Tetrahedron Letters 51 (2010) 4287–4290

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Salviatalin A and salvitrijudin A, two diterpenes with novel skeletons from roots of *Salvia digitaloides* and anti-inflammatory evaluation

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ARTICLE INFO

Article history:
Received 29 April 2010
Revised 4 June 2010
Accepted 9 June 2010
Available online 15 June 2010

Keywords:
Salviatalin A
Salvitrijudin A
Salvia digitaloides
Anti-inflammatory effects

ABSTRACT

Salviatalin A (**1**) and salvitrijudin A (**2**), two diterpenes with novel skeletons, were isolated from the roots of *Salvia digitaloides*. Their structures were determined using 1D, 2D NMR, and HRESI-MS spectroscopic analyses. Salviatalin A (**1**) from bioassay-guided fractionation showed a potent inhibitory effect on superoxide anion production in GMLP/CB-activated human neutrophils as well as other anti-inflammatory effects. A plausible biosynthetic pathway is also discussed.

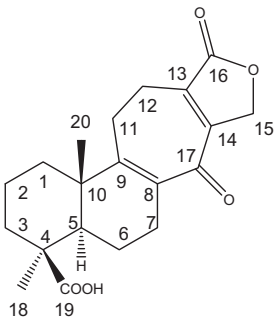
1. Introduction

Species of the genus *Salvia* (Labiatae) have been used worldwide in folk medicine from ancient times, because they exhibit various biological and pharmacological activities including antitumor,[1] antiallergic,[2] antioxidant,[3] antimicrobial,[4] and antiplatelet aggregation activities.[5] We previously reported a new antitumor agent, neotanshinlactone, which was isolated from *Salvia miltiorrhiza*,[6] and several notable new abietane diterpene alkaloids from *Schizothorsa yunnanensis*.[7]

Inflammation is a complex biological response of vascular tissues to harmful stimuli such as pathogens, damaged cells, or irritants. Inflammation can be classified as either acute or chronic. Although inflammation is a protective attempt by the organism to remove the injurious stimuli, chronic inflammation can also lead to a host of diseases such as hay fever, atherosclerosis, and rheumatoid arthritis. For this reason, inflammation is usually closely regulated. In our continuing investigation of *Salvia* species as anti-inflammatory agents, we recently investigated *S. digitaloides*, originally identified as the plant source of the traditional Chinese medicine 'Bai-Yun-Shen', later corrected to *Phlomis betonicoides* of the same plant family, from which a sweet glycoside named baiyunoside was isolated.[8] In the present study, we describe the isolation and the structure characterization of two new diterpenes, which have novel 6/6/7 and 6/5/3/7 tricyclic and tetracyclic skeletons, respectively, linked to a furan lactone ring, identified by using UV, IR, 1D and 2D NMR, and HRESI-MS spectroscopic analyses.

2. Results

The roots of *S. digitaloides* were extracted with MeOH. The concentrated extract was partitioned between water and chloroform. The chloroform extract (SDRC) was subjected to repeated silica gel chromatography to give salviatalin A (**1**) and salvitrijudin A (**2**).



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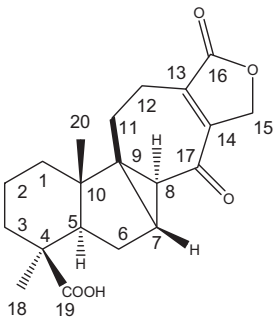

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doi:10.1016/j.tetlet.2010.06.048

Table 1
^{1}H (600 MHz), ^{13}C (150 MHz) NMR Data, and HMBC Correlations for **1** and **2** in $CDCl_3$

No.	**1**[a]			**2**[a]		
	δ_C (mult)	δ_H (mult, *J*, Hz)	HMBC	δ_C (mult)	δ_H (mult, *J*, Hz)	HMBC
1α	35.9 t	1.29 m	2, 5, 10, 20	33.6 t	1.21–1.26 m ovlp[b]	2, 5, 9, 10, 20
1β		2.03 br d (13.8)	9		1.62 dt (13.8, 4.2)	2, 3, 5, 10
2α	19.2 t	1.93 ddt (13.8, 13.8, 3.6)		19.8 t	1.74 ddt (13.8, 13.8, 4.2)	1
2β		1.62 dt (13.8, 3.6)			1.57–1.60 m ovlp[b]	3, 10
3α	37.0 t	2.24 dt (13.8, 3.6)	2, 4, 18, 19	36.8 t	0.90 dt (13.8, 4.2)	2, 4, 18
3β		1.04 ddd (13.8, 13.8, 3.6)			2.28 m	4, 5
4	43.7 s	—		43.3 s	—	
5	52.7 d	1.34 br d (13.0)	1, 4, 6, 7, 10, 19, 20	49.6 d	1.12 dd (12.9, 6.0)	1, 3, 4, 9, 10, 18, 20
6α	19.7 t	2.11 dd (13.0, 6.0)	5, 7, 10	26.5 t	1.93 dd (12.9, 6.0)	5, 7, 8, 9, 10
6β		1.73 ddd (13.0, 13.0, 6.0)	4, 5, 7, 10		2.26 m	4, 5, 7, 8
7α	27.9 t	2.01 m	6, 8, 9	31.4 d	2.17 t (3.0)	5, 17
7β		2.72 m	5, 8, 9, 17	—		
8	136.4 s	—		37.3 d	2.44 d (3.0)	6, 7, 10, 14, 17
9	157.4 s	—		47.4 s	—	
10	41.8 s	—		45.1 s	—	
11α	26.4 t	2.52 m	8, 9, 10, 12, 13	21.4 t	2.46 dt (14.8, 2.4)	7, 8, 9, 12, 13
11β		2.52 m			1.87 td (14.8, 2.4)	
12α	23.6 t	2.62 br dd (12.6, 1.2)	11, 13	25.1 t	2.26 m	
12β		2.39–2.47 m ovlp[b]	9, 11, 13		2.76 br d 19.8	
13	138.9 s	—		137.4 s	—	
14	152.5 s	—		151.0 s	—	
15α	70.1 t	4.82 ddd (18.0, 3.6, 1.2)	13, 14	69.8 t	4.71 dd (18.0, 3.0)	13, 14, 16
15β		4.98 dt (18.0, 3.6)	13, 14		4.98 dt (18.0, 3.0)	13, 14
16	173.9 s	—		173.6 s	—	
17	190.7 s	—		196.9 s	—	
18	28.5 q	1.29 s	2, 3, 4, 5, 19	28.9 q	1.19 s	3, 4, 5, 19
19	182.7 s	—		182.0 s	—	
20	16.7 s	0.98 s	1, 5, 9, 10	17.4 q	0.94 s	1, 5, 9, 10

[a] s: singlet, d: doublet, t: triplet, m: multiplet.
[b] Overlapping peaks.

Salviatalin A (**1**) was obtained as a colorless syrup and had a positive rotation ($[\alpha]_D^{25}$ +107, *c* 0.05, MeOH). The HRESI-MS of **1** shows a quasimolecular ion peak at *m*/*z* 367.1523, which is consistent with the molecular formula of $C_{20}H_{24}O_5Na$. This formula implies nine degrees of unsaturation. The UV spectrum of **1** shows absorption maxima at 247 and 312 nm, indicating the presence of α,β-unsaturated ketone moiety in the molecule. The IR spectrum shows strong absorption peaks for OH (3580 cm^{-1}), carbonyl group of a carboxylic acid (1696 cm^{-1}), furan ring (756 cm^{-1}), and a carbonyl group of a conjugated α,β-unsaturated γ-lactone (1762 cm^{-1}). The ^{13}C NMR spectrum of **1** (Table 1) shows 20 carbon signals, including four quaternary olefinic (δ 157.4, 152.5, 138.9, and 136.4), three carbonyl (δ 190.7, 182.7, and 173.9), two quaternary (δ 43.7 and 41.8), one methine (δ 52.7), eight methylene (δ 70.1, 37.0, 35.9, 27.9, 26.4, 23.6, 19.7, and 19.2), and two methyl (δ 28.5 and 16.7) carbons. These results were confirmed by the HSQC spectrum. The ^{1}H NMR spectrum of **1** (Table 1) showed signals for two methyls (δ 1.29 and 0.98) and an oxygen-bearing methylene (H-15, δ 4.98, and 4.82), which are the typical lactone protons. H-15 revealed ^{4}J long-range coupling to H-12α (δ 2.62, *J* = 16.2, 1.2 Hz). The COSY spectrum showed the following proton–proton cross-peaks: H-11 (δ 2.52) to H-12 (δ 2.43 and 2.62), H-6 (δ 1.73 and 2.11) to H-7 (δ 2.01 and 2.72)/H-5, (δ 1.34), and H-2 (δ 1.62, 1.93) to H-1 (δ 1.29, 2.03)/H-3 (δ 1.04, 2.24). The HMBC spectrum of **1** showed the conjugated cross-peaks of H-15 to C-13 (δ 138.9)/C-14 (δ 152.5)/C-17 (δ 190.7) and H-11 (δ 2.52) to C-13 (δ 138.9). The seven-membered C-ring was established by the correlations of H-7 (δ 2.01, 2.72) and H-11α (δ 2.52) with C-8 (δ 136.4) and C-9 (δ 157.4), of H-7β with C-17 (δ 190.7), and of H-1β (δ 2.03) and H-20 (δ 0.98) with C-9 in the HMBC spectrum. The ^{3}J HMBC correlation of the methyl protons (H-18) at δ 1.29 with C-3 (δ 37.0), C-5 (δ 52.7), and C-19 (δ 182.7) indicated that the quaternary C-4 (δ 43.7) was substituted with both methyl and carboxylic acid groups. The stereochemistry was confirmed by a NOESY experiment, which showed correlation between H-5, Me-18, and H-6α (Fig. 1). Thus, the methyl substitu-

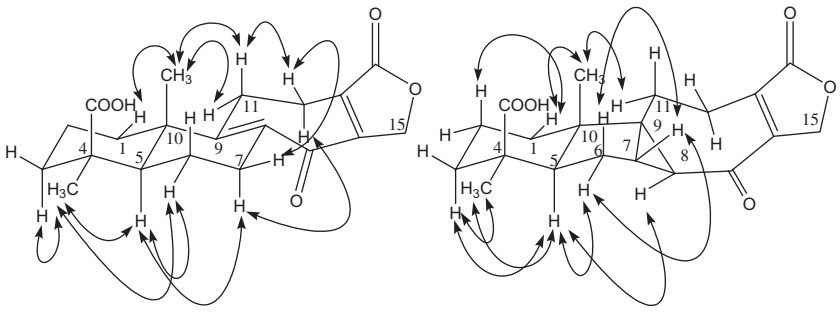


Figure 1. Selected NOESY correlations for **1** and **2**.

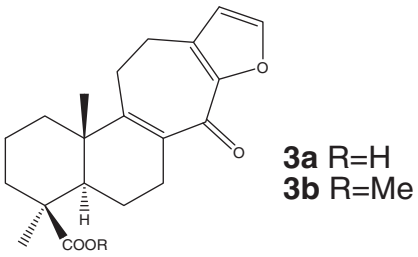


Figure 2. Structures of **3a** and **3b**.

ent at C-4 has an α-orientation. Rodriguez et al. isolated two compounds, hispanonic acid (**3a**) and its methyl ester (**3b**), with a furan ring α,β fused to a 6/6/7 tricyclic skeleton (Fig. 2) from *Ballota hispanica*. We confirmed that **1** has a different skeleton than **3a** and **3b** by HMBC correlations.[9] Based on the above-mentioned observations, the structure of salviatalin A was assigned as **1**, with a new diterpene skeleton.

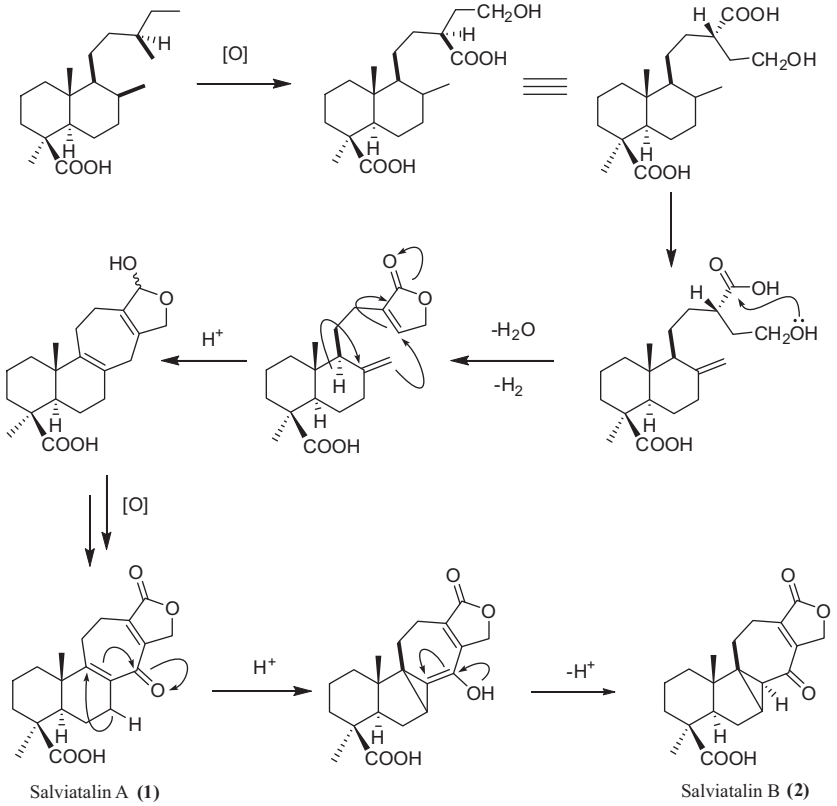
Salvitrijudin A (**2**) was also obtained as a colorless syrup with positive rotation ($[\alpha]_D^{25}$ +102, *c* 0.03, MeOH). The HRESI-MS of **2** shows a quasimolecular ion peak at *m*/*z* 367.1519, consistent with the molecular formula of $C_{20}H_{24}O_5Na$, and like **1**, implies nine degrees of unsaturation. The UV spectrum shows an absorption maximum at 238 nm and the IR spectrum shows strong absorptions at 3582, 1764, and 1642 cm^{-1} for hydroxy, α,β-unsaturated γ-lactone, and α,β-unsaturated ketone groups in the molecule, respectively. The NMR spectra of **2** were very similar to those of **1**, except for the presence of up-field carbon resonances for C-8 (δ 37.3) and C-9 (δ 47.4) rather than quaternary olefinic carbons, and a methine rather than a methylene for C-7 (Table 1). The methine proton (H-7) at δ 2.17 was correlated to a carbon at δ 31.4 in the HSQC spectrum. The HMBC spectrum showed ^2J or ^3J correlations of H-6 to C-4/C-5/C-7/C-8/C-9, H-8 to C-6/C-7/C-10/C-14/C-17, and H-15 to C-13/C-14/C-16. Furthermore, in the HMBC spectrum, the proton signal for H-11β (δ_H 1.87) showed cross-peaks with C-7/C-8/C-9/C-12/C-13 indicating a bond between C-7 and C-9, forming a three-membered ring between C-7/C-8/C-9.

From the above-mentioned data, we determined that **2** has a 6/5/3/7 tricyclic carbon skeleton. The orientation of the carboxylic and methyl groups at C-4 was assigned to be the same as that for **1** by using a NOESY experiment. H-8 was assigned α-configuration on the basis of the NOE correlation between H-5 (δ 1.12) and

Table 2
Inhibitory effects of extract fractions[a] and salviatalin A (**1**) on superoxide anion generation and elastase release by human neutrophils in response to FMLP/CB

	Superoxide	Elastase
	IC_{50} (μM) or (Inh%)	IC_{50} (μM) or (Inh%)
SDRM[a,b]	(64.77 ± 2.34)**	(36.13 ± 0.65)***
SDRC[a,b]	(84.35 ± 0.63)***	(131.59 ± 6.52)**
SDRB[a,b]	(65.79 ± 1.18)***	(60.50 ± 2.73)**
SDRW[a]	N.T.[d]	N.T.[d]
SDRP[a]	N.T.[d]	N.T.[d]
1[c]	(48.98 ± 1.60)***	(25.22 ± 4.41)**
2	N.T.[d]	N.T.[d]
DPI[e]	1.02 ± 0.35	N.T.[d]
PMSF[e]	N.T.[d]	95.00 ± 25

[a] SDR = *Salvia digitaloides* root; M = methanol, C = chloroform, B = *n*-BuOH, W = water, P = residue; fractionation of plant extract is described in Supplementary data.
[b] Percentage of inhibition (Inh%) at 10 mg/ml concentration.
[c] Percentage of inhibition (Inh%) at 30 μM concentration.
[d] N.T.: not tested.
[e] Diphenyleneiodonium (DPI, a NADPH oxidase inhibitor) and phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitor) were used as the positive controls in the generation of superoxide anion and release of elastase, respectively. Results are presented as mean ± S.E.M. (n = 2–4).
** p <0.01.
*** p <0.001 compared with the control value.



Scheme 1. Plausible biosynthetic pathway of salviatalin A (**1**) and salvitrijudin A (**2**).

H-8 (δ 2.44) (Fig. 1). H-7 was also identified as β-configuration, because of NOE correlation between H-7 (δ 2.17) and H-6 (δ 2.26, 1.93). Thus, compound **2** was named salviatalin B, and its novel diterpene skeleton was assigned as shown.

Neutrophils are active phagocytes that are a crucial component of innate immunity. Although antimicrobial functions of neutrophils are essential to host defense, their inappropriate or extensive activation often causes unwanted tissue damage, such as sepsis,[10] chronic obstructive pulmonary disease (COPD),[11] acute granule respiratory distress syndrome (ARDS),[12] granule, and other inflammatory processes.[13] In response to diverse stimuli, activated neutrophils secrete a series of cytotoxins, such as the superoxide anion (O_2^-), a precursor of other ROS, granule proteases, and bioactive lipids.[14] Therefore, it is crucial to restrain certain neutrophil functions, such as oxidative burst and degranulation, under physiological conditions while potentiating these functions in inflammatory tissues and organs.

Stimulation of neutrophils leads to their increased oxygen consumption through the activity of NADPH oxidase, which generates O_2^-. O_2^- production is linked to the killing of invading microorganisms, but it can also directly or indirectly cause damage by destroying the surrounding tissue. Degranulation also plays a pivotal role in most neutrophil functions. Neutrophil granules contain many antimicrobial and potentially cytotoxic substances. Neutrophil elastase is a major product secreted from stimulated neutrophils and a major contributor to the destruction of tissue in chronic inflammatory disease. Therefore, elastase is a target for the therapy of chronic inflammatory diseases.[15] In our search for new anti-inflammatory agents, salviatalin A (**1**), obtained by using bioassay-guided fractionation from extract fraction SDRC, was tested using DPI, a NADPH oxidase inhibitor, and PMSF, a serine protease inhibitor as positive control. Impressively, **1** showed inhibitory effects against the release of both O_2^- and elastase by human neutrophils, with IC_{50} values of 48.98 ± 1.60 and 25.22 ± 4.41 μM, respectively (Table 2). The anti-elastase release activity of **1** was fourfold higher than that of the PMSF control. Salviatalin A (**1**) also showed a potent inhibitory effect on O_2^- production in FMLP/CB-activated human neutrophils. These results suggested that **1** may represent a good candidate as a new anti-inflammatory agent.

Salviatalin A (**1**) and salvitrijudin A (**2**) were also evaluated for cytotoxicity against KB, A549, HCT-8, and DU145 cell lines. Neither of the compounds showed a significant cytotoxicity.

Because of their novel skeleton, the biosynthetic pathway to both compounds is of great interest. A plausible biosynthetic pathway is illustrated in Scheme 1.

Acknowledgments

The authors acknowledge the financial support from the National Science Council of Republic of China awarded to T.-S. Wu. This study was supported in part by Taiwan Department of Health Cancer Research Center of Excellence (DOH99-TD-C-111-005).

Supplementary data

Supplementary data (extraction and isolation procedures, bioassay methods, and ^1H and ^{13}C NMR, COSY, NOESY, HSQC, and HMBC spectra for **1** and **2**) associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2010.06.048.

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