*SHORT REPORT*

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# Heracleumate, A New Enynic Ester from the Roots of *Heracleum rapula* Franch

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**Abstract:** To discover new secondary metabolites and reveal the anti-inflammation effective ingredients of *Heracleum rapula*, a phytochemical investigation was performed, leading to one new enynic ester, heracleumate (**1**), and four polyacetylene diols, 9-epoxyfalcarindiol (**2**), 3(*R*), 8(*S*)-falcarindiol (**3**), oplopantriol B (**4**), and 18-*O*-acetyloplopantriol B (**5**) were isolated and identified. Their structures were established by spectroscopic analysis. Compounds **1**, **2**, **4**, and **5** exhibited NO inhibition activities with $IC_{50}$ values of 48.1 ± 1.9, 95.8 ± 6.2, 18.9 ± 2.0, and 88.7 ± 7.1 $\mu$M in RAW 264.7 cells treated with lipopolysaccharide. Among them, oplopantriol B (**4**) exhibited the best NO inhibition activity. Additionally, compounds **1**, **2**, and **4** can significantly reduce the levels of inflammatory cytokines, including COX-1, IL-6, iNOS, and TNF-$\alpha$.

**Keywords:** *Heracleum rapula* Franch; enynic ester; polyacetylene diol; anti-inflammation. 

## 1. Plant Source

The roots of *Heracleum rapula* Franch. (Apiaceae) were purchased from the Kunming Luosiwan medicinal material market in Yunnan Province, China. Prof. Yong Xiong, a botanist at the School of Ethnic Medicine, Yunnan Minzu University, authenticated them, and the voucher specimen (20231105) was kept at the department mentioned above.

## 2. Previous Studies

The roots of *Heracleum rapula* Franch. is a Yi ethnic medicine in the Yunnan province of China, with a background of folk and clinical application for thousands of years, and is widely used to treat rheumatic arthralgia, myalgia, chronic bronchitis, traumatic injury, and other conditions [1]. Modern pharmacological research indicates that the ethanol extract of the roots of *H. rapula* possesses diversified biological activities [2–5], including analgesia, anti-inflammatory, spasmolysis, and anti-rheumatoid arthritis properties. Phytochemical research has shown that coumarins [6,7] and steroids [7] are present in this plant, with coumarins being the main ingredients. Although the anti-platelet aggregation activity of the coumarins and their glycosides from the roots of *H. rapula* has been reported, an investigation of its anti-inflammatory effective ingredients has not been reported.

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## 3. Present Study

To discover new secondary metabolites and reveal the anti-inflammatory effective ingredients of *H. rapula* for illustrating its clinical application, a phytochemical separation of its ethanolic extract was performed, resulting in one new enynic ester, heracleumate (**1**), and four polyacetylene diols, 9-epoxyfalcarindiol (**2**) [8], 3(*R*), 8(*S*)-falcarindiol (**3**) [9], oplopantriol B (**4**) [10], and 18-*O*-acetyloplopantriol B (**5**) [10] were isolated and identified (Figure 1). Notably, they were first isolated from *H. rapula*, and compounds **2** and **4** were first isolated from the genus of *Heracleum*.

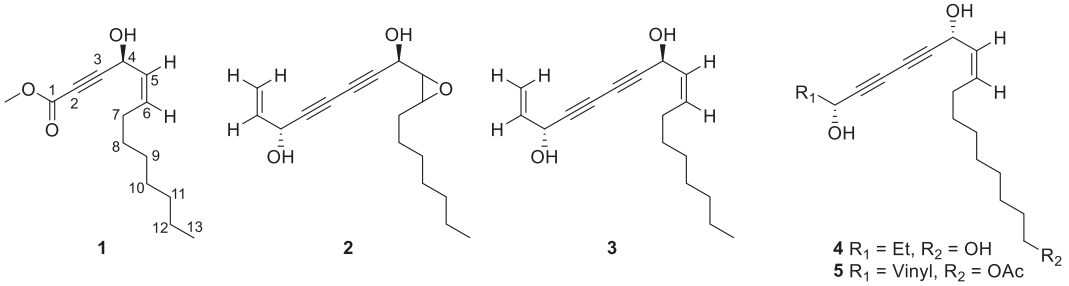


**Figure 1.** Structures of isolated compounds **1–5**

*Extraction and Separation*: The dried powder from the roots of *H. rapula* (20 kg) was soaked in 95% ethanol (50 L) and extracted under reflux three times consecutively to obtain the ethanol extract. After concentration, a residue weighing 1.31 kg was obtained. Then, the residue suspended in water (6 L) was extracted with organic solvents, petroleum ether (PE, 6 L × 3), EtOAc (EA, 6 L × 3), and *n*-butanol (6 L × 3), leading to three fractions of PE (169.4 g), EA (510.8 g), and *n*-butanol (523.6 g) were obtained. The silica gel column chromatography (SGCC) was used to separate the EA extracted fraction (510.8 g), resulting in five fractions: I (147.5 g), II (96.9 g), III (66.8 g), IV (32.6 g), V (52.6 g) employing $CH_2Cl_2$/MeOH (60:1, 30:1, 15:1, 8:1, 5:1, 2:1, 0:1; v/v; each 50 L) as the eluent. Subsequently, using a gradient solvent of $CH_2Cl_2$/MeOH (from 1:0 to 0:1; *v/v*; each 3 L) as the eluent for the separation of fraction II (96.9 g) by SGCC, resulting in fractions II1 (14.3 g), II2 (54.1 g), and II3 (24.8 g). Employing PE/EA (30:1, 20:1, 10:1, 5:1, 0:1; v/v; each 300 mL) as the eluent, fraction II1 (457.9 mg) was eluted by SGCC, yielding compounds **1** (44 mg) and **3** (107 mg). Similar to fraction II1, fraction II3 (24.8 g) was also eluted by SGCC, yielding compound **2** (59 mg). Subsequently, using $CH_2Cl_2$/MeOH (60:1, 40:1, 20:1, 10:1, 5:1, 0:1; *v/v*; each 3 L) as the eluent for the separation of fraction III (66.8 g) by SGCC, resulting in fractions III1 (26.5 g), III2 (18.1 g), and III3 (13.8 g). Then, a gradient solvent composed of PE/EA (20:1, 15:1, 10:1, 5:1, 0:1; v/v; each 3 L) was used as the eluent for the separation of fraction III1 (26.5 g) by SGCC, yielding compound **5** (4.6 mg). Fraction III3 (13.8 g) was fractioned by SGCC using PE/EA (10:1, 5:1, 2:1, 0:1; v/v; each 3L) as eluent, resulting in fractions III3-1 (3.7 g), III3-2 (2.6 g), and III3-3 (1.1 g). Using $CH_2Cl_2$/MeOH (1:1; v/v; each 3 L) as eluent, fraction III3-2 was separated by Sephadex LH-20, yielding compound **4** (112 mg).

*Heracleumate (**1**)*: Pale yellow oil, $[\alpha]_D^{28.1}$ − 46.0 (*c* 0.02, MeOH); CD (*c* 0.06, MeOH) $\lambda_{max}$ ($\Delta\varepsilon$) 216 (14.52) nm; IR (KBr) $\nu_{max}$: 3441, 2969, 2831, 2717, 2661, 2051, 2026, 1631, 1606, 1509, 1439, 1385, 1364, 1273, 1108, 1068, 860, 776, 683, 668, 638, 608, and 571 $cm^{-1}$; the $^1H$ and $^{13}C$-NMR data were shown in Table 1; HRESIMS: *m/z* 239.1641 $[M + H]^+$ (calculated for $C_{14}H_{23}O_3^+$, *m/z* 239.1641).

*ECD calculation:* The ECD for compound **1** was studied using DFT, where the structures were optimized at the B3LYP/6-31G(d) level using Gaussian 09. Then, 13 energy-minimized conformers were chosen for ECD calculations. For the ECD calculations, it was performed at B3LYP/6-311G(d,p) level of theory based on the optimized structures, and methanol was chosen as the solvent.

*Inhibition of NO production assay:* According to a reported method [11,12], the inhibitory effect of the isolated compounds on the production of nitric oxide (NO) was evaluated in RAW264.7 cells with a slight modification. After treating with lipopolysaccharide (LPS), each well was added the tested compounds (6.25, 12.5, 25, 50, and 100 $\mu$M) and NG-monomethyl-L-arginine acetate (L-NMMA, 3.13,

6.25, 12.5, 25, and 50 $\mu$M). After cultivating for 24 h, the Griess reagents were used to determine the level of NO.

**Table 1.** $^{1}$H (600 MHz) and $^{13}$C-NMR (150 MHz) data of compound **1** ($CDCl_3$)

| Position | $\delta_C$, mult. | $\delta_H$ (*J* in Hz) |
|---|---|---|
| 1 | 153.9, C | |
| 2 | 76.2, C | |
| 3 | 87.0, C | |
| 4 | 58.1, CH | 5.25, d (8.4) |
| 5 | 126.8, CH | 5.54, ddt (10.4, 8.4, 1.6) |
| 6 | 135.8, CH | 5.67, dtd (10.4, 7.5, 1.2) |
| 7 | 27.9, $CH_2$ | 2.14–2.10, m |
| 8 | 29.4, $CH_2$ | 1.40–1.37, m |
| 9 | 29.3, $CH_2$ | 1.33–1.25[a] |
| 10 | 29.2, $CH_2$ | 1.33–1.25 [a] |
| 11 | 31.9, $CH_2$ | 1.33–1.25 [a] |
| 12 | 22.8, $CH_2$ | 1.33–1.25 [a] |
| 13 | 14.2, $CH_3$ | 0.88, t (6.9) |
| OMe | 53.0, $CH_3$ | 3.78, s |

[a]Overlapped signal.

*Measurement of COX-1, iNOS, TNF-α, and IL-6:* According to the method mentioned above, LPS was used to stimulate RAW264.7 cells, and then the tested compounds (6.25, 12.5, 25, 50, and 100 $\mu$M) were added to each well. Then, the levels of COX-1, iNOS, TNF-$\alpha$, and IL-6 were measured by using ELISA kits.

Compound **1**, isolated as a pale yellow oil, has a molecular formula $C_{14}H_{22}O_3$, which was established by observing the ion peak at *m/z* 239.1641 $[M + H]^+$ in HRESIMS spectrum (Figure S2, calculated for $C_{14}H_{23}O_3^+$ 239.1642), which shows unsaturation degrees of four. In the $^{1}$H NMR spectrum (Figure S5), two olefinic proton signals [$\delta_H$ 5.67 (1H, dtd, $J$ = 10.4, 7.5, 1.2 Hz, H-6) and 5.54 (1H, ddt, $J$ = 10.4, 8.4, 1.6 Hz, H-5)], one oxymethine proton signal [$\delta_H$ 5.25 (1H, d, $J$ = 8.4 Hz, H-4)], one methoxy signal signal [$\delta_H$ 3.78 (3H, s, OMe)], six methylene signals [$\delta_H$ 2.14 − 2.10 (2H, m, H-7), 1.40 – 1.37 (2H, m, H-8), 1.33 − 1.25 (8H, overlapped), and one methyl signal [$\delta_H$ 0.88 (3H, t, $J$ = 6.9 Hz, $H_3$-13)] were observed. $^{13}$C NMR spectrum (Figure S6) showed 14 carbon signals, comprising one carbonyl group ($\delta_C$ 153.9), a double bond ($\delta_C$ 135.8, 126.8), a triple bond ($\delta_C$ 87.0, 76.2), an oxygenated methine ($\delta_C$ 58.1), a methoxy carbon ($\delta_C$ 53.0), six methylene carbons ($\delta_C$ 31.9, 29.4, 29.3, 29.2, 27.9, 22.8), and one methyl carbon ($\delta_C$ 14.2). The NMR data mentioned above suggested that compound **1** possesses a similar planar structure to (4*R*)-4-hydroxy-dodec-5-en-2-ynoic acid methyl ester reported by Trost et al. (2012) [13]. The difference is that more than one methylene is present in compound **1**. The presence of an aliphatic chain with a double bond was demonstrated by the $^{1}$H−$^{1}$H COSY correlations, as shown in Figure 2 (Figure S7). The HMBC correlations from OMe to $\delta_C$ 153.9 and $\delta_C$ 76.2 indicate that the OMe is connected with C-1 to form a methyl ester unit (Figures 2 and S9-3). The positions of the double bond and OH group were confirmed by the HMBC correlations (Figures 2, S9-1, S9-2, and S9-4) from $\delta_H$ 5.25 (H-4) to $\delta_C$ 135.8 (C-6), 126.8 (C-5), 87.0 (C-3), and 76.2 (C-2); from $\delta_H$ 5.54 (H-5) to C-6; from $\delta_H$ 5.67 (H-6) to $\delta_C$ 29.4 (C-8) and 22.8 (C-7); and from H-7 to C-5 and C-6. Then, the planar structure of compound **1** was established. The *cis* configuration of the $\Delta^{5,6}$ double bond in compound **1** was confirmed by comparing the coupling constants of the H-5 and H-6 between compound **1** and (4*R*)-4-hydroxy-dodec-5-en-2-ynoic acid methyl ester (Table S1), which is supported by the NOESY correlation (Figures 2 and S10-1) between $\delta_H$ 5.25 (H-4) and $\delta_H$ 2.14-2.10 (H-7). In addition, comparing the optical rotation value of compound **1** ($[\alpha]_D^{28.1}$ − 46.0, *c* 0.02, MeOH) with that of (4*R*)-4-hydroxy-dodec-5-en-2-ynoic acid methyl ester ($[\alpha]_D^{25}$ + 40.1, *c* 1.87, $CHCl_3$) [13] demonstrated that the absolute configuration at C-4 position was *S*. Meanwhile, the absolute configuration of **1** was also determined

by comparing the calculated ECD of two possible isomers, 4*R*-**1** and 4*S*-**1**, with the experimental ECD. The calculated ECD of 4*S*-**1** matched well with the measured one (Figure 3). Herein, the identification of compound **1** was successfully achieved, and it was named heracleumate.

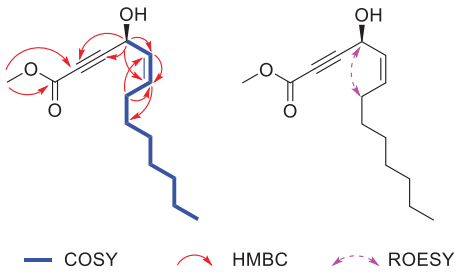


**Figure 2.** Key 2D NMR correlations of compound **1**

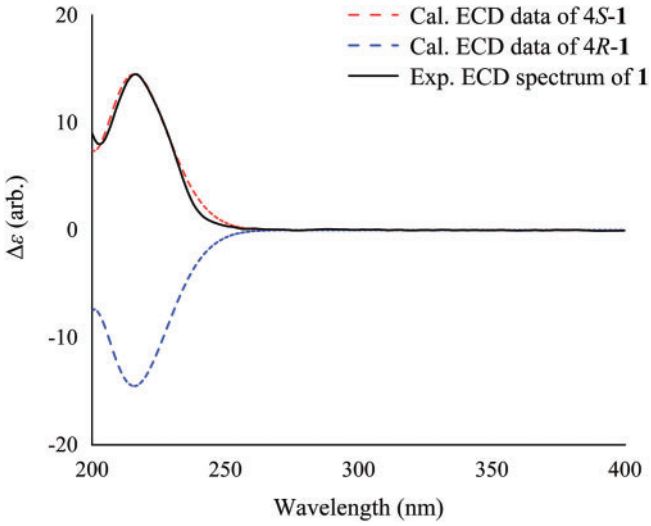


**Figure 3.** Experimental and calculated ECD curves of **1**

To elucidate the effective anti-inflammatory constituents from *H. rapula*, the effects of isolated compounds **1**, **2**, **4**, and **5** on NO inhibition were assessed using the LPS-stimulated RAW 264.7 cells assay, since compound **3** possesses significant anti-inflammatory activity [14]. It was demonstrated that L-NMMA (a positive control) showed significant NO inhibition activity (Table 2). Compounds **1**, **2**, **4**, and **5** exhibited NO inhibition activities with $IC_{50}$ values of 48.1 ± 1.9, 95.8 ± 6.2, 18.9 ± 2.0, and 88.7 ± 7.1 $\mu$M, respectively. Meanwhile, they also showed 50% cytotoxic concentration ($CC_{50}$) values greater than 200 $\mu$M.

**Table 2.** NO inhibition effects of compounds **1, 2, 4,** and **5**

| Compound | $CC_{50}$ ($\mu$M) | $IC_{50}$ ($\mu$M) |
|---|---|---|
| **1** | 215.3 ± 6.0 | 48.1 ± 1.9 |
| **2** | >400.0 | 95.8 ± 6.2 |
| **4** | 236.7 ± 13.6 | 18.9 ± 2.0 |
| **5** | >400.0 | 88.7 ± 7.1 |
| L-NMMA | >400.0 | 14.0 ± 1.3 |

Additionally, the effects of compounds **1**, **2**, and **4** on the levels of inflammatory cytokines were also evaluated. It was demonstrated that the levels of inflammatory cytokines, including COX-1,

iNOS, IL-6, and TNF-$\alpha$, were clearly increased by treating with LPS (Figure 4). However, they were significantly decreased by the treatment of compounds **1**, **2**, and **4** in a concentration-dependent manner (Figure 4). Compound **4** could outstandingly inhibit the levels of COX-1, iNOS, IL-6, and TNF-$\alpha$, with inhibitions of 50.3%, 70.6%, 61.8%, and 19.7%, respectively, at a concentration of 100 $\mu$M, implying its potential anti-inflammatory activity.

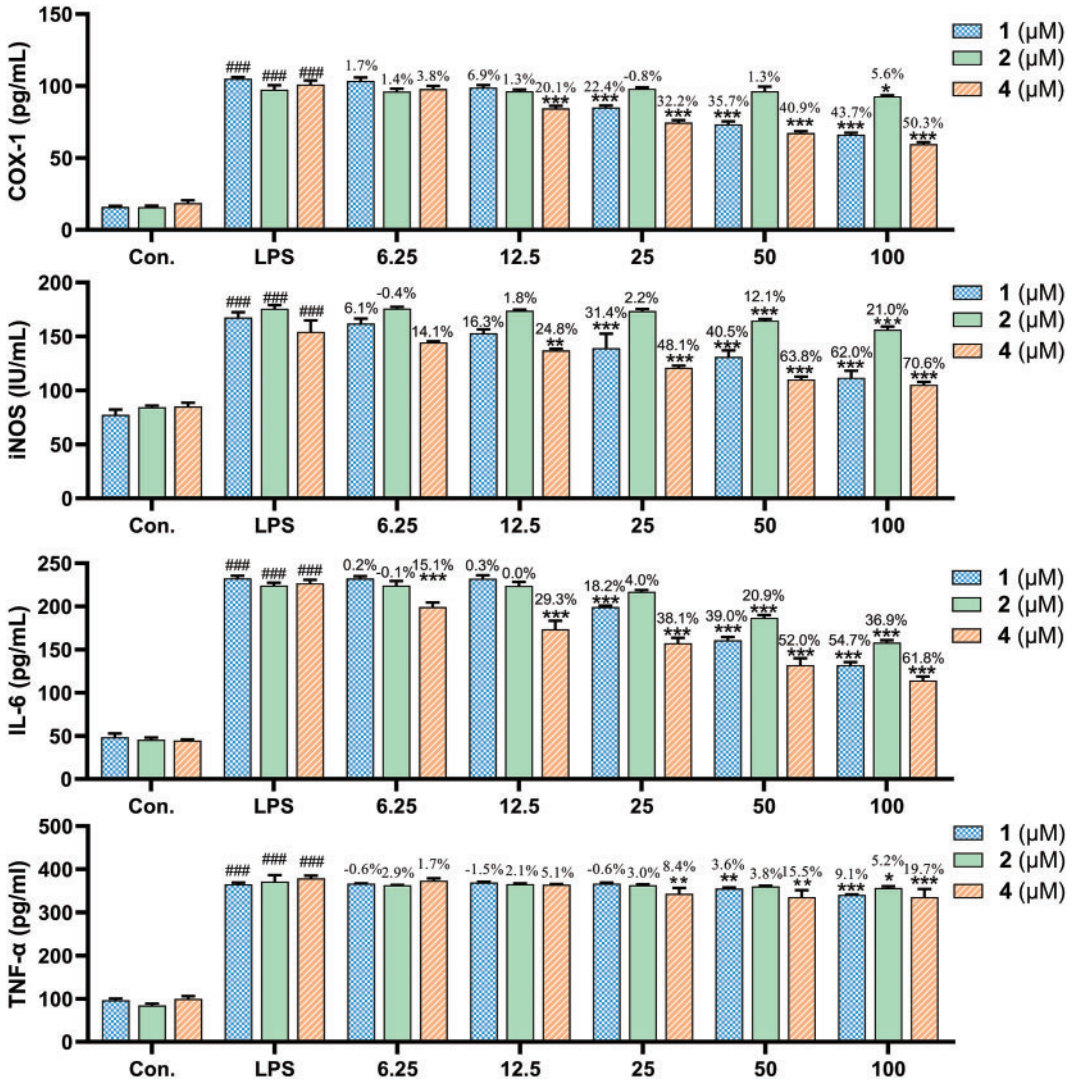


**Figure 4.** Anti-inflammatory effects of compounds **1**, **2**, and **4** *in vitro*. Data are expressed as mean ± s.e.m. ($n = 3$). $^{###}p < 0.001$ vs control group; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs LPS-treated group

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## Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products.

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