A New Phenolic Diterpene From the Leaves of \textit{Rosmarinus officinalis}

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Abstract
As part of our continuous studies on dietary supplements for diabetes, 1 new phenolic diterpene, 7-butoxyrosmanol (1), and 3 known ones (2-4) were isolated from \textit{Rosmarinus officinalis}. The new structure was elucidated based on comprehensive spectroscopic methods, including multiple nuclear magnetic resonance techniques, mass spectrometry, and x-ray diffraction analysis. In addition, compound 1 showed moderate activity against pancreatic lipase with an IC\textsubscript{50} value of 46.2 \textmu M.

Keywords
\textit{Rosmarinus officinalis}, diterpene, anti-obesity, phenolic, pancreatic lipase

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Introduction
Obesity is now a global public concern that exerts adverse effects on health and imposes a huge economic burden on society. Pharmacotherapy is the most common treatment option for obesity. Pancreatic lipase is well known to play an important role in lipid digestion.\textsuperscript{1} Thereby, the inhibition of pancreatic lipase is thought to be an effective therapy for obesity. Orlistat is a specific pancreatic lipase inhibitor and is clinically used as an anti-obesity drug in many countries.\textsuperscript{2} However, orlistat also causes gastrointestinal side effects.\textsuperscript{3} Therefore, it is necessary to find diet-derived anti-obesity compounds with excellent bioefficacy and long-term safety.\textsuperscript{4}

Rosemary (\textit{Rosmarinus officinalis} L.) is widely used as a food ingredient and culinary spice. Its extracts have been used for the treatment of Alzheimer’s disease, cancer, cardiovascular disease, obesity, and diabetes.\textsuperscript{5,9} The major constituents of rosemary have been reported to be phenolic acids, terpenoids, and flavonoids.\textsuperscript{5,10} In the present work, we report the isolation and pancreatic lipase inhibition of phenolic diterpenes from rosemary.

Results and Discussion
The methanol extract of rosemary was subjected to repeated column chromatography to yield one new phenolic abietane diterpene, 7-butoxyrosmanol (1), and 3 known compounds (Figure 1). Based on the spectroscopic analysis,\textsuperscript{11,12} the known compounds were identified as epirosmanol (2), carnosol (3), and carnosic acid (4), respectively.

Compound 1 was obtained as pale colorless crystals. Its negative high resolution electrospray ionization mass spectroscopy (HRESIMS) data showed an [M − H]\textsuperscript{+} ion at \textit{m}/\textit{z} 401.23291, which is in accordance with the molecular formula C\textsubscript{24}H\textsubscript{34}O\textsubscript{5}. The fragment ion peaks at \textit{m}/\textit{z} 329.1758 indicated the presence of a butoxyl group in 1 (Figure 2). The presence of hydroxy (3445 cm\textsuperscript{-1}) and carbonyl (1730 cm\textsuperscript{-1}) groups, and an aromatic ring (1592 cm\textsuperscript{-1}) was confirmed by its infrared spectroscopy (IR) spectrum. The nuclear magnetic resonance (NMR) spectroscopic data (Table 1) displayed an aromatic methane (\delta\textsubscript{H} 6.79, 1H, s; \delta\textsubscript{C} 120.81), 2 oxygenated methines [\delta\textsubscript{H} 4.66 (1H, d, \textit{J} = 3.0 Hz); \delta\textsubscript{C} 75.43 and 4.33 (1H, d, \textit{J} = 3.0 Hz); \delta\textsubscript{C} 76.14], an oxygenated methylene [\delta\textsubscript{H} 3.89 (2H, m); \delta\textsubscript{C} 70.92], 2 doublet methyls (\delta\textsubscript{H} 2.12, 6H, d, \textit{J} = 6.5 Hz; \delta\textsubscript{C} 22.45, 22.41), 2 singlet methyls (\delta\textsubscript{H} 1.00, 3H, s; \delta\textsubscript{C} 31.54, and \delta\textsubscript{H} 0.92, 3H, s; \delta\textsubscript{C} 22.17), and 1 triplet methyl (\delta\textsubscript{H} 0.96, 3H, t, \textit{J} = 3.0 Hz); \delta\textsubscript{C} 22.17), and a butoxyl group (\delta\textsubscript{H} 4.30, 2H, m; \delta\textsubscript{C} 69.10).

Table 1. \textit{H} and \textit{C} NMR Data of 1

| \text{H} NMR Data | \text{C} NMR Data |
|------------------|------------------|
| \text{\delta} | \text{\delta} |
| 6.79, 1H, s | 120.81 |
| 4.66 (1H, d, \textit{J} = 3.0 Hz) | 75.43 and 4.33 (1H, d, \textit{J} = 3.0 Hz) |
| 76.14 | |
| 3.89 (2H, m) | 70.92 |
| 2.12, 6H, d, \textit{J} = 6.5 Hz | 22.45, 22.41 |
| 1.00, 3H, s | 31.54 |
| 0.92, 3H, s | 22.17 |
| 0.96, 3H, t, \textit{J} = 3.0 Hz | 22.17 |
| 4.30, 2H, m | 69.10 |

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A carboxylic carbon at $\delta_C$ 179.29 and an aromatic ring ($\delta_C$ 142.61, 141.59, 134.72, 126.86, 124.59, and 120.81) were also observed. Collectively, these characteristic signals implied that 1 was an abietane diterpene. Further examination of the 2D NMR spectroscopic data of compound 1 (Figure 3) indicated that it is structurally similar to 7-methoxyrosmanol.11 The only difference in the NMR spectra of 1 and 7-methoxyrosmanol was that the 7-methoxy group of 7-methoxyrosmanol was replaced by a butoxyl group in 1. This observation was corroborated by the HMBC correlations from H-1′ to C-7 (Figure 3). Key ROESY correlations of 1 were not observed; the configuration of 1 was confirmed according to the x-ray single-crystal diffraction analysis with Cu Kα radiation (Figure 4). However, the Flack parameter [0.19(17)] was large and cannot assign the absolute configuration. Therefore, the structure of compound 1 was designated as (5α, 6β, 7α, 10β)7-butoxyrosmanol.

Compound 4 showed significant inhibitory activity against pancreatic lipase.13 Thus, compound 1 was evaluated for its inhibitory activity against pancreatic lipase and orlistat was used as a positive control. As expected, compound 1 showed good activity with an IC$_{50}$ value of 46.25 ± 3.85 µM; and the IC$_{50}$ value of orlistat was 10.13 ± 1.02 µM.

**Experimental**

**General**

The NMR data were acquired with Bruker Advance 500 MHz spectrometers (Bruker AVANCE NEO 500).
The HRESIMS were measured on a Thermo Exactive Orbitrap mass spectrometry (Thermo Fisher Scientific), the IR spectra on a Bruker VERTEX 70 FT-IR spectrometer, the ultraviolet (UV) spectra on a Shimadzu 160 UV/VIS Spectrometer, the optical rotations on an Anton Paar MCP 300 polarimeter with 1 dm cell, and the x-ray diffraction data on a dual-source Rigaku Oxford Diffraction Supernova diffractometer (using graphite-monochromated Cu Kα radiation). The structure was solved with the Superflip program using charge flipping and redefined with the ShelXL program. Sephadex LH-20 (Amersham Pharmacia Biotech), D101 macroporous resin (Sinopharm Chemical Reagent Co., Ltd) and YMC Gel (ODS-A, 12 nm, S-50 μm, YMC Co.) were used for column chromatography. Preparative high-performance liquid chromatography was carried out on a Waters 1525 instrument with a 2598 detector (Waters), using an YMC-Pack ODS-A column (250 mm x 20 mm, 5 μm). TLC was conducted on Merck silica gel 60 F 254 plates (Merck KGaA).

**Plant Material**

The leaves of rosemary were purchased from Yulin, in August 2015, and were authenticated by Professor Yin Li, School of Pharmacy, Southwest Minzu University, China. A voucher specimen (ID 20150803) has been deposited in the Herbarium of Materia Medica, School of Biotechnology and Health Sciences, Wuyi University, Jiangmen, China.

**Extraction and Isolation**

The dried leaves of*R. officinalis* (12.0 kg) were soaked (extracted) with EtOH (3 x 25 L) at room temperature. The solvent was evaporated under reduced pressure to yield 561.5 g of extract. The crude extract was suspended in water (2.5 L) and then successively solvent partitioned with n-hexane, acetate, and n-butanol. Four layers with increasing polarity were obtained: hexane-soluble (170.5 g), EtOAc-soluble (77.0 g), BuOH-soluble (85.0 g), and H2O-soluble (114.6 g) fractions. The hexane soluble was subjected to D101 resin elute with water-EtOH (1:0, 7:3, 1:1, 3:7, 1:9, and 0:1, vol/vol) to afford 12 fractions (H1-H12). Fraction H1 (1.61 g) was further separated by CC using silica gel (4:1, light petroleum-EtOAc) to give compound 2 (15 mg). Fraction H2 (30.5 g) was recrystallized with light petroleum—EtOAc to afford compound 3 (5.3 g), and the mother liquid was chromatographed on a Sephadex LH-20 column (CHCl3-MeOH, 2:1) to yield compound 1 (5 mg) and another impure yellow powder, which was purified by silica gel CC eluted with light petroleum-EtOAc (4:1) to give compound 4 (12.1 g).

### Table 1. 1H NMR (500 MHz) and 13C NMR (125 MHz) Spectroscopic Data of Compound 1 in CDCl3.

| Position | δ_C | δ_H mult (J in Hz) | Position | δ_C | δ_H mult (J in Hz) |
|----------|-----|--------------------|----------|-----|--------------------|
| 1        | 27.30, CH2 | 3.14, m            | 13       | 134.72, C         | 6.79, s |
| 2        | 19.55, CH2 | 1.44, overlap      | 14       | 120.81, CH         | 3.07, m |
| 3        | 38.08, CH2 | 1.44, overlap      | 15       | 27.42, CH         | 3.07, m |
| 4        | 31.57, C    | 2.26, s            | 16       | 22.45, CH3        | 1.21, d, 6.5 |
| 5        | 51.01, CH    | 4.66, d, 3.0       | 17       | 22.41, CH3        | 1.21, d, 6.5 |
| 6        | 75.43, CH    | 1.44, overlap      | 18       | 31.54, CH3        | 1.00, s |
| 7        | 76.14, CH    | 4.33, d, 3.0       | 19       | 22.17, CH3        | 0.92, s |
| 8        | 126.86, C    | 1.20, overlap      | 20       | 179.29, C         | 3.89, m |
| 9        | 124.59, C    | 1’                 | 2’       | 70.92, CH2        | 1.66, m, overlap |
| 10       | 47.11, C     | 2’                 | 3’       | 32.39, CH2        | 1.66, m, overlap |
| 11       | 141.59, C    | 4’                 | 4’       | 14.01, CH3        | 0.96, t, 7.5 |

**Abbreviation:** NMR, nuclear magnetic resonance.

![Figure 3. H-H COSY (blue bold lines) and key heteronuclear multiple bond correlations (HMBC) (red arrows) of 1.](image-url)
7-Butoxyrosmanol (1)
Colorless needle crystals; mp 232 to 235 °C; [α]D25 –60 (c 0.1, MeOH); UV (MeOH) λmax (log ε) 258 (3.02), 296 (3.14) 330 (3.22) nm; IR (neat) νmax 3323, 2916, 1654, 1453, 1355, 1280, 1000, 774 cm⁻¹; see Table 1 for 1H NMR (CDCl₃, 500 MHz) and 13C NMR (CDCl₃, 125 MHz); HRESIMS m/z 401.23291 [M – H]⁻ (calcd for C₂₄H₃₃O₅, 401.23225).

Crystallographic data for 1: colorless needle crystals, C₂₄H₃₄O₅, Mr = 402.51, orthorhombic, space group P21 21 21, a = 8.08066(15) Å, b = 11.5507(2) Å, c = 23.1107(5) Å, α = β = γ = 90°, V = 2157.08(8) Å³, Z = 4, Dx = 1.029 g/cm³, crystal size 0.4 × 0.2 × 0.03 mm³, F(000) = 872, Cu Kα radiation (λ = 1.54184 Å), T = 99.99(10) K. Flack parameter was 0.19(17). The final R1 was 0.2742 (I > 2σ[I]), and wR2 was 0.2751 (all data). Crystallographic data for 1 (CCDC 1964126) can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Pancreatic Lipase Assay
The pancreatic lipase activity was measured according to the previously reported method, with a minor modification. The pancreatic lipase (50 U/mL) and 4-methylumbelliferyl oleate (4-MU oleate, 0.1 mM) were dissolved in a buffer consisting of 13 mM Tris-HCl, 150 mM NaCl, and 1.3 mM CaCl₂ (pH 8.0). The tested sample (25 µL, dissolved in 10% DMSO) was preincubated with 25 µL of lipase solution (50 U/mL) at 25 °C for 10 min, and then, 50 µL of 0.1 mM 4-MU oleate solution was added. The reaction mixture was incubated at 25 °C for 30 min. Fluorescence was then analyzed at 25 °C with an excitation of 355 nm and an emission wavelength of 460 nm by using a microplate reader (Biotek NEO2).

Conclusions
A new phenolic diterpene, 7-butoxyrosmanol (1) was isolated from the leaves of R officinalis, along with 3 known ones, namely epirosmanol (2), carnosol (3), and carnosic acid (4). Their structures were determined by extensive analysis of HRESIMS and NMR spectral data. The relative configuration of compound 1 was determined by single crystal x-ray diffraction analysis. Compound 1 inhibited pancreatic lipase activity with an IC₅₀ value of 46.2 µM.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.
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Supplemental Material
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