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Inhibition of Human Neutrophil Elastase by Sesquiterpene Lactone Dimers from the Flowers of *Inula britannica* S

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A new sesquiterpene lactone dimer [**1**], together with five known compounds (**2**–**6**), was isolated from the flowers of *Inula britannica*. The structures of these compounds were established by extensive spectroscopic studies and chemical evidence. The inhibitory activities of these isolated compounds (**1**–**6**) against human neutrophil elastase (HNE) were also evaluated in vitro; compounds **1** and **6** exhibited significant inhibitory effects against HNE activity, with IC_{50} values of 8.2 and 10.4 μM, respectively, comparable to that of epigallocatechin gallate (EGCG; IC_{50} = 10.9 μM). In addition, compounds **3** and **5** exhibited moderate HNE inhibitory effects, with IC_{50} values of 21.9 and 42.5 μM, respectively. In contrast, compounds **2** and **4** exhibited no such activity (IC_{50} > 100 μM). The mechanism by which **1** and **3** inhibited HNE was noncompetitive inhibition, with inhibition constant (K_i) values of 8.0 and 22.8 μM, respectively.

Keywords: *Inula britannica*, asteraceae, sesquiterpenes, human neutrophil elastase

Introduction

The genus *Inula* belongs to the family Asteraceae, which comprises about 100 species of perennial herbs distributed across warm and temperate parts of Asia, Europe, and Africa [1]. Many *Inula* species have been used as traditional herbal medicines throughout the world for the treatment of bronchitis, diabetes, intestinal ulcers, digestive disorders, and inflammation [2, 3]. Among them, *Inula britannica* Linnaeus is one of the most commonly used plants in traditional Chinese medicine. The flowers of this plant have long been used in traditional medicine to treat digestive disorders and various types of inflammation [4], and have been shown to possess various pharmacological activities, attributable to their antimicrobial, hepatoprotective, antidiabetic, hypolipidemic, antitumor, and anti-inflammatory properties [5–10]. Numerous bioactive substances, such as steroids, terpenoids (sesquiterpenes, diterpenes, and triterpenoids), phenolics, and flavonoids, have been isolated from *I. britannica* [11]. Of these, sesquiterpenes, and especially sesquiterpene lactones, have frequently been reported to be the active constituents, showing cytotoxic, apoptotic, and anti-inflammatory activities [10, 12–14].

In the course of searching for novel and naturally occurring inhibitors of human neutrophil elastase (HNE), we found that an EtOH extract of *I. britannica* flowers exhibited considerable HNE inhibitory activity (IC_{50} = 2.68 μg/ml). However, to date, there have been no reported studies of the HNE inhibitory activity of this plant or its secondary metabolites. Thus, we investigated the active compounds of *I. britannica* flowers, resulting in the isolation of a new sesquiterpene lactone dimer (**1**), together with five known compounds (**2**–**6**). This report describes the isolation and structural determination of these compounds, as well as the characterization of their HNE inhibitory effects.

Materials and Methods

General Experimental Procedures

Optical rotations were measured using a JASCO P-2000 digital polarimeter. The ECD spectra were measured on a JASCO J-715

spectrometer. ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) spectra were obtained using a Bruker DRX-400 spectrometer with tetramethylsilane as an internal standard. The 2D NMR experiments (COSY, HSQC, HMBC, and NOESY) were performed on a Bruker Avance 500 NMR spectrometer. HRESIMS was performed using a Shimadzu LCMS-IT-TOF spectrometer. Column chromatography was performed using silica gel (70–230 mesh; Merck, Germany) and YMC-gel ODS-A (S-75 μm; YMC, Japan). TLC was performed on pre-coated silica gel 60 F_{254} (0.25 mm; Merck, Germany) and RP-18 F_{254s} plates (0.25 mm; Merck, Germany). Spots were detected by UV light (254 nm) and spraying of 10% H_2SO_4 followed by heating. HPLC analysis was performed on an Agilent 1200 HPLC system with a binary pump (G1312A), a vacuum degasser (G1322A), a thermostatted column compartment (G1316A), a multiple wavelength detector (G1365B, MWD), and an autosampler (G1329A) using a Luna C18 column (250 × 4.6 mm, 5.0 μm; Phenomenex, USA).

Plant Material

The flowers of *I. britannica* were purchased from a traditional herbal medicine store in Daejeon, Republic of Korea, in August 2016, and identified by Prof. Ki Hwan Bae (College of Pharmacy, Chungnam National University, Republic of Korea). A voucher specimen (IB2016-001) has been deposited in the Herbarium of the Korea Institute of Oriental Medicine, Republic of Korea.

Extraction, Fractionation, and Isolation

The air-dried flowers of *I. britannica* (5 kg) were extracted using EtOH (50 L) at 80°C for 3 h, filtered, and concentrated to yield an ethanol extract (275 g, 5.5% yield). This extract was suspended in water (2.5 L) and partitioned successively using *n*-hexane (3 × 2.5 L), EtOAc (3 × 2.5 L), and *n*-BuOH (3 × 2.5 L) to afford *n*-hexane- (70 g), EtOAc- (110 g), and *n*-BuOH-soluble fractions (65 g), respectively. The EtOAc-soluble fraction (100 g), which significantly inhibited HNE activity, was subjected to silica gel column chromatography (70–230 mesh, 40 × 10 cm) and eluted with a gradient solvent system consisting of CH_2Cl_2:MeOH (100:1, 50:1, 20:1, 10:1, 1:1, 0:1; 3 L each). The resulting fractions from column chromatographic separation were combined into four fractions (A, 12 g; B, 10 g; C, 32 g; D, 12 g) based on the TLC results. These four column fractions inhibited HNE by 32%, 38%, 68%, and 41%, respectively, at 4 μg/ml. In this bioassay-guided study, the most active column fraction, C, was subjected to YMC RP-18 column chromatography (50 × 5 cm) and eluted with a MeOH:H_2O gradient (2:3, 1:1, 2:1, 4:1; 1 L each) to afford three subfractions [C1 (1 L, 5.3 g), C2 (1 L, 14.5 g), and C3 (2 L, 4.8 g)]. Fraction C1 (5.0 g) was subjected to further chromatography using a YMC RP-18 column (50 × 3 cm) and a MeOH:H_2O gradient (1:1, 2:1, 4:1; 1 L each) to obtain compounds **1** (39 mg) and **2** (374 mg). Chromatography of fraction C2 (14.0 g) on a YMC RP-18 column (50 × 5 cm) using a MeOH:H_2O gradient (1:1, 2:1, 4:1; 1 L each) afforded three subfractions [C2.1 (1 L, 3.2 g), C2.2 (1 L, 5.5 g), and C2.3 (1 L, 2.2 g)], one of which, C2.2 (5.0 g), was further chromatographed on a YMC RP-18 column (50 × 3 cm), eluting with a MeOH:H_2O gradient (1:1, 2:1, 4:1; 0.7 L each) to obtain compounds **3** (41 mg), **4** (65 mg), and **5** (21 mg). Fraction C3 (4.5 g) was chromatographed separately on a YMC RP-18 column (50 × 3 cm), eluting with a MeOH:H_2O gradient (1:1, 2:1, 4:1; 1 L each), to yield compound **6** (104 mg). The purity of the isolated compounds ranged from 95.0% to 99.4%, as determined by analytical HPLC [Agilent 1200 HPLC system; Luna C18 column (250 × 4.6 mm, 5.0 μm), Phenomenex; 70% acetonitrile in water; UV detection, 215 nm; flow rate, 1.0 ml/min].

Inulanolide E1 (1): white amorphous powder; $[\alpha]^{25}_{D}$ +99.2 (*c* 0.1, $CHCl_3$); UV (MeOH) λ_{max} 202, 235 nm; ECD ($CHCl_3$) λ_{max} ($\Delta\varepsilon$) 215

Table 1. ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) data for **1** (in $CDCl_3$).

C	δ_c	δ_H (*J* in Hz)
1	64.8 t	4.00 m, 3.98 m
2	27.1 t	1.60 m, 1.49 m
3	33.7 t	1.64 m, 1.62 m
4	34.7 d	2.96 m
5	145.5 s	
6	124.6 d	7.23 d (2.1)
7	139.8 s	
8	124.4 d	7.14 dd (8.0, 2.1)
9	130.3 d	7.06 d (8.0)
10	134.0 s	
11	60.6 s	
12	177.4 s	
13	42.9 d	2.87 m, 2.34 m
14	19.2 q	2.27 s
15	21.8 q	1.21 d (5.6)
1′	61.3 s	
2′	82.0 d	3.47 d (2.0)
3′	55.0 d	3.75 d (2.0)
4′	135.5 s	
5′	139.1 s	
6′	26.2 t	2.08 m, 2.94 m
7′	45.9 d	2.71 m
8′	82.5 d	4.21 ddd (12.0, 9.0, 3.2)
9′	36.1 t	2.10 m, 2.36 m
10′	29.9 d	2.15 m
11′	139.7 s	
12′	170.3 s	
13′	119.4 t	6.24 d (3.2), 5.49 d (3.2)
14′	16.9 q	0.96 d (6.8)
15′	14.5 q	1.83 s
1′′	170.8 s	
2′′	20.4 q	1.20 s

(−3.8), 245 (+1.0) nm; IR (KBr) ν_{max} 3377, 2973, 1729, 1710, 1629, 1381, 1260, 1067 cm^{-1}; ^{1}H- and ^{13}C-NMR, see Table 1; HRESIMS *m/z* 559.2672 $[M + Na]^+$ (calcd for $C_{32}H_{40}O_7Na$, 559.2671).

HNE Inhibitory Assay

HNE inhibitory activity was evaluated following a previously described procedure [15]. Briefly, 100-μl reaction mixtures containing 10 mM Tris-HCl buffer (pH 7.5), 1.0 mM MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide (Sigma, USA), 0.18 U HNE (CalbioChem, USA), and various concentrations of sample were incubated in a 96-well plate for 1 h at 37°C in the dark. Each reaction was stopped by the addition of 100-μl soybean trypsin inhibitor (0.2 mg/ml; CalbioChem, USA), and the absorbance at 405 nm was immediately measured using a microplate reader. EGCG (99% purity; Sigma, USA) was used as a positive control. Experiments were performed in triplicate. The IC_{50} was estimated from the least-squares regression line of the logarithmic concentration plotted against remaining activity using GraphPad 5.0 Prism software (GraphPad Software, Inc.).

Kinetic Analysis

A kinetic study was conducted in the same reaction medium in the presence of various concentrations of sample, at substrate concentrations ranging from 0.1 to 0.8 mM. Reactions were started by adding diluted substrate and were recorded every 2 min for 10 min at 37°C. The maximum velocity (V_{max}) and Michaelis constant (K_m) were determined by means of Lineweaver–Burk plots. The inhibition constant (K_i) was calculated from the Dixon plot. Graphs were plotted using SigmaPlot 12.5 software (Systat Software, Inc.).

Results

HNE Inhibitory Activity of Solvent Fractions of the Flowers of *I. britannica*

To search for active compounds in the flowers of *I. britannica*, an EtOH extract of these flowers was systematically divided into three solvent fractions (*n*-hexane-, EtOAc-, and *n*-BuOH-soluble) (Fig. 1A) and their HNE inhibitory activities were evaluated. The EtOAc-soluble fraction exhibited the strongest activity (IC_{50}, 1.49 μg/ml), followed by *n*-BuOH- (IC_{50}, 3.42 μg/ml) and *n*-hexane-soluble fractions (IC_{50}, 6.01 μg/ml) (Fig. 1B).

Structure Determination of the Isolated Compounds

The EtOAc-soluble fraction, which exhibited the most potent activity, was subjected to a series of chromatographic separation steps guided by HNE inhibitory activity, leading to the isolation of compounds **1**–**6** (Fig. 2). Based on comparison of the physicochemical and spectral data of these compounds with those in the literature, five known compounds were identified: japonicone A (**2**) [16], japonicone

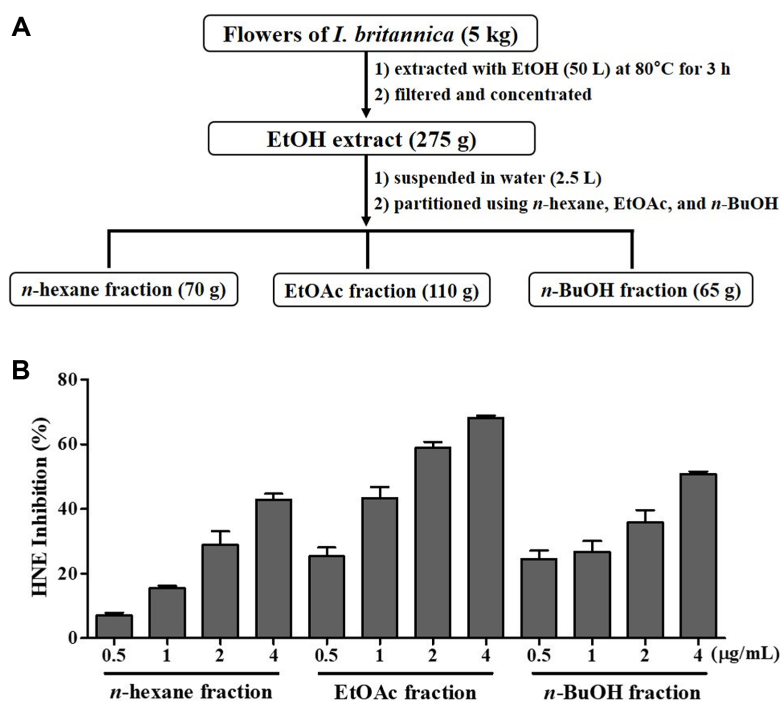

Fig. 1. Scheme of extraction and fractionation with different solvents of *Inula britannica* flowers (**A**) and HNE inhibitory activity of solvent fractions (**B**).

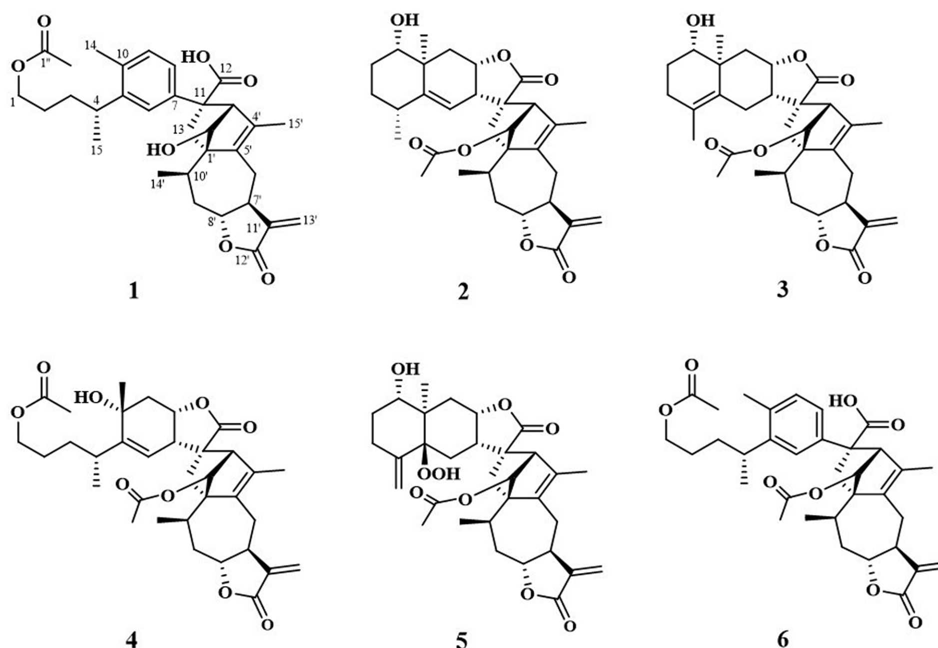

Fig. 2. Chemical structures of compounds **1**–**6** isolated from the flowers of *I. britannica*.

B (**3**) [16], japonicone D (**4**) [16], japonicone E (**5**) [17], and inulanolide E (**6**) [18].

Compound **1** was obtained as an amorphous white powder and showed a molecular ion peak at *m/z* 559.2671 $[M + Na]^+$ in HRESIMS, establishing the molecular formula as $C_{32}H_{40}O_7$. The IR spectrum showed characteristic absorption bands for carbonyls (1,729 and 1,710 cm^{-1}) and olefinic moieties (1,629 cm^{-1}). The ^{13}C-NMR data (Table 1), combined with the DEPT data, indicated 32 carbon signals consisting of 5 methyls, 6 methylenes, 10 methines, 8 quaternary, and 3 carbonyl carbons, of which the signals at δ_C 170.8 and 20.4 were assigned to an acetoxy group; the remaining 30 carbon signals suggested that **1** is a dimeric sesquiterpene lactone consisting of two different sesquiterpene types, designated as units A and B. One of them was nearly identical to a guaianolide skeleton (unit B) [16–18], which was supported by the presence of an α-methylene-γ-lactone functionality characterized by carbon signals at δ_C 170.3 (C=O, C-12′), 139.7 and 119.4 ($C=CH_2$, C-11′ and C-13′), and 82.5 (C-8′), and also by the characteristic ^1H-NMR data for an exocyclic methylene of H-13′a and H-13′b (δ_H 6.24 and 5.49 each d, *J* = 3.2 Hz) (Table 1). The other unit (unit A) was assumed to be an eudesmane sesquiterpene with a *seco*-ring, according to a detailed analysis of various 2D NMR spectra, such as ^1H-^1H COSY, HSQC, and HMBC. In the ^1H-NMR spectrum, the characteristic signals for an ABX aromatic system at δ_H 7.23 (1H, d, *J* = 2.1 Hz, H-6), 7.14 (1H, dd, *J* = 8.0, 2.1 Hz, H-8), and 7.06 (1H, d, *J* = 8.0 Hz, H-9), an acetoxyl group at δ_H 1.20 (3H, s, H-2′′), and the signals for a branched pentanol moiety, were observed. The presence of a branched pentanol moiety was supported by the ^1H-^1H COSY correlations observed between H_2-1/H_2-2, H_2-2/H_2-3, H_2-3/H-4, and H-4/H_3-15 (Fig. 3A). The placement of this pentanol moiety on C-5 was deduced from HMBC correlations among H-4/C-5, C-6, and C-10 (Fig. 3A). Furthermore, the HMBC correlations between H_2-1/C-1′′ and H-13/C-12 indicated the presence of a methyl ester group at C-1 and a carboxyl group at C-11. The linkage between the two units, via two C-C single bonds between C-13/C-1′ and C-11/C-3′, was deduced from the key HMBC correlations among H-2′/C-1′, C-11, and C-13; H-3′/C-7, C-11, and C-13; and between H_2-13/C-11, C-1′ and C-5′ (Fig. 3A). Detailed comparison of these spectral data with those of reported sesquiterpenes isolated from this plant revealed that the ^1H- and ^{13}C-NMR data of **1** were very similar to those of inulanolide E (**6**) [18], except for the additional signals consistent with the presence of an oxygenated methine group [δ_H 3.47, d, *J* = 2.0 Hz; δ_C 82.0 (d)] instead of an acetoxy group at C-2′ in **6**. The position of a hydroxyl group at C-2′ was further supported by the ^1H-^1H COSY correlation of H-2′/H-3′, as well as the HMBC correlations among H-2′/C-1′, C-3′, C-11, and C-13 (Fig. 3A).

The relative configuration of **1** was determined on the basis of a NOESY experiment and a comparison of the

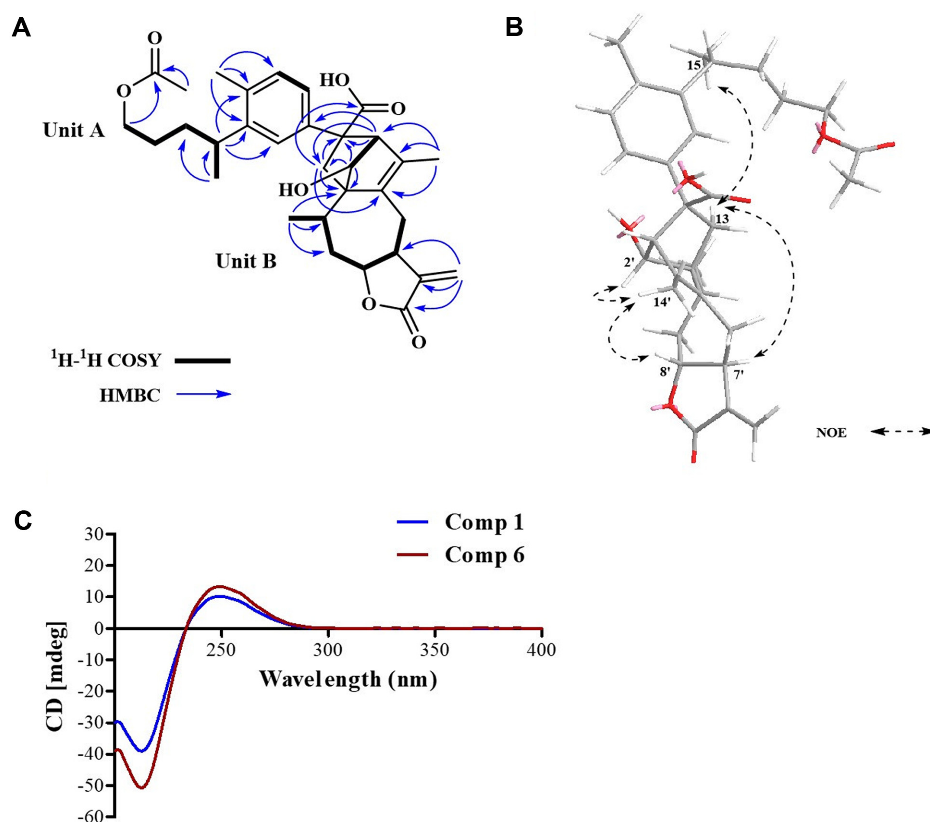

Fig. 3. Structure elucidation of compound **1**.
Key ^{1}H-^{1}H COSY and HMBC correlations (**A**), key NOE correlations (**B**), and ECD spectrum of **1** and **6** (**C**).

coupling constants with the literature. The large coupling constant between H-7′ and H-8′ (J = 12.0 Hz) suggested a *trans*-fused guaianolide skeleton [17, 19]. The orientation of the C-15 and C-14′ methyl groups was assigned as α and β, respectively, from the NOE correlations observed between H-7′/H_2-13 and H_2-13/H_3-15 and H-2′/H_3-14′ and H-8′/H_3-14′ (Fig. 3B). In the electronic circular dichroism (ECD) spectrum of **1**, a negative Cotton effect appeared at around 215 nm, while a positive Cotton effect was observed around 245 nm. This ECD feature was identical to those of **6** (Fig. 3C) and japonicones U and V [20], indicating that these compounds possessed the same absolute configuration. The absolute configuration of **1** was therefore assigned to be 4*R*, 11*S*, 1′*R*, 2′*R*, 3′*S*, 7′*S*, 8′*R*, 10′*R*. Finally, the structure of **1** was established as depicted in Fig. 2, and it was named inulanolide E1.

HNE Inhibitory Activity of the Isolated Compounds

The inhibitory activities of the isolated compounds (**1**–**6**) on HNE were evaluated in vitro according to a procedure described previously [15]. As shown in Table 2, all tested compounds except for **2** and **4** exhibited significant inhibitory effects against HNE activity, with IC_{50} values ranging from 8.2 to 42.5 μM. Among the compounds tested,

Table 2. HNE inhibitory effects of compounds **1-6** from the flowers of *I. britannica.*

Compounds	IC_{50} (95% CI) μM[a]	Inhibition mode (K_i[b], μM)
1	8.2 (7.2–9.2)	Noncompetitive (8.0)
2	>100	NT[c]
3	21.9 (20.2–23.8)	Noncompetitive (22.8)
4	>100	NT
5	42.5 (36.0–49.1)	NT
6	10.4 (9.4–11.4)	NT
EGCG[d]	10.9 (10.3–11.5)	NT

[a] IC_{50} indicates the concentration (μM) at which the inhibition percentage of the enzyme was 50%, and values in the parentheses represent the 95% confidence intervals of the IC_{50} values.

[b] Values of inhibition constant.

[c] NT is not tested.

[d] Epigallocatechin gallate (EGCG) was used as a positive control.

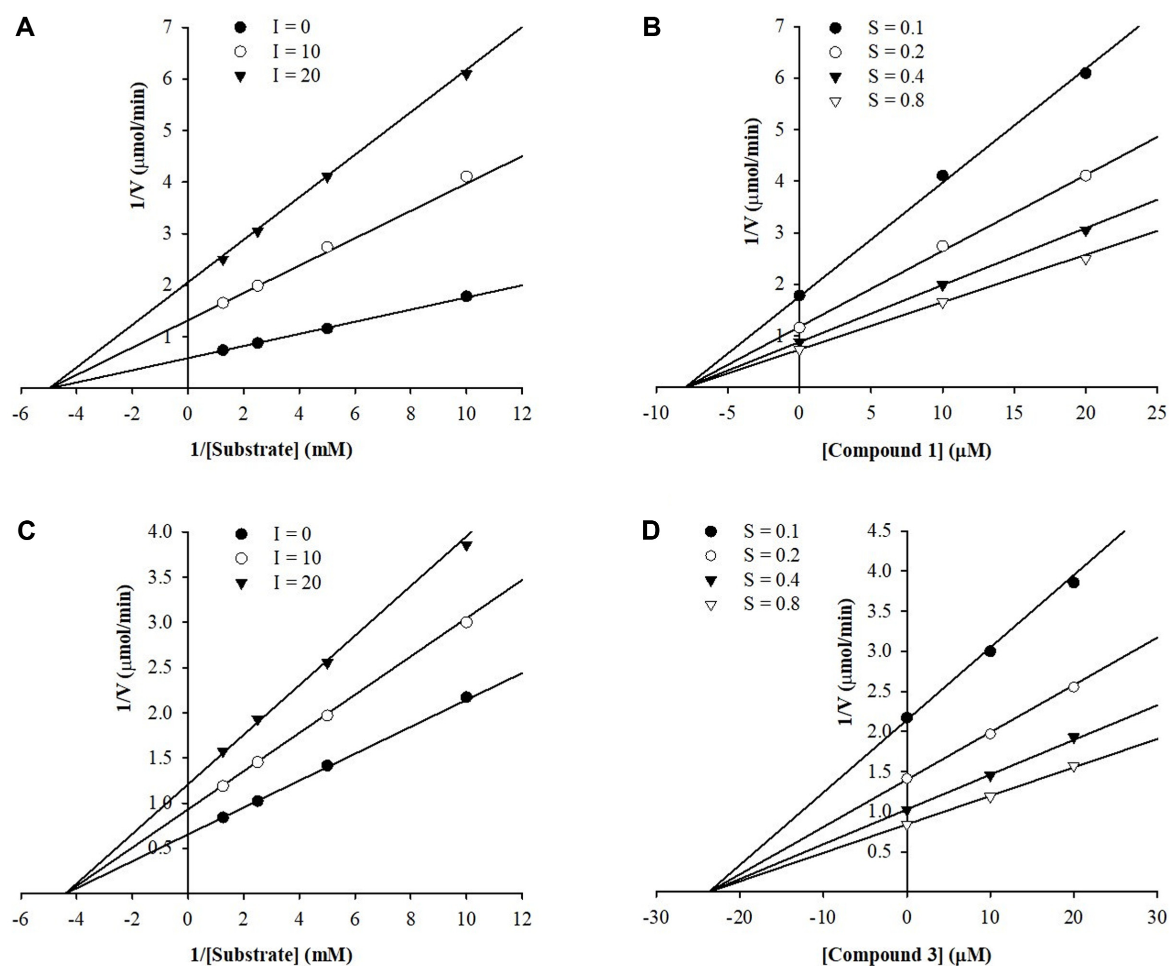

Fig. 4. Kinetic studies on HNE inhibition by compounds **1** and **3**.

Double-reciprocal Lineweaver–Burk plots of **1** (**A**) and **3** (**C**). The concentrations of **1** and **3** were 0 (●), 10 (○), and 20 μM (▼); Dixon plots of **1** (**B**) and **3** (**D**). The concentrations of substrate were 0.1 (●), 0.2 (○), 0.4 (▼), and 0.8 mM (▽).

inulanolide E1 (**1**) exhibited the most potent HNE inhibitory activity with an IC_{50} value of 8.2 μM, comparable to that of epigallocatechin gallate (EGCG; IC_{50} = 10.9 μM). Inulanolide E (**6**), which differs from **1** only in the substituent on C-2′, also showed considerable HNE inhibitory activity (IC_{50} = 10.4 μM). In addition, japonicone B (**3**) and japonicone E (**5**) exhibited moderate HNE inhibition, with IC_{50} values of 21.9 and 42.5 μM, respectively.

Kinetic Studies on HNE Inhibition by Compounds 1 and 3

To further characterize the HNE inhibitory behavior of the isolates, an enzymatic kinetic study was performed over a series of concentrations of two potent inhibitors (**1** and **3**) with different structural features and substrates. Under the experimental conditions, the oxidation of HNE by **1** and **3** followed Michaelis–Menten kinetics. As shown by the Lineweaver–Burk plot (Figs. 4A and 4C), the x-intercept ($-1/K_m$) was unaffected by increasing concentrations of **1** and **3** (from 0 to 20 μM), whereas $1/V_{max}$ increased gradually. Therefore, the mechanism by which **1** and **3** inhibited HNE was noncompetitive inhibition. Moreover, the Dixon plot analysis indicated that the inhibition constant (K_i) values of **1** and **3** were 8.0 and 22.8 μM, respectively (Figs. 4B and 4D, Table 2).

Discussion

HNE (E.C. 3.4.21.37) is a member of the chymotrypsin family of serine proteases primarily found in the azurophilic granules of neutrophils. It has broad substrate specificity and can degrade not only elastin, but also a variety of extracellular matrix proteins such as fibronectin, laminin,

collagen, and proteoglycans [21]. Under normal physiological conditions, HNE activity is tightly regulated by its endogenous protease inhibitors, such as secretory leukocyte protease and α1-protease inhibitors [22]. However, large amounts of oxygen radicals and proteases released by leukocytes that are recruited to sites of inflammation can overwhelm and inactivate these endogenous inhibitors [23]. This imbalance between HNE and its endogenous inhibitors can lead to abnormal degradation of healthy tissue, resulting in the development of chronic inflammatory diseases, such as rheumatoid arthritis, pulmonary emphysema, adult respiratory distress syndrome, cystic fibrosis, and delayed wound healing. Thus, the development and investigation of HNE inhibitors, particularly natural anti-HNE agents without side effects, may constitute a therapeutic approach for the prevention and treatment of inflammation-related diseases.

The present study was undertaken to identify natural products that inhibit HNE and revealed that *I. britannica* flowers are a potent HNE inhibitor. Thus, we propose *I. britannica* flowers as a new candidate for anti-HNE agents. We isolated six sesquiterpene lactone dimers, one of which is novel, as active compounds from this plant. Sesquiterpene lactones are an important class of compounds produced by many plants with a broad range of biological activities. Although sesquiterpene lactone monomers have been reported to exhibit HNE inhibitory activity [24–26], the HNE-inhibiting properties of the dimeric forms are reported here for the first time. Our findings suggest that the flowers of *I. britannica* and its active sesquiterpene lactone dimers would be an excellent source of natural HNE inhibitors for cosmetic and medicinal uses.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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