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Ceramides and terpenoids from *Russula austrodelica* Singer

[Ceramidas y terpenoides desde *Russula austrodelica* Singer]

Julio ALARCÓN, Nicolás VILLALOBOS, Claudio LAMILLA & Carlos L. CÉSPEDES

Laboratorio de Síntesis y Biotransformación de Productos Naturales, Departamento de Ciencias Básicas, Facultad de Ciencias, Universidad del Bío-Bío, Chillán, Chile
Contactos | Contacts: Julio ALARCÓN - E-mail address: jualarcon@ubiobio.cl

Abstract
A mixture of ceramides and known terpenes, was obtained from the fruiting bodies of *Russula austrodelica*. The structures were determined from chemical and spectroscopic evidence. *R. austrodelica* is a mycorrhizal fungus that grow in the *Nothophagus* forests of southern Chile. This is the first report of the isolation of ceramides in Chilean mushrooms.

Keywords: ceramides, chilean mushrooms, *Russula austrodelica*

Resumen
Una mezcla de ceramidas y de terpenos conocidos, se obtuvo de los cuerpos fructíferos de *Russula austrodelica*. Las estructuras fueron determinadas a partir de evidencias químicas y espectroscópicas. *R. austrodelica* es un hongo micorrícico que crecen en los bosques de *Nothophagus* del sur de Chile. Este es el primer informe del aislamiento de ceramidas en hongos chilenos.

Palabras Clave: ceramidas, hongos chilenos, *Russula austrodelica*

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INTRODUCTION

The Russulaceae family is one of largest in the subdivision Basidiomycotina (Order: Russulales) in Whitthaker's Kingdom of Fungi and comprises hundreds of species (Whitthaker, 1969). Although secondary metabolites occurring in the fruiting body of European Lactarius species have been investigated, the *Russula* mushrooms have received less attention, notwithstanding the larger number of existing species (Vidari *et al*., 1998). Recently some new terpenoids and ceramides from *Russula* species have been reported (Vidari *et al*., 1998; Tan *et al*., 2000; Tan *et al*., 2001; Tan *et al*., 2002; Gao *et al.*, 2001; Clericuzio *et al.*, 2012).

The genus *Russula* in Chile is represent by *R. austrodelica* Singer, *R. fuegina* Singer; *R. major* Singer, *R. nothofaginia* Singer, *R. sardonia*, and *R. pectinatoides* Peck (Garrido, 1985; Palfner, 2011). *R. austrodelica* (Russullaceae) is a fungus linked to the arrival of ectomycorrhizal trees introduced in forests ecosystems, parks and gardens have been established adventive species of this genus (Singer 1969, Garrido 1985, Valenzuela 1998), most specifically linked to certain kinds of phytobionts.

Ceramides are lipid species that exert biological effects through cellular proliferation, differentiation, and cell death, and interact with several pathways involved in insulin resistance, oxidative stress, inflammation, and apoptosis, all of which are linked to nonalcoholic fatty liver disease (NAFLD) (Pagadala *et al.*, 2012). These compounds plays an important role in many fields of biochemistry; while their functions as structural lipids in membranes, in epidermis, hair and nails of humans and animal has been known for the long time, recently the interest have been focused on its role as important second messengers for various cellular processes including cell cycle arrest, differentiation, senescence, apoptosis, and others (Cremestri and Fischi, 2000).

In previous papers, we have reported the isolation and structural elucidation of seven compounds from the Chilean mushroom (Aqueveque et *al.*, 2006). Continuing with search for the bioactive constituents from Chilean mushrooms, the chemical constituents of *R. austrodelica* were investigated.

MATERIAL AND METHODS

Chemical and solvent

All reagents used were either analytical grade or chromatographic grade, ether petroleum 30-60, MeOH, CH_2Cl_2, $CHCl_3$, Ethyl Acetate, NaOH, HCl, silica gel GF254 analytical chromatoplates, silica gel grade 60 (70-230, 60 A°) for column chromatography were purchase from Merck-Chile S.A., Santiago, Chile.

Instruments

The IR spectra were recorder on a Shimadzu FTIR 8400 spectrometer with KBr disks. ^{1}H NMR and ^{13}C NMR spectra measured on a Bruker AM-200 spectrometer with TMS as internal standar. The GC-MS analyses were made on gas chromatography-mass selective detector (GC: Shimadzu GC-17A, injection temperature 270 °C, split/splitness injector, MS: Shimadzu QP5050A)

Plant material

The fruiting bodies of R. *austrodelica* were collected during autumn in the forests near to Chillan city, at the field of "Piedras las Comadres" Route 55, on road to Termas de Chillan, (36° 54 '12.17 ", 71° 32' 21.85), VIII Region, Chile. Voucher specimen has been deposited in the herbarium of the Departamento de Ciencias Básicas, Universidad del Bío-Bío, Chillán, Chile.

Extraction of plant material

The fresh fruit bodies of *R. austrodelica* (683 g) were extracted five times with Me OH (2.5 L) at room temperature for 8 d. The resulting methanol extract was filtered and concentrated under vacuum at 40 °C and 180 mb to obtain a crude residue (85 g). Total methanolic extract of R. *austrodelica* was solvent partitioned by dissolving in a mixture of MeOH:H_2O, transferred to a separator funnel and extracted 15 times with n-hexane (100 ml per extraction), the n-hexane phase combined and concentrated under reduced pressure. An identical process was repeated with ethyl acetate and finally obtained residual water. The n-hexane soluble fraction was subjected to CC containing silica gel (Merck 60, 0.063-0.2 µm; column 3 x 30 cm), and elution with a solvent mixture of petroleum ether: ethyl acetate. Final purification was achieved by preparative TLC (Merck, Silica gel 60 F_{254}) to give the compounds **1**, **2** and **3**. The similar form ethylacetate fraction was concentrated to afford a residue (3 g), which was subjected to silica gel chromatography (Merck 60, 0.063-0.2 µm; column 3 x 30 cm) and eluted with n-hexane, CH_2Cl_2, CH_2Cl_2-MeOH (50:1), CH_2Cl_2-MeOH (30:1), CH_2Cl_2-MeOH (10:1), and MeOH. Compound **4** was eluted with CH_2Cl_2-MeOH (50:1), and ceramide **5** with CH_2Cl_2-

MeOH (10:1). To elucidated the structures was used the conventional methods as IR, UV, ^{1}H-RMN, ^{13}C-RMN and Mass spectrum.

Acetylation of Ceramide

A solution of ceramide **5** (50 mg) in pyridine (1 ml) was treated with Ac_2O (1 ml) was and the mixture was left standing at room temperature for 24 h, then poured into ice-water and extracted with ethyl acetate. Work-up ethyl acetate extracted in the usual manner gave the product, which was purified by column chromatography to furnish compound **6**.

Methanolysis of ceramide

Ceramide **5** (50 mg) was refluxed with 12 N HCl (2 ml) and dry MeOH (25 ml) for 3 h. The reaction mixture was adjusted to pH 8 with 10% NaOH-MeOH and the whole was extracted with petroleum ether, and petroleum ether layer was concentrated gave the products **7** and **8**. The compounds **8** was analyzed by GC-MS. GC-MS:0.53 x 30 m capillary column (SUPELCO SPB-1); column temperature 120-240 °C (5 °C/min); He flow rate, 20 m/s; ionization 22 eV. The methanol-water layer was neutralized with saturated Na_2CO_3 and concentrated to dryness, and then heated with Ac_2O/pyridine (1:1) for 1.5 h at 70 °C. The reaction mixture was diluted with H_2O and extracted with ethyl acetate. The solvent was evaporated and the obtained residue was further purified by preparative TLC as eluent to furnish of compound **9**.

RESULTS AND DISCUSSION

The *n*-hexane soluble fraction of MeOH extract from the fruiting bodies of *R. austrodelica* was subjected to column chromatography and preparative TLC to yield **1**, **2**, and **3**. Based upon comparison of spectroscopic (MS, IR, ^{1}H and ^{13}C NMR) and physical data with literature, the structure those compound were identifications as known compound furanol **1**, lactaral **2**, which were previosly isolated from *Fomitopsis insulari* (Nozoe *et al*., 1971) and *Lactarius vellereus* and *L. pergamenus* (Backens *et. al*, 1984) respectively. The compound **3** a lactarane sesquiterpene was reporter by first time by Vidari et al (1976). Compound **4** was isolated as a white powder from CH_2Cl_2:MeOH (50:1) eluent of the ethyl acetate soluble fraction, and was readily identified as brassicastrol (Hayee-Memon *et al.,* 1991), by comparison of their NMR spectroscopic and mass spectral data and their fragmentation pattern with literature values.

The ceramide **5a, 5b, and 5c** were obtained as a color less amorphous powder. The IR spectrum of 5a showed the presence of hydroxyl (3310 cm^{-1}), amide (1640 cm^{-1}), and aliphatic (2918, 2849, 1469 cm^{-1}) functionalities. The ^{1}H NMR spectrum of **5a** showed signals due two terminal methyl groups (δ 0.85, 3H, t, *J* = 7.0 Hz and δ 0.86, 3H, t, *J* = 7.0 Hz), aliphatic methylenes (δ 1.25, br s), an oxygenated methylene group (δ 4.44, 1H, m; 4.52, 1H, m), two oxygenated methine groups (δ 4.30, 1H, m; 4.37, 1H, ddd, *J* = 6.6,6.2, 4.8 Hz), a methine group (δ 5.12, 1H, m) and an amide proton (δ8.60, 1H, d, *J* = 9.2 Hz). The ^{13}C-NMR spectrum showed characteristic signals appearing due to an amide carbonyl at δ_C 175.3 and methine carbon linked to amide nitrogen at δ_C 53.0. These spectral data were virtually identical with ceramides isolated from *Grifola frondosa* (Yaoita *et al.,* 2000) and *Acanthaster planci* (Inagaki et al., 1998), except for the lengths of the long chain base and the fatty acid. To determine the numbers of hydroxyl groups, compound **5a** was acetylated with Ac2-piridine at room temperature to afford the corresponding tri-acetylated product **6**. The triacetate **6** showed four ester methyl proton signals at δ2.17, 2.06, 2.04, and 2.02 ppm in the ^{1}H NMR spectrum; thus the presence of three hydroxyl groups in the original structure of **5a** was confirmed. Furthermore, methanolysis of **5a** liberated of long-chain bases **7** and fatty acid esters **8** (Figure 2). Similarly structure of ceramide **5b** and **5c** were determined. Ceramide methanolisis indicate that the difference between them is in the type of fatty acid to form amide linkage. The length of long chain of fatty acid was determined by GC-MS analysis, defined the composition as a mixture of C_{16} (hexadecanoic acid methyl ester (m/z 270) 17.25 min), C18 (octadecanoic acid methyl ester (m/z 298) 19.36 min), and C_{20} (eicosanoic acid methyl ester (m/z 326) 21.14 min) saturated fatty acid methyl esters. Acetylations of **7** yield the compound **9**, confirming structure of the amine. The relative stereochemistry at C-2, C-3, C-4 was proposed as 2S,3S,4R, since the chemical shifts and coupling constant of 1-H, 2-H, 3-H, and 4-H in **5a** were in good agreement with the information on literature.

Phytosphingosine-type ceramides similar to **5a**-**5c** have been reported from the soft coral *Sinularis leptoclados* (Bala *et al.,* 1999), *Tuber indicum* (Gao *et al.,* 2004), *Grifola frondosa* (Yaoita *et al.,* 2000), *Ceratodictyon spongiosum* and *Sigmadocia symbiotica*

(Lo *et al.*, 2001). Similar ceramides were isolated by Gao (2001) from *R. cyanoxantha*; however these ceramides contained α-hydroxy moiety fatty acid.

Spectroscopic data

Furanol 1: IR: 3600, 3000, 2890, 1730, 1470, 1370, 1120, 1050, 1040, 880. ^{1}H NMR ($CDCl_3$) δ (ppm): 7.43(s,1H), 7.14(s,1H), 4.4 (d, 1H, *J* = 11.1 Hz), 3.34, 288 (ABq, *J* = 16 Hz, an allylic methylene), 1.74 (s,3H), 1.13(s, 3H),0.89(s,3H).

Lactaral 2: IR: ν_{max} 3140, 3050, 2740, 1700, 1590, 1540, 1385, 1380, 1150, 1050, 875, 815, 755 cm^{-1}. ^{1}H NMR($CDCl_3$) δ (ppm) : 10.2 (bs, 1H), 7.65 (m,1H), 7.45 (m,1H), 5.20 (bs, 1H), 2.07 (m,4H), 1.10 (s,3H), 0.95 (s,3H), 0.85 (s,3H). ^{13}C NMR ($CDCl_3$) δ (ppm): 184.9(d), 152.7 (d), 146.9 (s), 142.2 (d), 127.5 (s), 122.7 (s), 121.6 (d), 47.1 (t), 47.1 (t), 38.2 (s), 35.1 (d), 29.9 (q), 29.7 (q), 29.3 (t), 18.9 (q). MS: m/z (int. rel.) 232 (M^+, 16), 214 (15), 199 (19), 123 (100), 81 (60).

Compound 3: IR: ν_{max} 3600-3300, 1745 cm^{-1}6.45 (1H, d, *J* = 8.0 Hz, OH-13), 6,19 (1H, dd, *J* = 8.0, 2.0 Hz, H-13),4.52 (1H, d, *J* = 8.0 Hz, OH-8), 4.17 (1H, m, H-8), 3.15 (1H, d, *J* = 20 Hz, H-4), 2.78 (1H, d, *J* = 20 Hz, H-4'), 2.26 (1H, d, *J* = 14 Hz, H-1'), 2.11 (1H, d, *J* = 14 Hz, H-1), 1.96 (1H, dd, *J* = 13.0, 7.0 Hz, H-10'), 1.76(3H, s, H-12), 1.56(1H, dd, *J* = 13, 4.0 Hz, H-10), 1.12 (3H, s, H-14), 0.91 (3H, s, H-15). MS: m/z (relative intensity) 264(M^+,1.7), 246(8.3), 244 (9.2), 229(31), 201(63), 185(32), 173(32), 142(35), 128(27), 115(35), 57(30), 43(100).

Ergosta-5,22-dien-3β-ol (**4**): ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.35 (1H, d, *J* = 6.5 Hz, 22-H), 3.51 (1H, m, 3β-H), 1.24 (1H, d, *J* = 7 Hz, 21-Me), 0.99 (3H, s, 19-Me), 0.67 (3H, s, 18-Me). MS: m/z 398 (M^+ - $C_8H_{15}.H_2O$), 356(M^+-C_3H_6), 314(M^+-C_6H_{12}), 314(M^+-C_6H_{12}), 300(M^+ -C_7H_{13}-H), 269(M^+ -$C_8H_{15}.H_2O$), 255(M^+ -C_9H_{17} – H_2O), 239, 213, 157, 133, 95, 69.

2-Acetoamino-1,3,4-triacetoxyoctadecane (**9**): ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.97 (1H, d, *J* = 9.2 Hz, NH), 5.10 (1H, dd, *J* = 0.5, 3.1 Hz, 3-H), 4.93 (1H, dt, *J* = 9.8, 3.1 Hz, 4-H), 4.47 (1H, m, 2-H), 4.29 (1H, dd, *J* = 11.6, 4.3 Hz, 1-Ha), 4.00 (1H, dd, *J* = 11.6, 3.1 Hz, 1-Hb), 2.08 (3H, s, 3-OAc), 2.05 (6H, s, 1-OAc, 4-OAc), 2.03 (3H, s, HNAc), 1.12-1.70 (26H, m), 0.88 (3H, t, *J* = 6.1 Hz, CH_3). EI-MS (70 eV) *m/z* (relative intensity %): 486 $[M+1]^+$(1), 426 [M+1 - HOAc]$^+$(2), 366 [M+1 - 2 x HOAC]$^+$(9), 305 [M-3 x HOAc]$^+$(24.5), 245 [M+1 – 4 x HOAc]$^+$(0.5).

Table 1
^{1}H and ^{13}C NMR ($CDCl_3$) spectral data of ceramide 5a

	5a	
N°	δ_C	δ_H
1	62.1	4.51 (dd, 10.6, 4.5) 4.43 (dd, 10.6, 4.5)
2	54.6	5.10 m
3	74.7	4.35 (dd, 6.5, 4.0)
4	73.1	4.28 m
5	34.1	2.26 m 1.93 m
6	26.7	1.70 (m)
7-16	29.6-30.4	1.25-1.41 m
17	22.5	1.25-1.41 m
18	14.3	0.88 (t, 6.7)
NH	-	8.57 (d, 8.8)
1'	174.0	-

The assignment were based on ^{1}H-^{1}H and ^{1}H-^{13}C COSY experiments; TMS as internal standard. Coupling constant (*J* in Hz) are given in parentheses.

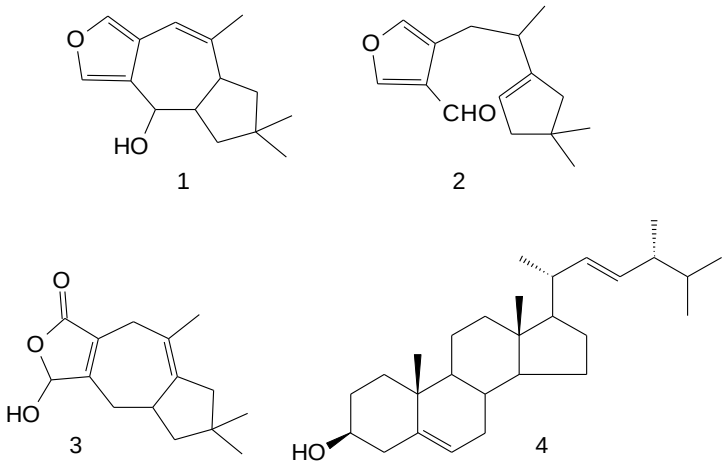

Figure 1
Terpenoids isolated from *Russula austrodelica* Singer.

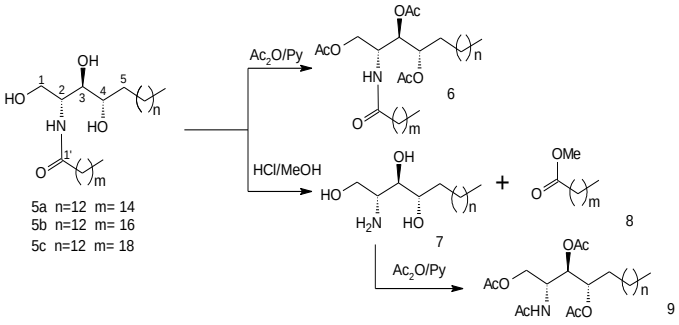

Figure 2
Ceramides isolated from *Russula austrodelica* Singer

CONCLUSIONS

Based on the above data was possible to determine the chemical structures of ceramides **5a**-**5c** present in the fruiting body of *R. austrodelica*. Ceramides **5a-5c** have same long chain base, 2-amino-1,3,4-octadecanetriol, and differ in the chain length of the fatty acids. This is the first report about the isolation and structural elucidation of each ceramide from *R. austrodelica*

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