Antibacterial Activities of Ramalin, Usnic Acid and its Three Derivatives Isolated from the Antarctic Lichen *Ramalina terebrata*

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The development of new antibacterial compounds is an urgent issue to meet the evolution of resistivity of pathogenic bacteria against the available drugs. The objective of this study was to investigate the antibacterial compounds from the Antarctic lichen species *Ramalina terebrata*. A total of five compounds, usnic acid, usimine A, usimine B, usimine C, and ramalin, were isolated by bioactivity guided-fractionation of the methanol extract of *R. terebrata* after several chromatographic procedures. The qualitative antibacterial activities of the crude extract and isolated compounds were determined by the disk diffusion method while the minimum inhibitory concentration (MIC) determination assay gave the quantitative strength of the test samples. All the test samples showed antibacterial activity against *Bacillus subtilis*. The crude extract and usnic acid showed antibacterial activity against *Staphylococcus aureus*. The MIC values of the isolated compounds against *B. subtilis* were in the range of 1 to 26 μg/mL. These observed experimental data showed the strong antibacterial potential of these compounds against *B. subtilis*.

Key words: Antimicrobial, Antarctic Lichen, *Ramalina terebrata*, Usnic Acid

Introduction

Lichens are symbiotic associations of a fungus (mycobiont) and one or more photosynthetic partners (photobiont). They are cosmopolitan in distribution from arctic to tropical regions and from plains to the highest mountains and, even, some survive in the extreme environment of deserts. Lichens are used for several purposes since ancient times such as food, dyes, decoration, and several folk medicines. In addition, several experiments have shown that lichens consist of various secondary metabolites having antibiotic, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative, cytotoxic effects and antioxidant properties (Ingólfdóttir, 2002; Kumar and Müller, 1999). More than 800 lichen metabolites are reported from several classes: aliphatic acids, pulvinic acid derivatives, depsides and depsidones, dibenzofurans, diterpenes, anthraquinones, naphthoquinones, xanthones as well as epidithiopiperazinediones (Müller, 2001).

Several pathogenic microbes, especially Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis*, cause several human infectious diseases. Despite the reports on the development of several new antibiotics each year, microbes with antibiotic resistance capacities are evolving day by day causing serious challenges in health care settings all over the world. Therefore, it is very urgent to explore novel antibiotics against specific pathogenic bacteria to meet the rate of evolution of a superpathogen. Several lichen metabolites were found active against Gram-positive bacteria and mycobacteria. In this report, we describe the quantitative antibacterial activities of recently reported ramalin and three usnic acid derivatives, usimine A, usimine B, and usimine C, isolated from the Antarctic lichen *Ramalina terebrata* for the first time.

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Material and Methods

Collection and identification of lichen species

Ramalina terebrata was collected from the Korean Antarctic Research Station site (King Sejong Station) on King George Island (61°50' to 62°15' S and 57°30' to 59°01' W), in early February 2006 and in early January 2008. The species was identified morphologically as well as by analyzing the ribosomal DNA sequence of the total internal transcribed spacer (ITS) region as described previously (Paudel *et al.*, 2008a). A voucher specimen was deposited in the Polar Lichen Herbarium, Korea Polar Research Institute (KOPRI), Incheon, South Korea.

Extraction and isolation of the antibacterial compounds

A completely freeze-dried and ground lichen sample (672 g) was extracted in a mixture of methanol and water (80:20 v/v). The extract was filtered and the solvent evaporated at 45 °C *in vacuo*. This extraction procedure was repeated three times to ensure the complete extraction of extractable compounds. Finally, 83 g of crude extract were obtained after freeze-drying, and the extract was stored at −20 °C until further use.

Purification and characterization of the active secondary metabolites

Initially, 83 g of crude extract were dissolved in 1 L of distilled water. Then, the hexane-soluble extract was first extracted with 1 L hexane three times to extract low polar compounds. The final weight of the hexane extracts was 12.7 g after freeze-drying. The remaining aqueous phase was extracted with 1 L chloroform three times to extract low or moderately polar compounds. The final weight of the chloroform extract was 9.1 g after freeze-drying. Finally, the remaining extract was only the water-soluble one and the yield was 52 g after freeze-drying. Then, the antimicrobial activities of all three extracts were performed. Among them, the chloroform-soluble and water-soluble extracts were active against the Gram-positive bacteria *B. subtilis* and *S. aureus*. Thus, these two fractions were preceded for further purification.

Purification of the water-soluble fraction

The water-soluble fraction (5 g) was subjected to automated mild pressure liquid chromatography (MPLC) using a C18 ODS silica column (150 cm × 3 cm). Ramalin was isolated using various chromatographic techniques as described previously (Paudel, 2009). The identification of the compound was performed by comparing HPLC (retention time) and various spectroscopic data (HRESIMS, ^{1}H NMR and ^{13}C NMR) with those of an authentic probe as described previously (Paudel, 2009).

Purification of the chloroform-soluble fraction

The chloroform-soluble fraction was subjected to semi-preparative reversed-phase HPLC using a C18 ODS column (250 mm × 10 mm). The gradient HPLC solvent system was as follows: 20–40% acetonitrile in water (0.1% formic acid) over 20 min, followed by 60% acetonitrile over 30 min, and 100% acetonitrile over 31 min. The total run time was 50 min. The flow rate was 2 mL/min. Three compounds usimine B, usimine C and usimine A were isolated at 29.6, 32.7 and 36.6 min, respectively. Similarly, yellow crystalline needles were also purified from the chloroform-soluble fraction and further purified by repetitive crystallization. The identification of these compounds was performed by comparing various spectroscopic data such as HRESIMS, ^{1}H NMR and ^{13}C NMR with those of authentic probes as described previously (Rashid *et al.*, 1999; Seo *et al.*, 2008).

Antimicrobial assays

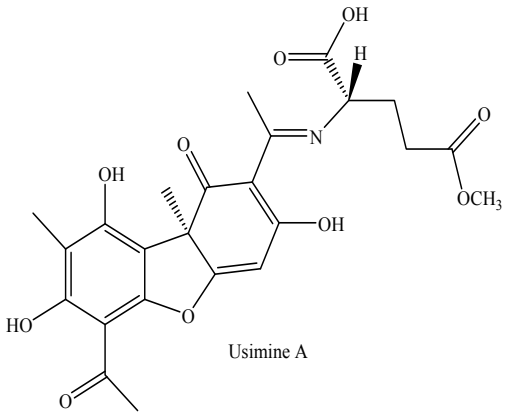
Antimicrobial assays of the crude extract of *R. terebrata* and isolated compounds were performed against five clinical microorganisms, including two Gram-positive (*Bacillus subtilis* KCTC1022 and *Staphylococcus aureus* KCTC3881) and two Gram-negative (*Escherichia coli* KCTC1039 and *Pseudomonas aeruginosa* KCTC1636) bacteria and one fungus (*Candida albicans* KCTC 7965). All strains were purchased from Korean Collection of Type Culture (KCTC), Deajeon, South Korea. Bacterial strains were grown on nutrient agar (NA) at 30–37 °C and *C. albicans* was grown on yeast mannitol (YM) agar at 25 °C. The qualitative antibacterial assay was performed by the disk diffusion assay as described previously (Bhattarai

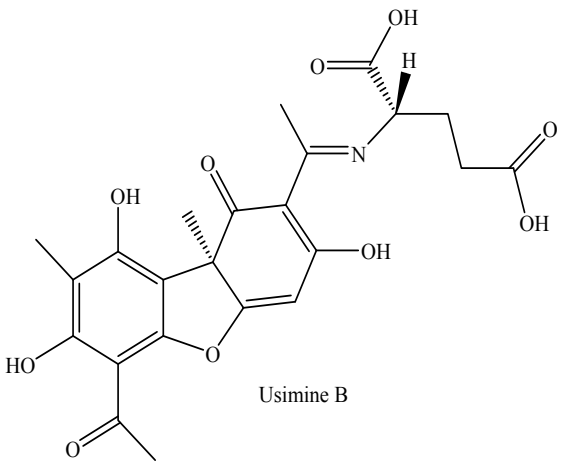
et al., 2006) to measure the zone of inhibition of the target microorganism. The quantitative antibacterial assay was performed by determining the minimum inhibitory concentration (MIC) of the test sample against the target microorganism as described previously (Swenson *et al.*, 1982).

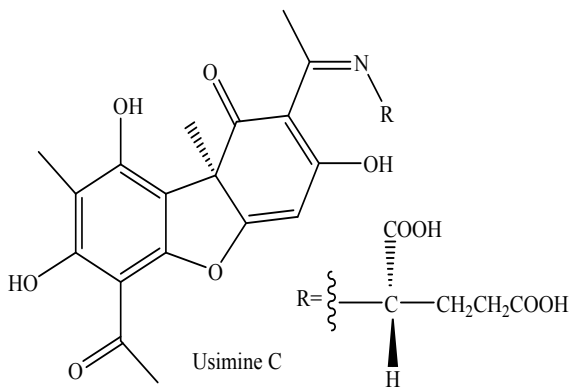
Results and Discussion

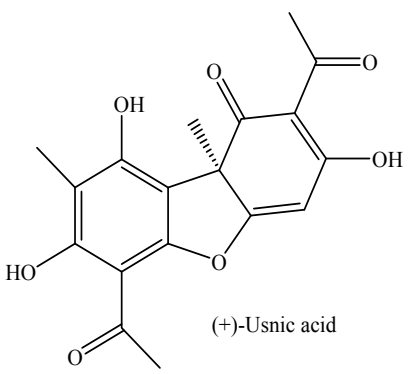
Identification of the antibacterial compounds

A total of five compounds, ramalin, usnic acid, and its three derivatives usimines A–C, were isolated (Fig. 1). Ramalin was the major constitu-









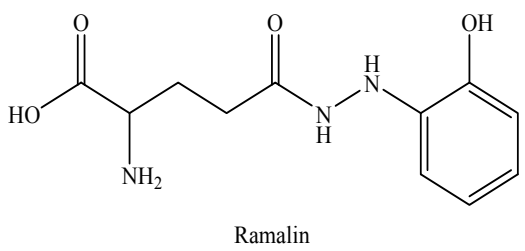


Fig. 1. Antibacterial compounds, ramalin, usimines A–C, and usnic acid, isolated from *R. terebrata*.

ent in the water-soluble fraction of the *R. terebrata* extract. The molecular formula of ramalin was determined as $C_{11}H_{15}N_3O_4$ by analysis of its HRESIMS data (*m/z* = 254.1141 $[M + H]^+$); its ^1H and ^{13}C NMR data (D_2O + acetone d_6) were comparable to previously reported data (Paudel, 2009). Usnic acid was the major constituent in the chloroform fraction of the *R. terebrata* extract. It was obtained as yellow needles when the saturated chloroform fraction was mixed with methanol. Its molecular formula, $C_{18}H_{16}O_7$, was deduced from HRESIMS (*m/z* = 345 $[M + H]^+$); its ^1H and ^{13}C NMR data ($CDCl_3$) were comparable to previously published data (Rashid *et al.*, 1999). Usimine A was obtained as a yellow gum. The molecular formula, $C_{24}H_{25}NO_{10}$, was deduced from HRESIMS (*m/z* = 488.1522 $[M + H]^+$); its ^1H and ^{13}C NMR data ($CDCl_3$) were comparable to previously published data (Seo *et al.*, 2008). Usimine B was also obtained as a yellow gum. The molecular formula, $C_{23}H_{23}NO_{10}$, was deduced from HRESIMS (*m/z* = 474.1392 $[M + H]^+$); its ^1H and ^{13}C NMR data ($CDCl_3$) were comparable to previously published data (Seo *et al.*, 2008). Usimine C was obtained as a yellow gum. The molecular formula was $C_{23}H_{23}NO_{10}$, as deduced from HRESIMS (*m/z* = 474.1393 $[M + H]^+$); its ^1H and ^{13}C NMR data ($CDCl_3$) were comparable to previously published data of usimine C, a geometrical isomer of usimine B (Seo *et al.*, 2008).

Antibacterial activities of the isolated compounds

During our previous preliminary screening study (Paudel *et al.*, 2008b), the methanol extract of *R. terebrata* showed antibacterial activity against Gram-positive pathogenic bacteria. The further work of isolation of bioactive compounds gave five compounds as described above. The antibacterial activities of usnic acid, usimines A – C, and ramalin in terms of inhibition zones and MIC values against two Gram-positive bacterial strains, *Bacillus subtilis* and *Staphylococcus aureus*, are presented here (Table I). In this experiment, none of the isolated compounds was found to be active against the Gram-negative strains *E. coli* and *P. aeruginosa* and the yeast *C. albicans*. The obtained experimental data indicated that usimines A–C and ramalin were active against *B. subtilis* only. Usnic acid showed antibacterial activity against both Gram-positive strains. However, the antibacterial activity against *B. subtilis* was stronger than that against *S. aureus*.

Among the five isolated compounds, usnic acid showed the strongest antibacterial activity against *B. subtilis* followed by usimines A and B, ramalin and usimine C. The minimum inhibitory concentrations (Table I) of the compounds show the quantitative strengths of the antibacterial activity. Several lichen metabolites have been described to be active against Gram-positive pathogenic bacteria. The observed antibacterial activity of usnic acid and usimines A and B was stronger than that of other lichen metabolites described in the literature such as atranorin (MIC against *B. subtilis*, 15.6 μg/mL, and against *S. aureus*, 500 μg/mL) from *Cladonia foliacea* (Yilmaz *et al.*, 2004), methyl *ß*-orsellinate and a mixture of methyl and ethyl orsellinates (MIC against *B. subtilis*, 160–330 μg/mL, and against *S. aureus*, 80–330 μg/mL) from *Sterocaulon alpinum* and *Peltigera aphthosa*, respectively (Ingólfsdóttir *et al.*, 1985).

Table I. Antimicrobial activities[a] of crude extract and compounds isolated from *R. terebrata*.

Sample	Inhibition zone [mm] (30 μg/disk)		MIC [μg/mL]	
	B. subtilis	*S. aureus*	*B. subtilis*	*S. aureus*
Extract[b]	38 ± 2.2	14 ± 1.2	33.8 ± 2.3	69.1 ± 6.7
Ramalin	32 ± 2.5	na	24.8 ± 1.5	nt
Usnic acid	32 ± 3.5	10 ± 0.2	1.2 ± 0.1	5.6 ± 0.8
Usimine A	27 ± 2.3	na	11.14 ± 0.9	nt
Usimine B	22 ± 1.8	na	12.73 ± 1.1	nt
Usimine C	20 ± 2.1	na	26.4 ± 2.7	nt
Ampicillin	–	–	0.4 ± 0.01	0.35 ± 0.01

[a] There was no antimicrobial activity of the lichen extract against *E. coli*, *P. aeruginosa*, and *C. albicans*.
[b] 500 μg/disk of crude extract was tested.
na, not active at test concentration.
nt, not tested because these compounds were not active in the disk diffusion assay.

Previously, various biological activities of usnic acid and usimines A–C were reported. Usnic acid was isolated from several species of lichens and showed several biological activities such as antitumour (Kupchan and Kopperman, 1975), antibacterial (Klosa, 1953; Lauterwein *et al*., 1995), antimycobacterial (Ingólfsdóttir *et al*., 1998), analgesic and antipyretic (Okuyama *et al*., 1995), antiviral (Yamamoto *et al*., 1995), antiproliferative (Kumar and Müller, 1999) and anti-inflammatory. Usimines A–C from *R. terebrata* showed moderate inhibitory activity against the therapeutically targeted protein tyrosine phosphate 1B (PTP1B) (Seo *et al*., 2008). Similarly, ramalin from *R. terebrata* also showed strong antioxidant activities *in vitro* without giving any cytotoxic effects against two human cell lines, keratinocyte and fibroblast (Paudel, 2009). Antibacterial activities of ramalin and usimines A–C are reported here for the first time. In conclusion, the described natural compounds from the Antarctic lichen *R. terebrata* showed potential antibacterial activity especially against the Gram-positive bacterium *B. subtilis*, and these compounds merit for future research in the field of new drug discovery.

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