the inhibition test method (14). The metabolite 5, which contains an OH group at C-11 and a carboxylic acid moiety at the C-13 position, exhibited antibacterial activity against

*Bacillus subtilis* and *Pseudomonas aeruginosa* (Table 2).

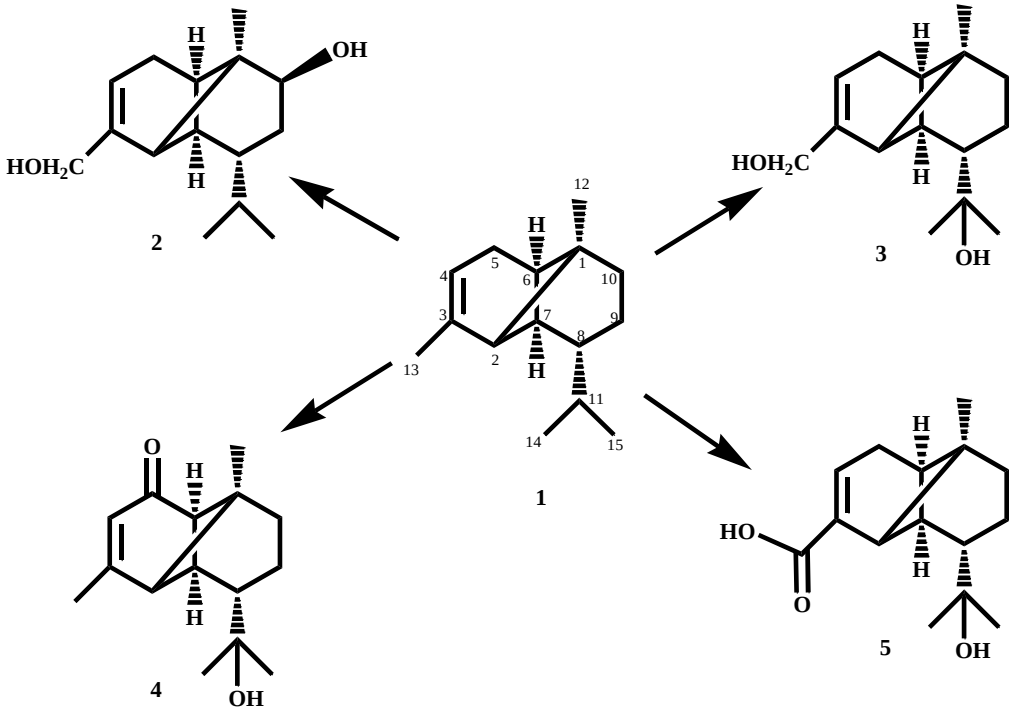


**Figure 1** Biotransformation of α-Copaene (**1**) by a fungal culture of *Cunninghamella elegans*.

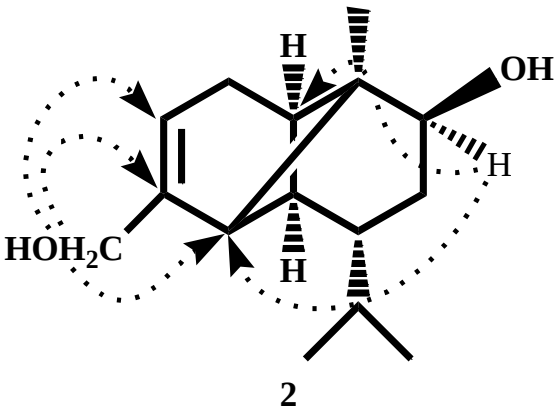


**Figure 2** HMBC correlations observed for the compound **2** in $CDCl_3$.

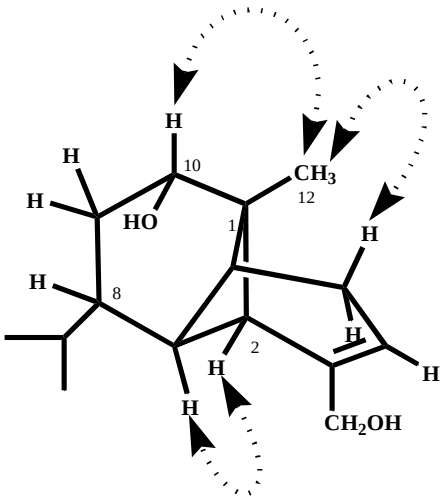


**Figure 3** Main NOESY correlations observed for the compound **2** in $CDCl_3$.

**Table 1** $^{13}$C-NMR data of metabolites **2-5** at 150 MHz in $CDCl_3$; δ in ppm.

| **Carbon No.** | **2** | | **3** | | **4** | | **5** | |
|---|---|---|---|---|---|---|---|---|
| | $\delta_C$ | **Multiplicity** | $\delta_C$ | **Multiplicity** | $\delta_C$ | **Multiplicity** | $\delta_C$ | **Multiplicity** |
| 1 | 39.7 | C | 39.0 | C | 31.9 | C | 38.7 | C |
| 2 | 48.1 | CH | 51.2 | CH | 54.6 | CH | 48.4 | CH |
| 3 | 148.3 | C | 147.1 | C | 169.8 | C | 169.0 | C |
| 4 | 118.8 | CH | 117.9 | CH | 121.4 | CH | 138.7 | CH |
| 5 | 29.6 | $CH_2$ | 29.7 | $CH_2$ | 203.7 | C | 30.8 | $CH_2$ |
| 6 | 33.8 | CH | 37.2 | CH | 57.1 | CH | 36.6 | CH |
| 7 | 44.5 | CH | 42.3 | CH | 50.2 | CH | 42.0 | CH |
| 8 | 41.5 | CH | 49.6 | CH | 53.5 | CH | 49.3 | CH |
| 9 | 32.4 | $CH_2$ | 20.5 | $CH_2$ | 29.7 | $CH_2$ | 20.3 | $CH_2$ |
| 10 | 75.7 | CH | 35.7 | $CH_2$ | 36.6 | $CH_2$ | 35.4 | $CH_2$ |
| 11 | 32.1 | CH | 74.0 | C | 73.4 | C | 73.9 | C |
| 12 | 14.0 | $CH_3$ | 19.4 | $CH_3$ | 20.7 | $CH_3$ | 19.2 | $CH_3$ |
| 13 | 65.9 | $CH_2$ | 65.9 | $CH_2$ | 23.7 | $CH_3$ | 177.0 | C |
| 14 | 19.5 | $CH_3$ | 25.2 | $CH_3$ | 25.1 | $CH_3$ | 25.2 | $CH_3$ |
| 15 | 20.0 | $CH_3$ | 27.6 | $CH_3$ | 28.0 | $CH_3$ | 27.7 | $CH_3$ |

**Table 2:** Antibacterial Activities of Compounds **1-5**.

| **Compound** | ***Escherichia coli*** | ***Bacillus subtilis*** | ***Shigella flexneri*** | ***Staphylococcus aureus*** | ***Pseudomonas aeruginosa*** | ***Salmonella typhi*** |
|---|---|---|---|---|---|---|
| **1** | - | 15 | - | 12 | 15 | - |
| **2** | - | - | - | - | - | - |
| **3** | - | - | - | - | - | - |
| **4** | - | - | - | - | - | - |
| **5** | - | 12 | - | - | 11 | - |
| Imipenem | 30 | 33 | 27 | 33 | 24 | 25 |

## 4.0 Conclusion

In summary, we presented a new study that has been conducted on the biotransformation of α-Copaene (1) by *C. elegans,* producing four new transformed products 2-5 after an 11-day fermentation process. Spectroscopic techniques were used to identify the structures of all new metabolites. Antibacterial activities were tested on all metabolites 2-5, including substrate 1. Metabolite 5 was found to be active against two bacterial strains. The study focused on the fermentation of α-Copaene with *C. elegans*. To understand the potential of *C. elegans* in transforming terpenoids, it is important to explore the biotransformation of different substrates, including other terpenoids such as ursane-type and other oleanane-type pentacyclic triterpenoids. Insight into *C. elegans*' capacity for producing a wide range of physiologically active chemicals would be greatly enhanced by such a development. Biotransformation is an important process, and it would be useful to compare *C. elegans* to other microbes like bacteria and fungi. This would help determine the unique advantages and limitations of using *C. elegans* as a biotransformation agent and identify potential synergistic interactions between microorganisms. Furthermore, it would benefit potential pharmaceutical and agricultural applications to comprehend the active metabolite's mechanism of action.

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## Conflict of interest

The authors declare no conflict of interest.

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