

## Cytokine-Suppressive Activity of a Hydroxylated Alkylamide from *Echinacea purpurea*

Martha Leyte-Lugo<sup>1</sup>, Daniel A. Todd<sup>1</sup>, Travis V. Gulledge<sup>2</sup>, Monika Juzumaite<sup>2</sup>, Frederick S. Carter<sup>1</sup>, Scott M. Laster<sup>2</sup>, Nadja B. Cech<sup>1</sup>

<sup>1</sup> Department of Chemistry and Biochemistry, The University of North Carolina at Greensboro, Greensboro, NC, United States

<sup>2</sup> Department of Biological Sciences, North Carolina State University, Raleigh, NC, United States

### Abstract

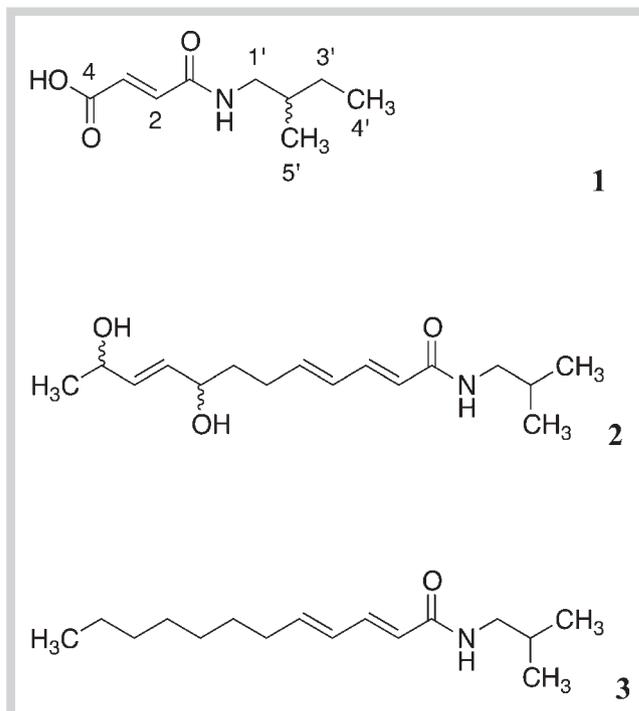
*Echinacea purpurea* has been widely used to treat upper respiratory tract infections. Bioassay-guided fractionation of hexane and ethanol extracts of this plant yielded 4[(2-methylbutyl)amino]-4-oxo-2-butenoic acid (**1**) and 8,11-dihydroxy-dodeca-2*E*,4*E*,9*E*-trienoic acid isobutylamide (**2**), both of which are new to *Echinacea*. Compound **2** suppressed the production of TNF- $\alpha$  from RAW 264.7 cells with an IC<sub>50</sub> value of 6.8  $\mu$ M, while **1** was inactive. Neither compound showed cytotoxicity at concentrations up to 100  $\mu$ M. These findings support a growing body of literature demonstrating that *E. purpurea* contains an array of compounds that suppress cytokine secretion by macrophage-type cells *in vitro*.

### Key words

*Echinacea purpurea* · Asteraceae · alkylamides · alkamides · anti-inflammatory · tumor necrosis factor- $\alpha$

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Preparations from *Echinacea purpurea* (L.) Moench (Asteraceae) are used in the treatment and prevention of upper respiratory infections [1]. The mechanisms that underlie the purported efficacy of *E. purpurea* have been subject to debate. However, recent studies have indicated that some *E. purpurea* extracts and constituents possess *in vitro* activity consistent with an anti-inflammatory effect [2, 3]. Along these lines, our group has shown that *E. purpurea* root extracts suppress the secretion of inflammatory cytokines by cultured immune cells. The activity of the extracts could not entirely be explained by the presence of known compounds [4, 5]. Thus, we sought to isolate and identify additional compounds with cytokine-suppressive activity from *E. purpurea*. Bioassay-guided fractionation of *E. purpurea* root extracts yielded two alkylamides new to *E. purpurea* (Fig. 1). From a hexane extract, 4[(2-methylbutyl)amino]-4-oxo-2-butenoic acid (**1**) was isolated, while a mixture of diastereoisomers (as indicated by LC-MS, Fig. 1 S, Supporting Information), 8,11-dihydroxy-dodeca-2*E*,4*E*,9*E*-trienoic acid isobutylamide (**2**), was obtained from an aqueous ethanol extract. Compound **1** is commercially available as a synthetic compound (Aldlab Chemicals, Woburn, MA; Aurora Building Blocks, San Diego, CA), but its characterization has not been described and it has not, to our knowledge, been reported in the literature. Compound **2** is a mixture of diastereoisomers, for which the NMR spectroscopic data matches



**Fig. 1** Alkylamides from the roots of *E. purpurea*. Two compounds were isolated and identified, 4[(2-methylbutyl)amino]-4-oxo-2-butenoic acid (**1**) and 8,11-dihydroxy-dodeca-2*E*,4*E*,9*E*-trienoic acid isobutylamide (**2**). The structure of an additional known alkylamide from *E. purpurea*, dodeca-2*E*,4*E*-dienoic acid isobutylamide (**3**), is shown for comparison.

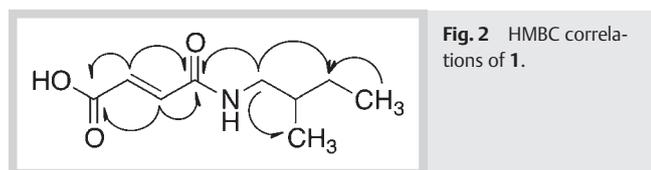
that of a known constituent of *Spilanthes callimorpha* A.H. Moore (Asteraceae), 8,11-dihydroxy-dodeca-2*E*,4*E*,9*E*-trienoic acid isobutylamide [6]. Interestingly, although at least 17 different alkylamides have been reported from *E. purpurea* [4, 7–12], **1** and **2** are the first carboxylated and hydroxylated alkylamides, respectively, to be identified from this species. Importantly, although **1** and **2** were originally isolated and purified from extracts of different polarity (hexane and aqueous ethanol, respectively), both of these compounds were detected in both the hexane and aqueous ethanol crude extracts (Fig. 2 S, Supporting Information). The presence of **1** and **2** in the aqueous ethanol extract is particularly interesting given that it is common to use aqueous ethanol extracts of *E. purpurea* as dietary supplements. However, it should also be noted that **1** and **2** appear, based on LC-MS data (Fig. 2 S, Supporting Information), to be present only at relatively low levels compared to other alkylamides.

Compound **1** was obtained as a yellow powder with a molecular formula of C<sub>9</sub>H<sub>15</sub>NO<sub>3</sub> at *m/z* 186.1126 (calcd. for [M + H]<sup>+</sup>, C<sub>9</sub>H<sub>16</sub>NO<sub>3</sub>, 186.11302), indicating three degrees of unsaturation. Spectral data (<sup>1</sup>H and <sup>13</sup>C NMR, Table 1) revealed that the compound is an  $\alpha$ -unsaturated N-methylbutylamide. Characteristic <sup>1</sup>H-NMR signals at  $\delta$  3.10 (dd, H-1'), 1.56 (m, H-2'), and ~0.88 (H-4' and H-5') further supported a methylbutylamide moiety, as did the HMBC correlations observed from H-1' to C-1, C-3', and C-5' (Fig. 2). Signals of olefinic protons at  $\delta$  6.92 (d, H-2) and 6.64 (d, H-3) confirmed the presence of one double bond conjugated with the amide carbonyl. The *E* configuration was assigned based on the coupling constants (*J* = 15.6). The terminal carboxylic acid was confirmed with the HMBC correlations from H-2 to C-4, and from H-3 to C-1 and C-4 (Fig. 2).

**Table 1** NMR spectroscopic data<sup>a</sup> (400 MHz for <sup>1</sup>H and 100 MHz for <sup>13</sup>C) for 4[(2-methylbutyl)amino]-4-oxo-2-butenic acid (**1**).

Position	$\delta_{\text{H}}$ , m (J in Hz)	$\delta_{\text{C}}$
1		166.6
2	6.92, d (15.6)	137.1
3	6.64, d (15.2)	132.3 <sup>b</sup>
4		168.1
1'	3.10, dd (7.2, 6.0)	46.4
2'	1.56, m	36.1
3'	1.39, m	28.1
1.13, m		
4'	0.87, t (7.6)	11.6
5'	0.88, d (6.4)	17.5

<sup>a</sup> <sup>1</sup>H and <sup>13</sup>C chemical shifts with reference to methanol-*d*<sub>4</sub> ( $\delta_{\text{H}}$  = 3.31 ppm) and methanol-*d*<sub>4</sub> ( $\delta_{\text{C}}$  = 49.01 ppm), respectively; <sup>b</sup> Obtained from HSQC



**Fig. 2** HMBC correlations of **1**.

The inflammatory response is mediated, in part, by the secretion of proinflammatory cytokines by macrophages. Thus, to explore potential anti-inflammatory activity of **1** and **2**, we evaluated their influence on the secretion of the cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) by RAW 264.7 macrophage-type cells *in vitro*. To mimic an inflammatory state, these cells were concomitantly treated with lipopolysaccharide (LPS), which induces the production of TNF- $\alpha$ . No significant suppression of LPS-induced TNF- $\alpha$  secretion was observed for **1**, while a dose-dependent suppressive effect was observed for **2** ( $\text{IC}_{50}$  = 6.8  $\mu\text{M}$ ) (● Fig. 3). The potency of **2** was higher than that of the positive control, dodeca-2E,4E-dienoic acid isobutylamide (**3**). Notably, the activity observed in ● Fig. 3 is not attributable to cytotoxic effects. The three

compounds tested were shown to have very little toxicity up to a concentration of 100  $\mu\text{M}$  in the lactate dehydrogenase (LDH) assay (Table 1 S, Supporting Information).

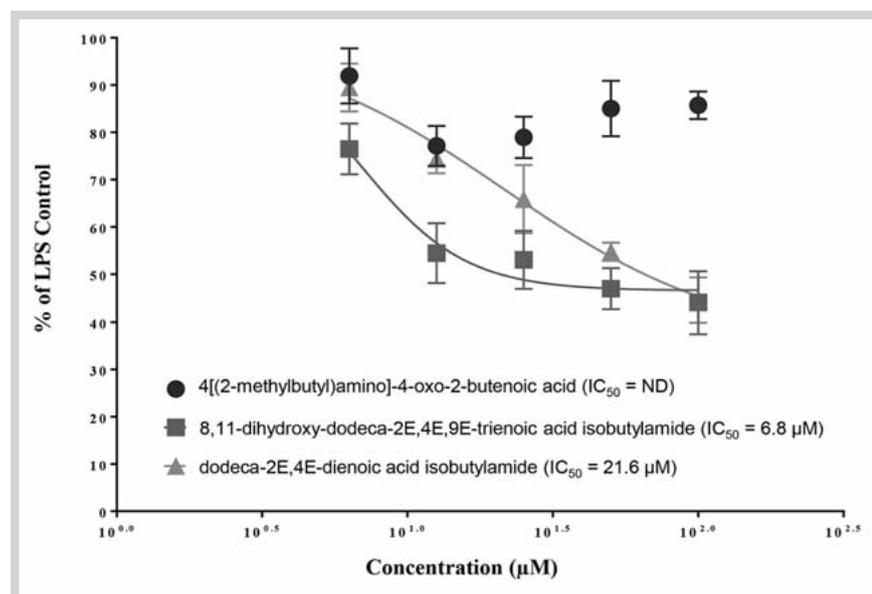
## Materials and Methods

Optical rotations of pure compounds were recorded in MeOH on a Rudolph Research Autopol (II) Polarimeter. NMR was conducted using a JEOL ECS-400 NMR spectrometer operating at 400 MHz for <sup>1</sup>H and 100 MHz for <sup>13</sup>C. High-resolution electrospray ionization mass spectrometry (HRESIMS) was performed on a Thermo LTQ Orbitrap XL mass spectrometer with an electrospray source. A Varian ProStar HPLC with a photodiode array detector was used with a Phenomenex Gemini-NX preparative C<sub>18</sub> column (5  $\mu\text{m}$ ; 250  $\times$  21.2 mm). Flash chromatography was performed on a Teledyne ISCO CombiFlash<sup>®</sup>R<sub>f</sub> using 120 g RediSep RF Gold<sup>®</sup> silica columns (20–40  $\mu\text{m}$  particle size). All reagents and solvents were obtained from Fisher Scientific.

The roots of *E. purpurea* were purchased from Pacific Botanicals in Grants Pass, Oregon and species was verified by Nadja B. Cech. In support of the correct assignment of the genus and species, the alkylamide profile for the samples under investigation, as reported in [5] and indicated in Fig. 2 S, Supporting Information, was consistent with that observed for *E. purpurea*. The alkylamide profile differs among *Echinacea* species [7], and is, therefore, a useful indicator for species identification. A voucher specimen (NCU633811) is deposited in the University of North Carolina Herbarium. Two extracts were prepared from these roots, one in hexane and the other in ethanol:water (75:25), as described previously [5].

The hexane extract (4.9 g) was fractionated via flash chromatography with a hexane/chloroform/methanol gradient (flow rate 20 mL/min) to yield 11 primary fractions (FI-FXI). Fraction VII (42 mg) was subjected to purification with reverse-phase preparative HPLC with acetonitrile/water following a gradient of 30–90% acetonitrile over 20 min. Compound **1** (4[(2-methylbutyl)amino]-4-oxo-2-butenic acid) eluted at 7 min (1.0 mg, 96% purity, Fig. 3 S, Supporting Information).

Residue from the ethanolic extract was fractionated into 13 fractions according to previously described methods [5]. Fractions 9



**Fig. 3** Influence of **1** and **2** on TNF- $\alpha$  secretion by murine RAW 264.7 macrophage-type cells. Included as a positive control is the *Echinacea* isobutylamide dodeca-2E,4E-dienoic acid isobutylamide (**3**), which has been previously shown to suppress TNF- $\alpha$  secretion in this assay [4]. The data presented are the means of two independent experiments, with error bars representing the standard deviation. Percent of control was determined according to the formula: compound treatment (ng/mL)/LPS treatment (ng/mL)  $\times$  100. Compounds **1–3** were shown not to be cytotoxic up to a concentration of 100  $\mu\text{M}$  (see Table 1 S, Supporting Information).

and 10 were combined based on a similar bioactivity and LC-MS profile for a combined yield of 747 mg. This combined sample was then separated with a hexane/chloroform/methanol gradient (flow rate 40 mL/min) and pooled into five fractions (A–E). Fraction C (330 mg) was subjected to the third stage of separation with a hexane/ethyl acetate/methanol gradient (flow rate 30 mL/min) and pooled into three fractions (I–III). Fraction II (50 mg) was then subjected to further purification with an acetonitrile/water gradient using reversed-phase preparative HPLC (40–50% acetonitrile over 20 min). Compound **2**, 8,11-dihydroxy-dodeca-2E,4E,9E-trienoic acid isobutylamide (1.9 mg, 97% purity as mixture of diastereomers, **Fig. 1 S**, Supporting Information) eluted at 6.4 min.

4[(2-methylbutyl)amino]-4-oxo-2-butenic acid (**1**): pale yellow amorphous powder;  $[\alpha]_D^{25} = +2.4$  (c 0.0025, MeOH);  $^1\text{H}$  (400 MHz) and  $^{13}\text{C}$  NMR (100 MHz) (**Table 1**; **Figs. 4 S** and **5 S**, Supporting Information); HRESIMS  $m/z$  186.1126  $[\text{M} + \text{H}]^+$  (calcd. for  $\text{C}_9\text{H}_{16}\text{NO}_3$  186.1125).

8,11-dihydroxy-dodeca-2E,4E,9E-trienoic acid isobutylamide (**2**): light yellow oil;  $^1\text{H}$  (400 MHz) and  $^{13}\text{C}$  NMR (100 MHz) data were consistent with the literature (**Table 2 S**, **Figs. 9 S** and **10 S**, Supporting Information) [8]; HRESIMS  $m/z$  282.2056  $[\text{M} + \text{H}]^+$  (calcd. for  $\text{C}_{16}\text{H}_{28}\text{NO}_3$  282.2064).

Cytokine assays were performed using RAW 264.7 cells in 96-well plates. TNF- $\alpha$  secretion was stimulated with LPS from *Salmonella minnesota* R595 (List Biological Laboratories) at 10 ng/mL. TNF- $\alpha$  levels were measured in supernatants after 18 h using an ELISA kit (R&D Systems). Dodeca-2E,4E-dienoic acid isobutylamide (**3**) (Chromadex) served as a positive control. To evaluate cytotoxicity, RAW 264.7 cells were seeded into 96-well plates, treated for 18 h, and the supernatants were analyzed for LDH activity using a commercially available kit (Thermo Fisher Scientific).

### Supporting information

NMR spectra, chromatograms for **1** and **2** and the crude *E. purpurea* extracts, and cytotoxicity results are available as Supporting Information.

### Acknowledgements

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### Conflict of Interest

The authors report no conflict of interest.

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### Correspondence

**Nadja B. Cech**

Department of Chemistry and Biochemistry  
The University of North Carolina at Greensboro  
301 Mclver St.  
Greensboro, NC 24702  
United States  
Phone: +1 336 334 3017  
Fax: +1 336 334 5402  
[nadja\\_cech@uncg.edu](mailto:nadja_cech@uncg.edu)

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