ChemPubSoc Europe

DOI: 10.1002/chem.201904420

CHEMISTRY A European Journal Full Paper



Structure Assignment

# Humulene Diepoxides from the Australian Arid Zone Herb *Dysphania*: Assignment of Aged Hops Constituents

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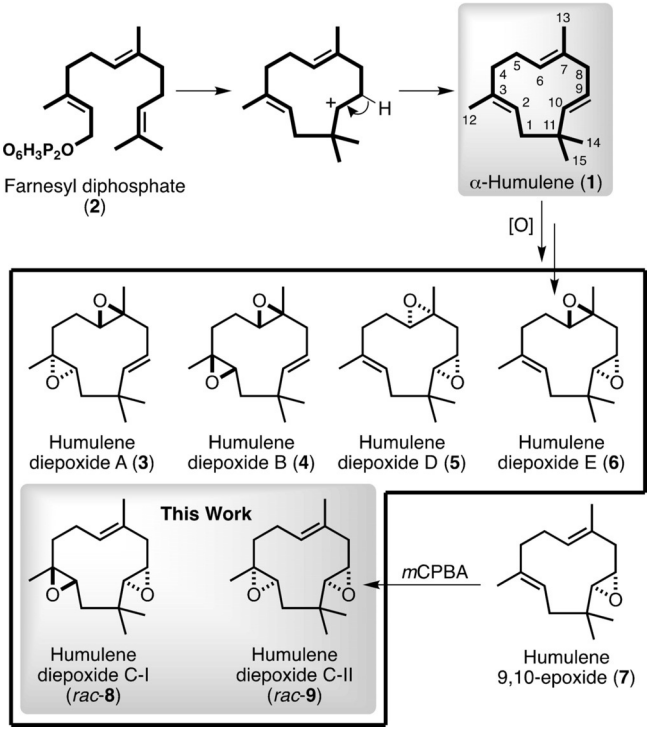
**Abstract:** *Dysphania* is an abundant genus of plants, many of which are endemic to the Australian continent, occurring primarily in arid and temperate zones. Despite their prevalence, very few investigations into the phytochemistry of native *Dysphania* have been undertaken. Described herein, is the isolation and elucidation of two enantiomeric diastereomers of humulene diepoxide C from *D. kalpari* and *D. rhadinostachya*, of which unassigned diastereomers of humulene diepoxide C have been previously reported as components in beer brewed from aged hops. In addition, two (+)-humulene diepoxiols (humulene diepoxiol C-I and C-II) were isolated from *D. rhadinostachya*. Analysis of Chinook hops oil confirmed the presence of both humulene diepoxide C-I and C-II as trace components, and in turn enabled GC-MS peak assignment to the relative stereochemistry. Anticancer assays did not reveal any significant activity for the (+)-humulene diepoxides. Antifungal assays showed good activity against a drug-resistant strain of *C. auris*, with $MIC_{50}$ values of 8.53 and 4.91 μM obtained for (+)-humulene diepoxide C-I and C-II, respectively.

## Introduction

Humulene (**1**) is a sesquiterpene found in many aromatic plants, such as sage[1] and ginseng,[2] and is biosynthetically derived from farnesyl diphosphate (**2**) (Scheme 1). It was first isolated as the major sesquiterpene constituent of *Humulus lupulus*,[3] or hops, which is a key component in the brewing of most modern beers.[4] Under aerobic storage and during the process of wort boiling, the humulene present in hops undergoes various stages of aerial oxidation, producing humulene diepoxide[5] A (**3**),[6] B (**4**),[6a,c,e–g] C,[6a,c,e–g] D[6a,c] and E[6a,c] (Scheme 1), amongst other oxidation products.[7] Beyond being derived from aged hops, naturally derived humulene oxides are quite rare, occurring primarily in fungi.[8] Of the humulene diepoxides, only humulene diepoxide A (**3**) has ever been reported in plants.[9] Moreover, because both mono- and diepoxide formation derived from aged hops is not believed to be governed by enzymatic mechanisms (i.e., aerial oxidation),




**Scheme 1.** Biosynthesis of the humulene (**1**) skeleton starting from farnesyl diphosphate (**2**), including oxidation of **1** to the diepoxides A–E shown with relative stereochemistry.

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these epoxides are by default racemic mixtures of diastereomers.[7a] Surprisingly, however, the relative stereochemistry of the humulene diepoxides C–E remained unknown for a considerable period of time.[10] Subsequently, Hayano and Mochizuki[11] demonstrated that pure diastereoisomers of humulene diepoxides D (**5**) and E (**6**) could be obtained from humulene-9,10-epoxide (**7**) on treatment with *meta*-chloroperoxybenzoic acid (*m*CPBA), which further revealed that humulene diepoxide C was produced as two racemic diastereoisomers, that is, racemic C-I (*rac*-**8**) and racemic C-II (*rac*-**9**) (Scheme 1). However, since this time there have been no attempts to assign relative stereochemistry to the humulene diepoxide C diastereomers found in hops essential oil.

As part of our continued efforts in surveying the phytochemical composition of Australian arid zone flora,[12] a particular focus has been the Chenopodiaceae,[13] which are a subfamily of the cosmopolitan amaranth family of flowering plants. They are exceptionally adapted to saline, dry or disturbed habitats of temperate and subtropical climates, and are thus found throughout the Australian continent. Within Australia, one of the more prominent genera is that of *Dysphania*,[14] which are described as annual or short-lived perennial herbs. *Dysphania* consists of approximately 43 species worldwide, including 19 known to exist within Australia, 16 of which are endemic.[15]

Considering that *Dysphania* species native to Australia have not previously undergone phytochemical investigation, a $^1$H NMR-guided fractionation study of *D. kalpari*[16] and *D. rhadinostachya* was undertaken, which unearthed a series of highly oxygenated α-humulenes as disclosed herein.

## Results and Discussion

*D. kalpari* collected from a Western Queensland desert region was extracted and subjected to fractionation that revealed alkene and oxygenated methine shifts in the proton NMR spectrum ($\delta$(H) = 5.0–6.8 and 2.2–3.2 ppm). These fractions were then further purified to give pure isolates, which were elucidated as detailed below.

(+)-Humulene diepoxide C-I [(+)-**8**] showed a molecular ion at $m/z$ = 259.1667 $[M+Na]^+$ (−0.2 Δmmu) in the positive high-resolution (HR) ESI-MS mode. From the HR-ESI-MS data, a molecular formula of $C_{15}H_{24}O_2$ was determined, which revealed a ring double bond equivalents (RDBE) value of four. The $^1$H NMR spectrum revealed four singlet methyl groups at $\delta$(H) = 1.25 (Me-12), 1.71 (Me-13), 1.09 (Me-14) and 0.84 ppm (Me-15), and one alkene proton at $\delta$(H) = 5.28 ppm (H-6). The $^{13}$C NMR spectrum revealed fifteen carbon signals including trisubstituted double-bond resonances at $\delta$(C) = 132.6 (C-7) and 125.4 ppm (C-6), four oxygenated carbon atoms at $\delta$(C) = 61.6 (C-2), 60.9 (C-3), 56.4 (C-9) and 65.4 ppm (C-10), and four methyl signals at $\delta$(C) = 16.3, 17.8, 28.9 and 18.1 ppm (Table 1).

The COSY spectrum revealed coupling between H-2 ($\delta$(H) = 2.58 ppm) and H-1 ($\delta$(H) = 1.58, 1.68 ppm, 2H), H-6 ($\delta$(H) = 5.28 ppm) and H-4 ($\delta$(H) = 1.16, 2.12 ppm, 2H) as well as H-5 ($\delta$(H) = 2.31, 2.07 ppm, 2H), and H-9 ($\delta$(H) = 3.01 ppm) with H-8 ($\delta$(H) = 1.62, 2.68 ppm, 2H) as well as H-10 ($\delta$(H) = 2.36 ppm).

**Table 1.** $^1$H and $^{13}$C NMR assignment for (+)-humulene diepoxide C-I [(+)-**8**].

| No. | $^{13}$C[a] | $^1$H[b] | Multiplicity (*J* [Hz]) |
|---|---|---|---|
| 1a | 38.6 | 1.58 | dd (15.4, 7.6) |
| 1b | | 1.68 | d (15.4) |
| 2 | 61.6 | 2.58 | dd (7.9, 1.2) |
| 3 | 60.9 | – | – |
| 4a | 38.3 | 1.16 | m |
| 4b | | 2.12 | m |
| 5a | 23.4 | 2.31 | m |
| 5b | | 2.07 | m |
| 6 | 125.4 | 5.28 | t (7.9) |
| 7 | 132.6 | – | – |
| 8a | 41.4 | 1.62 | dd (12.9, 9.9) |
| 8b | | 2.68 | dd (12.9, 3.7) |
| 9 | 56.4 | 3.01 | ddd (9.8, 3.5, 2.3) |
| 10 | 65.4 | 2.36 | d (2.3) |
| 11 | 33.2 | – | – |
| 12 | 16.3 | 1.25 | s |
| 13 | 17.8 | 1.71 | s |
| 14 | 28.9 | 1.09 | s |
| 15 | 18.1 | 0.84 | s |

[a] Chemical shifts (ppm) referenced to $CDCl_3$ ($\delta$(C) = 77.0 ppm) at 125 MHz. [b] Chemical shifts (ppm) referenced to $CDCl_3$ ($\delta$(H) = 7.26 ppm) at 500 MHz

This in turn provided C1–C2, C4–C5–C6 and C8–C9–C10 connectivity as shown in bold in Figure 1.

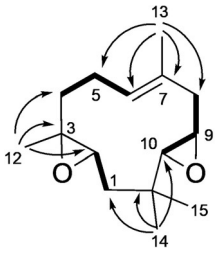


**Figure 1.** Selected COSY (bold bonds) and HMBC (curved) correlations (left).

The HMBC spectrum showed correlations arising from Me-14 and Me-15 to $\delta$(C) = 38.6 ppm (C-1), a quaternary carbon atom at $\delta$(C) = 33.2 ppm (C-11) and an oxygenated carbon atom at $\delta$(C) = 65.4 ppm (C-10). This indicated C1–C11–C10 connectivity with two methyl groups attached at C-11. Furthermore, Me-12 showed HMBC correlations to the oxygenated carbon atom C-2, the oxygenated quaternary carbon atom C-3 and a methylene carbon atom at $\delta$(C) = 38.3 ppm (C-4). This information confirmed the attachment of Me-12 to C-3 as well as establishing a relationship to C-4. Furthermore, the two oxygenated carbon atoms at C-2 and C-3 suggested an epoxide ring moiety. The methyl group, Me-13, also showed HMBC correlations to $\delta$(C) = 23.4 ppm (C-5), an olefinic carbon atom at $\delta$(C) = 125.4 ppm (C-6), an olefinic carbon atom at $\delta$(C) = 132.6 ppm (C-7) and a methylene carbon atom at $\delta$(C) = 41.4 ppm (C-8). These correlations suggested Me-13 was attached to C-7 with correlations to alkene C-6, C-5 and C-8. Furthermore, an additional epoxide was confirmed at C-9 and C-10 due to COSY correlations, and oxygenated carbon atom shifts at $\delta$(C) = 56.4 and 65.4 ppm, respectively. These data in combination with the RDBE value of four indicated an 11-membered carbon ring with two epoxides and was therefore determined to be a humulene diepoxide. This was further confirmed by $^1$H and $^{13}$C NMR comparison to that previously reported for humulene diepoxide C-I (*rac*-**8**).[8b,11]

The X-ray structure analysis of (+)-**8** confirmed the proposed structure and established the absolute configuration as (6*E*)-2*S*,3*S*,9*S*,10*S*-humulene-2,3;9,10-diepoxide that is, (+)-humulene diepoxide C-I [(+)-**8**] (Figure 2).

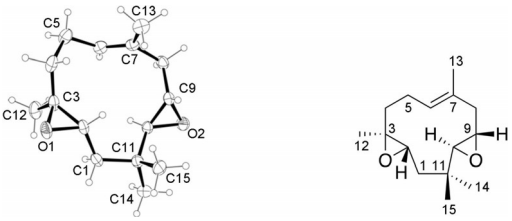


**Figure 2.** ORTEP view (30% probability ellipsoids) of (+)-humulene diepoxide C-I [(+)-**8**] (left) and line drawing showing the absolute stereochemistry (right).

When analysed by using HR-ESI-MS in the positive mode, (+)-humulene diepoxide C-II [(+)-**9**] gave a similar molecular ion to that of (+)-**8**, with $m/z$ = 259.1669 $[M+Na]^+$ (±0.0 Δmmu). A molecular formula of $C_{15}H_{24}O_2$ was therefore calculated, which revealed a RDBE value of four. The $^1$H NMR spectrum revealed four methyl groups at $\delta$(H) = 1.14 (Me-12), 1.77 (Me-13), 1.30 (Me-14) and 0.70 ppm (Me-15), and one alkene proton at $\delta$(H) = 5.10 ppm (H-6). The $^{13}$C NMR spectrum also revealed fifteen carbon signals including double-bond resonances at $\delta$(C) = 131.2 and 127.9 ppm, four oxygenated carbon atoms at $\delta$(C) = 60.2 (C-2), 60.4 (C-3), 54.8 (C-9) and 64.6 ppm (C-10), and four methyl signals at $\delta$(C) = 17.0, 17.7, 26.8 and 22.9 (Table 2).

Proton coupling correlations were established by using COSY NMR spectroscopy from H-2 ($\delta$(H) = 2.79 ppm) to H-1 ($\delta$(H) = 1.53, 1.49 ppm, 2H), H-6 ($\delta$(H) = 5.10 ppm) to H-4 ($\delta$(H) = 1.19, 2.18 ppm, 2H) and H-5 ($\delta$(H) = 2.15, 2.32 ppm, 2H), and from H-9 ($\delta$(H) = 2.92 ppm) to H-8 ($\delta$(H) = 1.65, 2.66 ppm, 2H) and H-10 ($\delta$(H) = 2.67 ppm). This indicated connectivity of C1–C2, C4–C5–C6 and C8–C9–C10. Connectivity between C-9 and C-10, in addition to chemical shift values [$\delta$(C) = 54.8 (C-9) and 64.6 ppm (C-10)] further suggested the presence of an epoxide residue at this position.

From the HMBC spectrum, both methyl carbon atoms Me-14 and Me-15 were revealed to have correlations to $\delta$(C) = 40.8 ppm (C-1), a quaternary carbon atom at $\delta$(C) = 32.4 ppm (C-11), as well as an oxygenated carbon atom at $\delta$(C) = 64.6 ppm (C-10). This led to the formation of a partial structure, comprised of C1–C11–C10, with two methyl groups attached at C-11. Furthermore, Me-12 showed HMBC correlations to the oxygenated carbon atom C-2 ($\delta$(C) = 60.2 ppm), an oxygenated quaternary carbon atom C-3 ($\delta$(C) = 60.4 ppm) and a methylene carbon at $\delta$(C) = 38.4 ppm (C-4). The two oxygenated carbon atoms C-2 and C-3 indicated an epoxide ring moiety in the structure with Me-12 attached to C-3, which suggested a relationship to C-4. The methyl group Me-13 exhibited HMBC correlations to $\delta$(C) = 24.7 ppm (C-5), an olefinic carbon atom at $\delta$(C) = 127.9 ppm (C-6), a quaternary olefinic carbon atom at $\delta$(C) = 131.2 ppm (C-7) and a methylene carbon atom at $\delta$(C) = 43.3 ppm (C-8). This reinforced that Me-13 was connected to a protonated double bond (H-6) with an attachment to C-7, in addition to connections with C-5 and C-8. In essence, COSY and HMBC correlations observed for (+)-**8** were identical with (+)-**9** (Figure 1).

Further confidence in this structure was garnered through $^1$H and $^{13}$C NMR comparison to that previously reported for humulene diepoxide C-II (*rac*-**9**).[8b, 11]

X-ray crystallographic analysis revealed the absolute configuration as (6*E*)-2*S*,3*S*,9*R*,10*R*- humulene-2,3;9,10-diepoxide that is, (+)-humulene diepoxide C-II [(+)-**9**] (Figure 3).

**Table 2.** $^1$H and $^{13}$C NMR assignment for (+)-humulene diepoxide C-II [(+)-**9**].

| No. | $^{13}$C | $^1$H | Multiplicity ($J$ [Hz]) |
|---|---|---|---|
| 1a | 40.8 | 1.53 | dd (15.12, 2.5) |
| 1b | | 1.49 | dd (15.12, 5.5) |
| 2 | 60.2 | 2.79 | dd (5.4, 2.7) |
| 3 | 60.4 | – | – |
| 4a | 38.4 | 1.19 | dd (13, 4.5) |
| 4b | | 2.18 | dt (13.1, 3.5) |
| 5a | 24.7 | 2.15 | d (13.1) |
| 5b | | 2.32 | m |
| 6 | 127.9 | 5.10 | br.dd (11.9, 2.1) |
| 7 | 131.2 | – | – |
| 8a | 43.3 | 1.65 | dd (12.3, 9.9) |
| 8b | | 2.66 | dd (12.2, 3.3) |
| 9 | 54.8 | 2.92 | ddd (9.8, 3.6, 2.6) |
| 10 | 64.6 | 2.67 | d (2.7) |
| 11 | 32.4 | – | – |
| 12 | 17.0 | 1.14 | s |
| 13 | 17.7 | 1.77 | s |
| 14 | 26.8 | 1.30 | s |
| 15 | 22.9 | 0.70 | s |

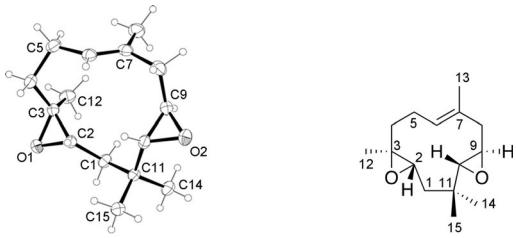


**Figure 3.** ORTEP view (30% probability ellipsoids) of (+)-humulene diepoxide C-II [(+)-**9**] (left) and line drawing showing the absolute stereochemistry (right).

Encouraged by these findings a similar $^1$H NMR-guided fractionation approach was applied to the closely related species *D. rhadinostachya*, which afforded (+)-humulene diepoxiol C-I (**10**) and C-II (**11**), elucidated below.

The HR-ESI-MS (positive mode) of (+)-humulene diepoxiol C-I (**10**) located a molecular ion at $m/z$ = 275.1659 $[M+Na]^+$ (−0.8 Δmmu), which allowed for the molecular formula to be determined as $C_{15}H_{24}O_3$ (RDBE = 4). The $^1$H NMR spectrum revealed four singlet methyl groups at $\delta$(H) = 1.21 (Me-12), 1.73 (Me-13), 1.08 (Me-14) and 0.82 ppm (Me-15), as well as one alkene proton at $\delta$(H) = 5.42 (Table 3). The $^{13}$C NMR spectrum

**Table 3.** $^{1}$H and $^{13}$C NMR assignment for (+)-humulene diepoxiol C-I (**10**).

| No. | $^{13}$C | $^{1}$H | Multiplicity (*J* [Hz]) |
|---|---|---|---|
| 1a | 38.5 | 1.50 | dd (15.4,8.3) |
| 1b | | 1.66 | d (15.3) |
| 2 | 61.3 | 2.50 | d (8.1) |
| 3 | 60.9 | – | – |
| 4a | 37.7 | 2.10 | m |
| 4b | | 1.20 | s |
| 5a | 22.7 | 2.36 | m |
| 5b | | 2.06 | s |
| 6 | 124.9 | 5.42 | t (7.8) |
| 7 | 136.1 | – | – |
| 8 | 79.6 | 3.67 | d (8.1) |
| 9 | 60.4 | 3.00 | dd (8.2, 2.5) |
| 10 | 63.9 | 2.42 | d (2.5) |
| 11 | 32.9 | – | – |
| 12 | 16.3 | 1.21 | m |
| 13 | 12.0 | 1.73 | s |
| 14 | 28.7 | 1.08 | s |
| 15 | 18.2 | 0.82 | s |

exhibited fifteen carbon signals, including one trisubstituted double bond resonances at $\delta$(C) = 136.1 and 124.9 ppm, five oxygenated carbon atoms at $\delta$(C) = 61.3 (C-2), 60.9 (C-3), 60.4 (C-9), 63.9 (C-10) and 79.6 ppm (C-8), and four methyl signals at $\delta$(C) = 16.3, 12.0, 28.7 and 18.2 (Table 3). Comparison of the chemical shifts and splitting patterns showed that the $^{1}$H and $^{13}$C NMR data for **10** strongly resembled that of (+)-humulene diepoxide C-I [(+)-**8**] (Table 1).

Evaluation of the carbon shifts between (+)-**8** and **10** unearthed the key structural difference resided at position C-8. In (+)-**8**, this is a methylene carbon atom at $\delta$(C) 41.1 ppm, whereas in **10** this carbon atom was found to have a chemical shift of $\delta$(C) = 79.6 ppm, suggesting it to be oxygenated, presumably in the form of a hydroxyl moiety. This was further supported by the calculated molecular formula for **10**, as well as by its IR spectrum, which exhibited a broad absorption band at $\tilde{\nu}$ = 3431 cm$^{-1}$. The HMBC spectrum indicated that Me-13 was correlated to an olefinic carbon atom at $\delta$(C) = 124.90 ppm (C-6), an olefinic carbon atom at $\delta$(C) = 136.1 ppm (C-7) and an oxygenated carbon atom at $\delta$(C) = 79.6 ppm (C-8). HMBC resonances similar to that of (+)-humulene diepoxide C-I [(+)-**8**] were located from both methyl carbon atoms (Me-14 and Me-15) to C-1 ($\delta$(C) = 38.5 ppm), a quaternary carbon atom at $\delta$(C) = 32.9 ppm (C-11) and C-10, in addition to Me-12 correlations to C-2, C-3 and a methylene carbon atom at $\delta$(C) = 37.7 ppm (C-4) (Figure 4a). COSY correlations established coupling relationships for H-2 ($\delta$(H) = 2.50 ppm) with H-1 ($\delta$(H) = 1.50,1.66 ppm), in addition to H-5 ($\delta$(H) = 2.06, 2.36 ppm) with H-6 ($\delta$(H) = 5.42 ppm) and H-4 ($\delta$(H) = 1.20, 2.10 ppm), and H-9 ($\delta$(H) = 3.00 ppm) with H-8 ($\delta$(H) = 3.67 ppm) and H-10 ($\delta$(H) = 2.42 ppm) (Figure 4a).

Unfortunately, efforts towards crystallisation of **10** resulted in its decomposition. Therefore, the relative configuration was determined by NOE experiments, and comparing chemical shifts of the carbon atoms surrounding the epoxide ring (specifically C-2, C-3 and C-12) and that of (+)-humulene diepoxide C-I. The $^{13}$C NMR chemical shifts at C-2, C-3 and C-12 were

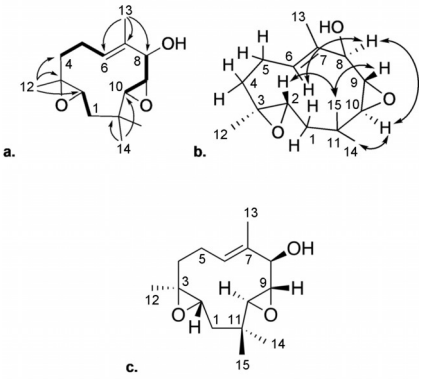


**Figure 4.** a) Selected COSY (bold bonds) and HMBC (curved arrows) correlations, b) NOE correlations and c) line drawing showing the proposed absolute stereochemistry of (+)-humulene diepoxiol C-I (**10**).

almost identical to (+)-humulene diepoxide C-I [(+)-**8**], which suggested that the epoxide moiety had a *trans* configuration, with H-2 in the $\beta$ orientation and Me-12 in the $\alpha$ orientation. Selective NOE irradiation of H-15 showed correlations to H-2 and H-9, confirming that H-5, Me-15 and H-9 adopted the $\beta$ orientation. Selective NOE irradiation of Me-14 showed correlations to H-10, and irradiation of H-8 showed a correlation to H-10, supporting the conclusion that H-10, Me-14 and H-8 exist in the $\alpha$ conformation. NOE irradiation of H-8 further revealed correlations to H-6, allowing for **10** to be assigned as an *E*-configured double bond (Figure 4b). These data suggested a relative stereochemical configuration of 6(*E*)-2*S**,3*S**,9*S**,10*S**-humulene-2,3;9,10-diepoxide-8-ol (Figure 4c).

(+)-Humulene diepoxiol C-II (**11**) gave a molecular ion at *m/z* = 275.1614 $[M+Na]^{+}$ (−0.4 Δmmu) in the positive HR-ESI-MS mode. The molecular formula was therefore determined to be $C_{15}H_{24}O_3$ with an RDBE value of four. The $^{1}$H NMR spectrum (Table 4) revealed four methyl groups at $\delta$(H) = 1.13 (Me-12), 1.84 (Me-13), 1.29 (Me-14) and 0.68 ppm (Me-15), as well as one alkene proton at $\delta$(H) = 5.16 ppm. The $^{13}$C NMR spectrum exhibited fifteen carbon signals, including trisubstituted double-bond resonances at $\delta$(C) = 134.7 and 130.7 ppm, five oxygenated carbon atoms at $\delta$(C) = 60.2 (C-2), 58.7 (C-3), 66.1 (C-5), 54.4 (C-9) and 64.5 ppm (C-10), and four methyl signals at $\delta$(C) = 18.1, 18.3, 26.7 and 22.8 ppm. The $^{1}$H and $^{13}$C NMR data strongly resembled (+)-humulene diepoxide C-II [(+)-**9**] (Table 2).

A comparison of the chemical shifts revealed that the methylene carbon of (+)-humulene diepoxide C-II [(+)-**9**] corresponded to C-5 ($\delta$(C) = 24.7 ppm), which exists more downfield in **11** ($\delta$(C) = 66.1 ppm). This was attributed to the presence of an oxygenated carbon atom at this position. As with **10**, this was confirmed based on the calculated molecular formula, in addition to the presence of a broad absorption band within the IR spectrum at $\tilde{\nu}$ = 3431 cm$^{-1}$. Moreover, HMBC correlations concerning Me-13 and olefinic carbon atoms at $\delta$(C) = 130.7 (C-6) and 134.7 ppm (C-7), as well as the oxygenated carbon atom at $\delta$(C) = 66.1 ppm (C-5) supported inclusion of a hydroxyl group. HMBC cross peaks between $\delta$(H) = 4.54 ppm (H-5) to C-7 and C-4 at $\delta$(C) = 46.9 ppm then confirmed the hydroxyl

**Table 4.** $^{1}H$ and $^{13}C$ NMR assignment for (+)-humulene diepoxiol C-II (**11**).

| No. | $^{13}C$[a] | $^{1}H$[b] | Multiplicity [*J* in Hz] |
|---|---|---|---|
| 1a | 40.6 | 1.51 | dd (15.12,1.8) |
| 1b | | 1.45 | dd (15.12,5.7) |
| 2 | 60.2 | 2.80 | dd (5.9,1.8) |
| 3 | 58.7 | – | – |
| 4a | 46.9 | 2.52 | dd (12.5,4.8) |
| 4b | | 1.19 | m |
| 5 | 66.1 | 4.54 | ddd (11.1, 10.2, 4.8) |
| 6 | 130.7 | 5.16 | d (10.3) |
| 7 | 134.7 | – | – |
| 8a | 43.6 | 2.67 | dd (11.7,3.7) |
| 8b | | 1.67 | ddd (11.7,9.5,1.0) |
| 9 | 54.4 | 2.87 | ddd (9.9,3.7,2.6) |
| 10 | 64.5 | 2.66 | d (2.6) |
| 11 | 32.4 | – | – |
| 12 | 18.1 | 1.13 | s |
| 13 | 18.3 | 1.84 | d (1.5) |
| 14 | 26.7 | 1.29 | s |
| 15 | 22.8 | 0.68 | s |

[a] Chemical shifts (ppm). referenced to $CDCl_3$ ($\delta$(C) 77.0 ppm) at 125 MHz. [b] Chemical shifts (ppm) referenced to $CDCl_3$ ($\delta$(H) 7.26 ppm) at 500 MHz.

moiety to be attached at the C-5 position (Figure 5a). The COSY spectrum established correlations from H-2 ($\delta$(H) = 2.80 ppm) to H-1 ($\delta$(H) = 1.45, 1.51, 2H), from H-5 ($\delta$(H) = 4.54 ppm) to H-6 ($\delta$(H) = 5.16 ppm) and H-4 ($\delta$(H) = 1.19, 2.52 ppm, 2H), and from H-9 ($\delta$(H) = 2.87 ppm) to H-8 ($\delta$(H) = 1.67, 2.67 ppm, 2H) and H-10 ($\delta$(H) = 2.66 ppm). Lastly, HMBC correlations for both Me-14 and Me-15 to $\delta$(C) = 40.6 ppm (C-1), the quaternary carbon atom at $\delta$(C) = 32.4 ppm (C-11) and C-10, and Me-12 to C-2, C-3 and a methylene carbon atom at $\delta$(C) = 46.9 ppm (C-4), were found to support a structure corresponding to an isomer of diepoxiol C-I (**10**).

The relative configuration of **11** was determined by 1D NOE experiments and from comparison of the epoxide ring carbon atoms. The $^{13}C$ NMR chemical shifts of C-9 and C-10 were found to be almost identical to that of (+)-humulene diepoxide C-II [(+)-**9**], suggesting that the epoxide had a *trans* configuration, that is, H-10β and H-9α. Selective NOE irradiation of H-2 revealed correlations to Me-14 and H-10, which confirmed H-10, Me-14 and H-2 as having β orientation. Irradiation of H-2 provided no correlations to Me-12, therefore indicating Me-12α (Figure 5b). Selective NOE irradiation of Me-12 showed correlations to H-5, supporting H-5 as α facing and the hydroxyl group in the β position. Moreover, NOE irradiation of H-6 showed correlations to H-8. With these structural attributes, **11** was confirmed as having an *E*-configured double bond. These data established **11** as 6(*E*)-2*S**,3*S**,9*R**,10*R**-humulene-2,3;9,10-diepoxide-5-ol (Figure 5c).

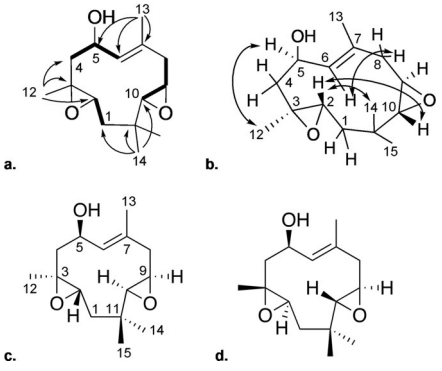


**Figure 5.** a) Selected COSY (bold bonds) and HMBC (curved arrows) correlations, b) NOE correlations, c) line drawing showing the proposed absolute stereochemistry of (+)-humulene diepoxiol C-II (**11**) and d) antrodol B (**12**).

Further support of the proposed structure came from a diastereomer of **11**, antrodol B [6(*E*)-2,3:9,10-diepoxy-humulen-5α-ol (**12**)], which was previously isolated from the fungus *Antrodiella albocinnamomea* (Figure 5d).[8c]

### Comparison to hops oil

Chinook hops were extracted giving a crude hop oil, which was subsequently fractionated by using silica gel column chromatography. Crude hop oil and fractions thereof were analysed by gas chromatography mass spectrometry (GC-MS) [and with gas chromatography flame ionisation detection (GC-FID)], and samples subsequently spiked with (+)-humulene diepoxide C-I [(+)-**8**] and C-II [(+)-**9**]. Sample spiking firstly revealed the presence of both humulene diepoxide C-I (*rac*-**8**) and C-II (*rac*-**9**) as trace components,[17] but also facilitated GC-MS peak assignment, that is, retention time (Rt) assignment of C-I (Rt = 89.65 min/Kováts RI: 1726) and C-II (Rt = 90.99 min/Kováts RI: 1737). Kováts indices (Kováts retention index)[18] were also determined as assigned above.

### Biological activity of (+)-humulene diepoxide C-I and C-II

The activity of (+)-humulene diepoxides C-I [(+)-**8**] and C-II [(+)-**9**] was assessed against eight human cancer cell lines and five species of pathogenic fungi. The diepoxiols **10** and **11** were not evaluated due to their susceptibility to decomposition, presumably facilitated by elimination of the hydroxyl group to give a reactive diene as proposed for the related diepoxiol phomanoxide.[19]

The cytotoxicity of (+)-humulene diepoxide C-I [(+)-**8**] and C-II [(+)-**9**] was evaluated against human fibroblast (NFF), melanoma (MM96L), breast cancer (MCF-7), hypopharyngeal cancer (FaDu) and four different tongue squamous cell carcinomas (CAL-27, SCC-9, SCC-15 and SCC-25). Results from this evaluation show neither (+)-**8** or (+)-**9** to possess any significant cytotoxic activity against the cell lines examined (see the Supporting Information, Table S2 and Figure S7).

Antifungal activity of (+)-**8** and (+)-**9** was evaluated through the determination of the 100 and 50% inhibitory concentration values (MIC and $MIC_{50}$, respectively) against four species of *Candida*: *C. albicans* (ATCC 90028), *C. glabrata* (ATCC 90030), *C. krusei* (ATCC 6258) and *C. auris* (CBS 10913 and CBS 12373). Results from these assays did not show significant activity across the majority of species, with the exception of *C. auris* (see the Supporting Information, Table S1 and Figure S6). In particular, good activity was observed for both (+)-**8** ($MIC_{50}$ = 8.53 μM)

and (+)-**9** ($MIC_{50}$ = 4.91 μM) against the drug-resistant *C. auris* strain CBS 12373.

## Conclusion

Two enantiomerically pure diastereomers of (+)-humulene diepoxide C (C-I [(+)-**8**], and C-II [(+)-**9**]) were isolated from *D. kalpari* and *D. radinostachya*, and used to assign the relative stereochemistry to peak retention time for humulene diepoxide C-I (*rac*-**8**) and C-II (*rac*-**9**) in Chinook hops oil. Intriguingly, although humulene diepoxide A (**3**) was also isolated from *D. kalpari*, none of the other diepoxides nor any humulene monoepoxides were detected from this species. This, in addition to the enantiopurity of the two diastereomers obtained (i.e., (+)-**8** and (+)-**9**), strongly suggests that the diepoxides in *D. kalpari* and *D. radinostachya* are generated through biosynthetic means rather than by aerial oxidation as is the case for hops.[20] In addition, although (+)-**8** and (+)-**9** displayed limited cytotoxic activity against human cancer cell lines, both were found to have good activity against a drug-resistant strain of *Candida auris* (CBS12373). Lastly, the wo novel (+)-humulene diepoxiols **10** and **11** were also isolated from *D. radinostachya*, and thus represent an intriguing class of highly oxygenated humulene compounds that are known in fungi,[8c] but previously unreported in plants.

## Experimental Section

### General

Both 1D and 2D $^{1}$H NMR spectra were acquired on either a Bruker Avance 500 or 700 MHz spectrometer at 298 K. All $^{13}$C NMR spectra were recorded at 125 MHz on a Bruker Avance. Coupling constants are given in Hertz (Hz) and chemical shifts are reported as $\delta$ values in parts-per-million (ppm), with the solvent resonance as the internal standard ($^{1}$H NMR: $CDCl_3$: $\delta$ = 7.26 ppm and $^{13}$C NMR: $CDCl_3$: $\delta$ = 77.0 ppm). The optical rotation measurements were carried out by using a JASCO P-2000 spectrophotometer. Infrared spectroscopy (IR) was undertaken by using a Perkin–Elmer-400 model. Ultraviolet spectroscopy (UV) was undertaken by using a Perkin–Elmer Lambda 35 model. Positive and negative ion electrospray mass spectra (low resolution (LR) ESI-MS) were measured by using a Bruker Esquire HCT or HR-ESI-MS by using a MicroTof Q instrument. Purification of fractions through preparative high-performance liquid chromatography (HPLC) was carried out by using a Shimadzu LC-20AD pump, with an SPD-M20A UV detector and ELSD-LT II light detector. The separation process was conducted by using a Phenomenex reverse-phase (RP) C-18(2) Luna 10 μM 100 Å column (250×10 mm) and a Phenomenex normal-phase (NP) Silica Luna 10 μM 100 Å column (250×10 mm). TLC was performed by using silica gel 60 F254 TLC plates (Merck).

Crystallographic data were collected on an Oxford Diffraction Gemini CCD X-ray diffractometer by using $Cu_{K\alpha}$ (1.54184 Å) radiation. The samples were cooled to 190 K with an Oxford Cryosystems Desktop Cooler. The structures were solved with SHELXS and refined with SHELXL[21] within the WinGX package.[22] The thermal ellipsoid diagrams were produced with ORtEP3.[23] CCDC 1955277 and 1955278 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre.

### Plant collection

*Dysphania kalpari* was collected in August of 2014 from the Cooper Creek basin in Western Queensland, Australia (S 25° 39.360′, E 143° 06.549′). *Dysphania rhadinostachya* was collected in August of 2016 in Welford National Park, Western Queensland, Australia (S 25° 08.801′, E 143° 07.123′). Plant identification was provided with assistance from the Queensland Herbarium, Brisbane.

### Plant extraction

Whole plant samples were extracted with ethanol (≈2 L) at room temperature for approximately six months. The extracts were filtered through a pad of Celite and the solvent was removed by using a rotary evaporator. Subsequently, the crude extract was partitioned between water (200 mL) and ethyl acetate (3×300 mL) to remove salts and water. The ethyl acetate extract was then concentrated to approximately 50 mL under reduced pressure, washed with brine and dried over anhydrous sodium sulfate ($Na_2SO_4$). Extracts were subsequently dried under high vacuum.

### *D. kalpari* fractionation and isolation

A 942 g sample was extracted as described above and concentrated to give 6.1 g of crude extract. This extract was then fractionated by flash chromatography on a NP silica gel 60 column (Ø 4.5× 16 cm). The column was then eluted with a series of solvent gradients including petroleum ether (PE)/EtOAc (90:10 (600 mL), 80:20 (400 mL), 70:30 (600 mL), 60:40 (400 mL), 50:50 (400 mL), 40:60 (400 mL), 30:70 (200 mL), 20:80 (200 mL), 10:90 (200 mL), 20:80 (200 mL), 10:90 (200 mL)), and then solvent systems of EtOAc/MeOH (100:0 (200 mL), 90:10 (400 mL), 80:20 (400 mL), 70:30 (200 mL), 50:50 (200 mL), and 30:70 (200 mL)). Fractions were pooled into 26 sub-fractions (E1P21SF1-SF26) based on TLC analysis (i.e., comparing $R_f$ value and colour of the bands observed after developing). A 40 mg portion of E1P21SF-3 was purified by semi-preparative NP HPLC for 20 min (hexane/isopropanol, with 98% isocratic run and a flow rate of 2.5 mL min$^{-1}$; collecting 5 mL per tube) to yield **3** (17.0 mg, Rt = 17.0 min). A 40 mg sample of E1P21SF6 was purified by semi-preparative NP HPLC for 25 min (hexane/isopropanol, with 98% isocratic run and a flow rate of 2.5 mL min$^{-1}$; collecting 5 mL per tube) to yield (+)-**8** (3 mg, Rt = 9.8 min) and (+)-**9** (2.8 mg, Rt = 10.3 min).

### *D. rhadinostachya* fractionation and isolation

An 834 g sample was extracted as described above and extracted to give 6.4 g of crude extract. This was fractionated by using flash chromatography with NP silica 60. A series of solvent gradients was applied containing 90:10 (600 mL), 80:20 (600 mL), 70:30 (600 mL), 60:40 (400 mL), 50:50 (400 mL), 40:60 (400 mL), 30:70 (200 mL), 20:80 (200 mL), 10:90 (200 mL), 20:80 (200 mL), 10:90 (200 mL) PE/EtOAc, and solvent systems of 100:0 (200 mL), 90:10 (400 mL), 80:20 (400 mL), 70:30 (200 mL), 50:50 (200 mL) and 30:70 (200 mL) of EtOAc/MeOH. Fractions were pooled into 26 sub-fractions (E2P9SF1-SF22) based on TLC analysis. A 28 mg sample of E2P9SF14 was purified by semi-preparative RP HPLC ($MeCN/H_2O$, with 50–30% MeCN for 25 min and a flow rate of 3 mL min$^{-1}$; collecting 5 mL per tube) to yield **10** (4.1 mg, Rt = 7.6 min). A 30 mg of E2P9SF19 was purified initially by semi-preparative RP HPLC ($MeCN/H_2O$, with 50–30% MeCN for 15 min with a flow rate of 2.5 mL min$^{-1}$; collecting 5 mL per tube) to yield a mixture (2.5 mg, Rt = 29.3 min). This was then subjected to further purification

through semi-preparative NP HPLC (hexane/isopropanol, with 90–80% for 20 min, followed by an 80% isocratic run for 10 min and a further 90% isocratic run for 5 min with a flow rate of 2.0 mL $min^{-1}$; collecting 5 mL per tube) to yield **11** (1.2 mg, Rt = 28.5 min). (+)-Humulene diepoxide C-I [(+)-**8**] and C-II [(+)-**9**] were also isolated from this plant (from E2P9SF-5-E2P9SF-7) in the same manner as described for *D. kalpari*.

### Hops extraction

Whole cone Chinook (bale format) from the 2015 harvest (Cornerstone Ranches, Washington State) was extracted by using the ASBC Hops-13 method of hydrodistillation.[24] In brief, coarsely ground hops (≈105 g) were boiled in distilled water (3 L) for 3 h.[25] Post-distillation, the total hop oil (≈1.72 mL oil per 100 g hop) was collected in amber vials with foil-lined closures (2.5 mL). After filling the amber vials, the access water was removed, the vial headspace was flushed with nitrogen and then capped. The hop oil was stored at −20 °C until subsequent analysis.

### Gas chromatography (GC) analysis of the hops

A 3.00 μL aliquot of crude hops oil in addition to samples containing either a humulene diepoxide C-enriched fragment (E2P9SF5-SF7), (+)-humulene diepoxide C-I [(+)-**8**] or C-II [(+)-**9**] was dissolved in *n*-hexanes (up to 1.00 mL). These samples, in addition to a C-7-C-30 alkane ladder (1000 μg $mL^{-1}$ in *n*-hexane) were run with a split injection (1.00 μL injection, with either 1:10 or 1:5 split ratio) on a Shimadzu QP2020 NX GC-MS with He as carrier gas, equipped with an Rxi-5ms column (30 m, 0.25 mm ID, 0.25 μM $d_f$). The injection port and interface temperatures were set to 250 °C, and oven set to 50 °C, ramping up to 250 °C at a rate of either 1.0 or 0.67 °C $min^{-1}$. A further two samples of crude Chinook hop oil (1:100 in *n*-hexane) spiked with 10 and 20 μL of a 1:100 *n*-hexane solution of E2P9SF5-SF7, (+)-humulene diepoxide C-I [(+)-**8**] or C-II [(+)-**9**] were run in the same manner. Retention indices were determined through GC-FID on a Shimadzu QC2030, equipped with an Rxi-5 ms column (30 m, 0.25 mm ID, 0.25 μM $d_f$) by using conditions identical to that used for GC-MS analyses, but with a 0.67 °C $min^{-1}$ ramp rate. (+)-Humulene diepoxide C-I and C-II were detected by using GC-MS with a Rt of 89.65 min (Kováts RI: 1726) and 90.99 min (Kováts RI: 1737).

### Antifungal assays

Cell-based antifungal assays were performed according to CLSI broth microdilution protocols against *Candida albicans* (ATCC 90028), *Candida glabrata* (ATCC 90030) and *Candida krusei*, (ATCC 6258).[26] In addition, two *Candida auris* strains were obtained from the Westerdijk Fungal Biodiversity Institute, Netherlands for inclusion in testing. Of these, one (CBS 10913) was a drug-susceptible-type strain isolated from a patient in Japan, and the other (CBS 12373) was an azole resistant strain isolated in Korea. All assays were performed by using YNB broth (without amino acids and ammonium sulfate) supplemented with 2% glucose, 100 mM $(NH_4)_2SO_4$, and 82.1 mM citrate-phosphate buffer (final concentration 64.2 mM $Na_2HPO_4$/ 17.9 mM citric acid).

### Cell proliferation assays

Cells (3000 well) seeded in triplicate in 96-well plates in (RPMI)-1640 medium containing 423 μM (final) equivalent of compound at the top well. Then, 1 in 10 dilutions to the bottom were made, reserving last row as no treatment control. After six days incubation at 37 °C, the sulforhodamine B (SRB) assay was performed according to previously reported inhouse methods.[26]

### Diepoxide A (3)

Isolated as a colourless oil; $^1$H NMR ($CDCl_3$, 500 MHz): $\delta$ = 5.52–5.44 (m, 1H), 5.36 (d, $J$ = 12.0 Hz, 1H), 2.74 (dd, $J$ = 10.4, 5.2 Hz, 1H), 2.65 (dd, $J$ = 12.2, 5.3 Hz, 1H), 2.49 (d, $J$ = 10.2 Hz, 1H), 2.09–2.23 (m, 2H), 1.65 (t, $J$ = 11.3 Hz, 1H), 1.61 (d, $J$ = 14.5 Hz, 1H), 1.41–1.36 (2H, m), 1.31 (s, 3H), 1.20 (s, 3H), 1.09–1.11 (m, 1H), 1.08 (s, 3H); $^{13}$C NMR ($CDCl_3$, 175 MHz): $\delta$ = 142.9, 122.6, 64.7, 63.3, 60.3, 60.1, 43.3, 38.3, 35.7, 34.9, 30.7, 25.2, 23.4, 16.5, 16.4 ppm; LR-ESI-MS: $m/z$: 479.3 $[M+Na]^+$.

### Humulene diepoxide C-I [(+)-8]

Isolated as colourless needles; m.p. 93–95 °C; $[\alpha]_D^{23}$ = +126.9 ($c$ = 0.12 in $CDCl_3$); IR: $\tilde{\nu}$ = 2961, 2919, 2864, 1388, 838 $cm^{-1}$; UV (MeOH): $\lambda_{max}$ (log $\varepsilon$) = 209 (0.87), 233 (0.12), 287 nm (0.03).

### Humulene diepoxide C-II [(+)-9]

Isolated as colourless needles; m.p. 115–116 °C; $[\alpha]_D^{23}$ = +34.2 ($c$ = 0.13 in $CDCl_3$); IR: $\tilde{\nu}$ = 2962, 2933, 2865, 1388, 838 $cm^{-1}$; UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) = 212 (1.07), 234 (0.40), 287 nm (0.10).

### Humulene diepoxiol C-I (10)

Isolated as colourless needles; $[\alpha]_D^{23}$ = +178.4 ($c$ = 0.02 in $CDCl_3$); IR: $\tilde{\nu}$ = 3432, 2958, 2927, 1456 $cm^{-1}$; UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) = 211 (1.03), 238 nm (0.59).

### Humulene diepoxiol C-II (11)

Isolated as a colourless oil; $[\alpha]_D^{23}$ = +4.3 ($c$ = 0.004 in $CDCl_3$); IR: $\tilde{\nu}$ = 3413, 2958, 2928, 1451, 1221 $cm^{-1}$; UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) = 213 (1.22), 231 (0.94), 281 nm (0.35).

## Acknowledgements

The authors thank the University of Queensland (UQ) and the QIMR Berghofer Medical Research Institute for financial support. The assistance of Dr. T. Le from UQ for assistance with NMR measurements, S. Hume from Welford National Park (Queensland Parks & Wildlife Service Department of Environment and Science), and A. Barnett from UQ for assistance with fractionation of one crude extract are gratefully acknowledged. C.M.W is grateful to the Australian Research Council for a Future Fellowship award (grant number FT110100851) and for assistance provided by the Queensland Herbarium. In addition, we thank Prof. R. Henry and G. Fox from the Queensland Alliance for Agriculture and Food Innovation (UQ), and Prof. B. Carroll and Dr. S. Fletcher from the School of Chemistry and Molecular Biosciences, for helpful discussions regarding phylogenetics and hops. We also thank Deschutes Brewery (Oregon, U.S.A.) for supplying aged hops.

## Conflict of interest

The authors declare no conflict of interest.

**Keywords:** diepoxides · epoxides · hops · humulenes · natural products

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Manuscript received: September 26, 2019
Revised manuscript received: November 13, 2019
Version of record online: January 21, 2020