ORGANIC LETTERS

2013
Vol. 15, No. 8
1862–1865

New Inhibitors of ROS Generation and T-Cell Proliferation from *Myrtus communis*

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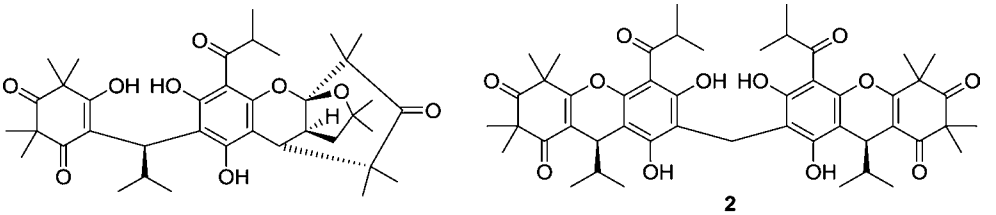
Received February 22, 2013

ABSTRACT



1 **2**

Phytochemical investigation on *Myrtus communis* Linn. afforded myrtucommuacetalone (1) with an unprecedented carbon skeleton and a new phloroglucinol-type compound, myrtucommulone M (2), along with four known constituents 3−6. Their structures were established by extensive analyses of NMR and mass spectral data as well as by single-crystal X-ray diffraction studies. These constituents were evaluated for their ability to modulate the immune response, based on their effects on various components of immune system. Compounds 1 and 5 exhibited significant inhibitory effect against nitric oxide (NO•) production. Compound 1 also exhibited significant antiproliferative activity (IC_{50} < 0.5 μg/mL) against T-cell proliferation. Myricetin (3) exerted a significant inhibition (IC_{50} = 1.6 μg/mL) on zymosan-stimulated whole blood phagocytes ROS production. Compounds 1 and 3 were active against PMA-stimulated ROS generation.

Myrtus communis Linn., commonly referred to as "True Myrtle", is an evergreen shrub widely spread over the Mediterranean region. It is an important medicinal herb of the family Myrtaceae.[1,2] Myrtle has been traditionally used in folk therapeutic practices as an anti-inflammatory, antibacterial, antioxidant, antihyperglycemic, and analgesic agent, as well as for the treatment of oral diseases, candidiasis, wounds, and urinary disorders.[3−6] The major constituents of the plant include monoterpenoids, triterpenoids, flavonoids, and phloroglucinol derivatives. Previously, we reported several phloroglucinol-type compounds as antibacterial and α-glucosidase inhibitors from

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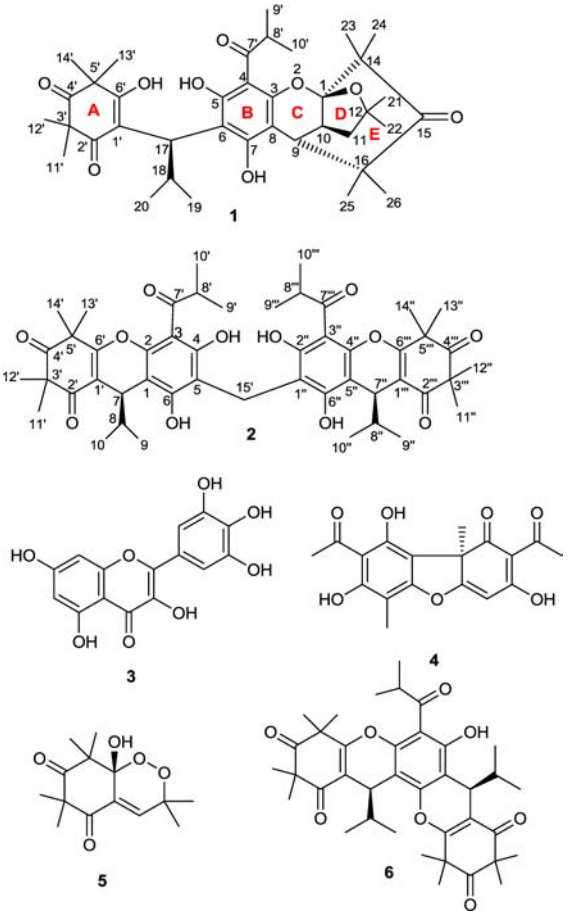
10.1021/ol4002976
Published on Web 04/03/2013

this plant.[7] A current study has led to the isolation of two new acylphloroglucinols derivatives (**1** and **2**), along with four known compounds, myricetin (**3**),[8] isousnic acid (**4**),[9] G3-factor (**5**),[10] and myrtocommulone E (**6**).[7] The effect of compounds **1**–**6** on the production of a short-lived nitric oxide radical, the end product of the enzymatic oxidation of L-arginine in macrophages,[11] was also evaluated. LPS-activated macrophages produce large amounts of nitric oxide, which is an essential mediator of the innate response.[12] Nitric oxide radicals, generated as an immune response, are toxic to bacteria.[13] Excess $NO^{\bullet}$ production is associated with the pathogenesis of various diseases, such as septic shock, colitis, diabetes mellitus, and ischemic neuronal damage.[14]



In the current study, we examined the effect of compounds **1**–**6** on the production of ROS by human peripheral blood phagocytes, generated by NADPH oxidase.[15] When phagocytes are exposed to an opsonized microbial particle, production of a series of ROS is initiated as an oxidative burst which promotes inflammatory responses and can cause tissue damage.[16] On the other hand, suppression of T-cell activity is required for the prevention or treatment of autoimmune disorders such as Parkinson's disease, diabetes mellitus, and rheumatoid arthritis.[16]

Compound **1** was obtained from *M. communis* as yellow crystals. The molecular formula of **1** was found to be $C_{38}H_{52}O_9$ on the basis of positive-ion $[M + 1]^+$ at m/z 653.3685 (calcd for $C_{38}H_{53}O_9$, 653.3689) based on a HRESIMS measurement. The novel structure of compound **1** was deduced by single-crystal X-ray diffraction (Figure 1), and NMR studies as a hybrid of syncarpic acid[17] with an unusual bridged tetracyclic ketal skeleton containing a furochromene moiety. NMR spectral data (Table S1) of **1** exhibited signals closely similar to those observed in bullataketals A and B, the constituents of *Lophomyrtus bullata* (Myrtaceae) .[17] However, the signals of the unsubstituted aromatic ring of bullataketals were lacking and additional signals of four quaternary methyls, two quaternary carbons, and one carbonyl group were observed in the NMR spectra of **1**.

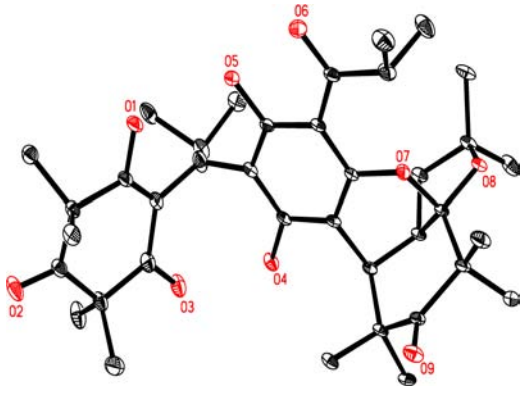


Figure 1. Computer-generated ORTEP diagram of compound **1**. H-atoms are omitted for clarity.

Single-crystal X-ray diffraction studies of **1** revealed that compound **1** contains an unprecedented bridged furochromene moiety. Thus unassigned NMR signals were assigned to the carbonyl and vicinal two *gem* dimethyl groups in ring E. The ^{1}H NMR spectrum of **1** displayed signals for 5 methine, 1 methylene, and 14 methyl groups. In the HMBC spectrum (Figure S1), four quaternary methyl groups (δ 1.33, 1.41, 1.37, 1.49) exhibited interactions with C-2′, C-3′, C-4′, C-5′, and C-6′, indicating the presence of an enolizable 1,1,3,3-tetramethylcyclohexatrione (syncarpic acid) moiety similar to that found in bullataketals.[17] The correlation of the H-17 methine proton (δ 3.73) with the

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H-18 methine proton (δ 2.92), and correlations of H-18 with C-19 (δ 0.68) and C-20 (0.80) *gem* dimethyl protons in the COSY spectrum (Figure S2), indicated the presence of an isobutylidene moiety in the molecule. The heteronuclear interactions of the H-17 methine proton of the isobutylidene moiety with quaternary C-5 (δ 162.0), C-6 (δ 108.2), C-1′ (δ 114), and C-6′ (δ 178.7) and the carbonyl carbon at C-2′ (δ 202.9) indicated the connectivity of the syncarpic acid moiety with the phloroglucinol group of **1**. This linkage is also found in other compounds such as semimyrtucommulone and bullataketals.[17] In the HMBC spectrum, the H-8′ methine proton (δ 4.09) showed interactions with the C-7′ carbonyl (δ 211.4) and C-9′ (δ 20.8) and C-10′ (δ 18.9) *gem*-dimethyl carbons. This supported the presence of a 2-methylpropionyl group in **1**. The tetracyclic fused structure was supported by COSY and HMBC techniques. In the COSY spectrum, H-9 methine (δ 3.43) showed a cross-peak with the H-10 methine (δ 3.40), which was in turn correlated with H_2-11 methylene (δ 1.86). The HMBC spectrum showed heteronuclear interactions of H-9 (δ 3.43) with C-1 acetal (δ 113.9), C-3 (δ 162.5), C-8 (δ 102.2), and C-10 (δ 34.9) of the furochromene moiety and also showed interactions with the C-16 (δ 50.0) of ring E. However H-10 (δ 3.40) exhibited interactions with C-1 acetal carbon (δ 113.9) and C-16 (δ 50.0) of ring E. The single methylene group (δ 1.86) of the skeleton exhibited correlations with the C-1 acetal carbon and C-10 (δ 34.9), thus supporting the fused tetracyclic part of compound **1**. The X-ray studies showed that compound **1** consists of a planner phenyl ring A, three six membered rings B, C, and D, and a five membered ring E. Rings B, C, and D possess a half chair/half chair and chair conformations, respectively, while ring E is in envelope conformation. All the bond angles and lengths were within the normal range. Thus the NMR and single-crystal X-ray data unambiguously deduced the novel structure of compound **1** (myrtucommuacetalone).

Compound **2** (myrtucommulone M) was obtained from *M. communis* as colorless crystals. NMR and single-crystal X-ray diffraction studies revealed the structure of **2** as a dimeric form of a known compound myrtucommulone B.[18] The molecular formula of compound **2** was deduced to be $C_{49}H_{60}O_{12}$ on the basis of $[M + 1]^+$ at m/z 841.4135 (calcd for $C_{49}H_{61}O_{12}$, 841.4163) in HRESIMS. The fragment ion at m/z 427 in EI-MS was due to the monomeric unit of compound **2** with an intact methylene group which acts as the bridge between the two monomers (Figure S3). The ^{1}H NMR spectrum of **2** showed eight resonances for methyl groups, an upfield multiplet at δ 1.96 and a downfield doublet at δ 4.39 ($J_{7,8}$ = 3.6 Hz) for methine protons of an isobutylidine group, a multiplet at δ 3.91 for an isopropyl methine, and a multiplet for a methylene proton at δ 3.85. The ^{13}C broad-band decoupled and DEPT NMR spectra showed 25 signals, including 8 methyl, 1 methylene, 3 methine, and 13 quaternary carbons. The COSY spectrum (Figure S4) showed the correlation of the H-7 methine proton (δ 4.39) with the H-8 methine proton (δ 1.96), which was further correlated with C-9 (δ 0.72) and C-10 (δ 0.94) methyl protons. These correlations indicated the presence of an isobutylidine group in the skeleton. The heteronuclear interactions of the H-7 methine proton with C-1′ (δ 111.9), C-6′ (δ 167.4), and C-1 (δ 105.9) indicated the linkage of the syncarpic acid moiety with the phloroglucinol group, as found in myrtucommulone B. The presence of the 2-methylpropionyl group was inferred from the COSY correlations of the H-8′ methine proton (δ 3.91) with C-9′ and C-10′ methyl protons and heteronuclear interactions of H-8′ with C-7′ carbonyl carbon (δ 209.7). The dimeric nature of the compound was further deduced by the HMBC correlations of methylene protons (δ 3.85) with quaternary carbons at δ 161.1 and 109.8 (Figure S5). Single-crystal X-ray diffraction analysis of **2** unambiguously established the structure for myrtucommulone M (**2**) (Figure 2). It showed that the monomeric units of the myrtucommulone M are linked together through a methylene bridge. Two monomers have three rings A/B/C and D/E/F with planar (rings A and F), boat (rings B and E), and half-chair (rings C and F) conformations. The isopropyl group adopts a *pseudo*-axial orientation on rings B and E. All the bond angles and lengths were within the normal range.

Myricetin (**3**) is a flavonol, commonly found in plants. It is a potent antioxidant as well as an anticarcinogenic, antidiabetic and antithrombotic agent.[19] It is also reported to affect glutamate induced apoptotic signaling *via* multiple distinct biochemical pathways.[20] Isousnic acid (**4**) is exclusively found in lichens.[21] Growth regulator G3 factor (**5**) was previously isolated from *Eucalyptus grandis*,[22] whereas myrtucommulone E (**6**) was obtained previously from *M. communis*.[7]

The effects of compounds **1**–**6** on ROS and RNS production, as well as on proliferation of T-cells, was also studied (Table S2). Compound **3** (myricetin) exhibited significant activity on professional phagocytes upon activation with the serum opsonized zymosan (as an antigen).

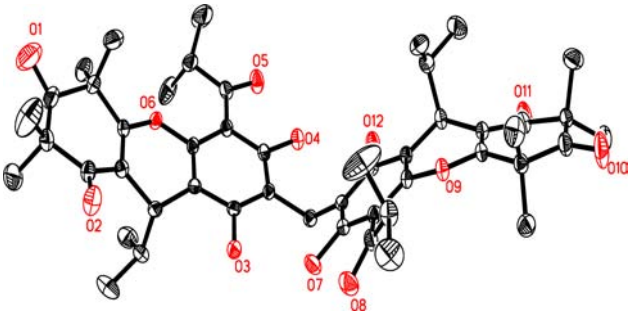

Figure 2. X-Ray structure. ORTEP generated diagram of compound 2. H-atoms omitted for clarity.

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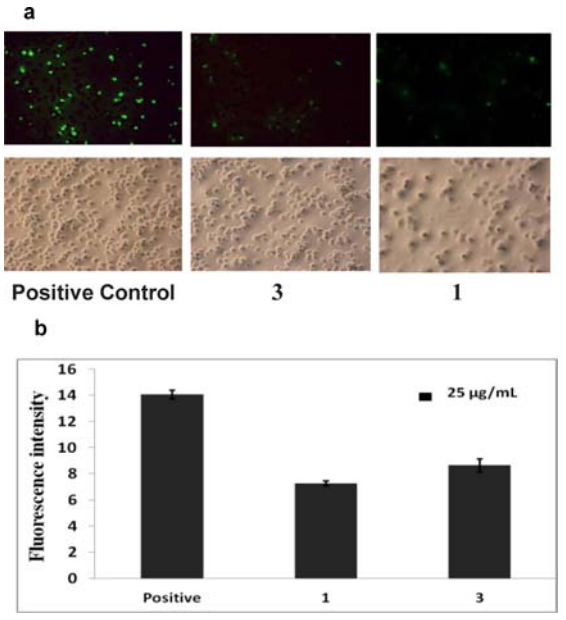

Figure 3. (a) Mouse macrophage J774.2 intracellular oxidative stress examined by fluorescent microscopy. The J774.2 cells, treated with phorbol 12-myristate 13-acetate (PMA) alone (positive control) or in combination with compounds **1** and **3** for 30 min and then incubated with 2′,7′-dichlorofluorescein DCFH; 20 μM for 30 min. The cells were examined at 20× magnification under a green channel. Brightfield images, showing the total number of cells present in the same field, were obtained under phase contrast at 20× magnification. (b) Histogram shows the mean intensity of fluorescence.

Compound **3** exhibited dose dependent inhibitory activity on ROS production of whole blood phagocytes (IC_{50} of 1.6 μg/mL). Thus **3** was identified as an inhibitor of the innate immune response. Compounds **1** and **5** inhibited $NO^{\bullet}$ production in mouse macrophages (82.3% and 59.36% respectively) at 25 μg/mL concentration. The results were compared with the standard inhibitor of $NO^{\bullet}$ (LNMA) (% Inhibition 65.65%) at 25 μg/mL (Table S2). iNOS produces large amounts of $NO^{\bullet}$ as a defense tool and is an important factor against parasite attack, bacterial infection, and tumor growth. In contrast, if over produced, it reacts with superoxide and gives rise to a toxic radical peroxynitrite, which is one of the main causes of septic shock and may play a role in many diseases with an autoimmune etiology. Therefore, compounds **1** and **5** may have some therapeutic potential against the diseases which involve nitric oxide associated oxidative stress.

In the PHA-induced T-cell proliferation assay, compound **1** showed potent inhibition (IC_{50} of $\leq$0.5 μg/mL) (Table S2). These results indicate that compound **1** has a highly significant inhibitory activity on cellular immune response and might be useful in suppressing various allergic, inflammatory, and autoimmune disorders.

The effect of compounds **1** and **3** on intracellular ROS was also studied by using the 2′,7′-dichlorofluorescein (DCFH) dye. PMA was employed as a stimulant to distinguish the activity of the oxidative burst from opsonized zymosan activation used in a chemiluminiscence assay.[23] PMA is also known to activate the NADPH oxidase *via* a protein kinase C pathway whereas zymosan is involved in phagocytosis. Compounds **1** and **3** were found to possess an inhibitory effect on ROS generation (Figure 3).

Compound **3** inhibits ROS in both stimulant systems. The possible mechanism of action of **3** could be through its action on myeloperoxidase dependent ROS generation as in the case of H_2O_2. Interestingly compound **1** showed inhibitory effects only in the PMA induced ROS generation, but not in zymosan-induced ROS. This suggests that compound **1** is inhibiting ROS production, particularly superoxide, by a myloperoxidase independent pathway.

Acknowledgment. The authors appreciate the financial support by the Higher Education Commission (HEC), Pakistan and OPCW for immunomodulatory work conducted at ICCBS, University of Karachi. The authors extend their appreciation to The Deanship of Scientific Research at King Saud University for funding the work through the research group Project No. RGP-VPP-207.

Supporting Information Available. Experimental procedures, NMR spectroscopic and X-ray crystallographic data of **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.