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Carnitine-esters from the mushroom *Suillus laricinus*

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Abstract

Carnitine-esters (**1-8**) including, (*R*)-3-hydroxybutanoyl-(*R*)-carnitine (**5**), were isolated from the mushroom *Suillus laricinus*. Their structures were determined by spectroscopic analyses and by total synthesis. One of these, (*R*)-3-hydroxy-2-methylpropanoyl-(*R*)-carnitine (**4**), promoted hyaluronan-degradation by human skin fibroblasts.

1. Introduction

Hyaluronan (HA) is a non-sulfated glycosaminoglycan composed of repeating disaccharide units of *N*-acetylglucosamine and glucuronic acid. There are many reports showing that hyaluronan is elevated in disorders such as fibrosis, diseases and tumors (Satoh et al., 2000). For example, during the progression of hepatitis, accumulated HA causes fibrosis and eventually cirrhosis of the liver (Satoh et al., 2000). An increase of HA in the endocervical canal at the appropriate stage of pregnancy can result in miscarriage (El Maradny et al., 1997). Overproduction of HA accelerates tumor growth and is associated with cancer metastasis (Itano et al., 1999; Jacobson et al., 2002). These results show that the balance of production and degradation of HA in tissues is strictly regulated. Regulators targeting HA degradation may be expected as useful drugs. We have already reported hyaluronan-degradation inhibitors, orirubenones, from the mushroom *Tricholoma orirubens*. (Kawagishi et al., 2004; Sakai et al., 2005).

In the present study, we found promoting activity in the extract from the mushroom *Suillus laricinus* against HA-depolymerization. We wish to report here the isolation, the structure determination and the activity against HA-degradation of the carnitine-esters.

2. Result and discussion

Fresh fruiting bodies of *S. laricinus* were extracted with EtOH and then acetone. The extracts were partitioned between $CHCl_3$ and H_2O and then between EtOAc and H_2O. Among the three fractions, the water-soluble fraction only showed significant activity. Therefore, this fraction was further separated using bioassay-guided fractionation. As a result, eight compounds (**1**–**8**) were purified.

Compounds **1**–**4** and **6**–**8** were identified as (*R*)-carnitine and its esters based on analysis of their NMR spectroscopic data (Tables 1 and 2) as well as from additional spectroscopic data; most of their structures were also confirmed by synthesis (Duncombe and Rising, 1972; Fukao, 2003; Guilbert and Chung, 1974; Holland and Sherratt, 1973; Koeberl et al., 2003; Paul, 2000; Quistad et al., 1978a,b; Quistad et al., 1978c; Quistad et al., 1986; Quistad et al., 1979; Rashed et al., 1995; Roeschinger et al., 1885; Shigematsu et al., 1994; Silva et al., 2001; Zytkovicz et al., 2001). The absolute configuration of **4** was determined by synthesis of both diastereomers of the (*R*)-carnitine-ester.

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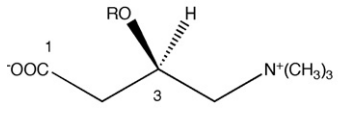
Table 1
^{1}H NMR spectroscopic data for **1**–**8** (in D_2O)

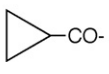
Position	1	2	3	4	5	6	7	8
2	2.30 (dd, 15.6, 6.6) 2.35 (dd, 15.6, 7.2)	2.39 (dd, 15.4, 7.6) 2.53 (dd, 15.4, 5.5)	2.35 (dd, 15.4, 7.7) 2.48 (dd, 15.4, 5.5)	2.40 (dd, 15.5, 7.5) 2.50 (dd, 15.5, 5.8)	2.39 (d, 7.0) 2.51 (m)	2.37 (dd, 15.6, 7.7) 2.51 (m)	2.38 (dd, 15.7, 7.8) 2.52 (dd, 15.7, 5.3)	2.36 (dd, 15.6, 7.6) 2.48 (dd, 15.6, 5.8)
3	4.47 (m)	5.49 (m)	5.47 (m)	5.54 (m)	5.51 (m)	5.50 (m)	5.49 (m)	5.49 (m)
4	3.32 (m)	3.50 (d, 14.4) 3.74 (dd, 14.4, 8.6)	3.47 (d, 14.3) 3.71 (dd, 14.3, 8.9)	3.50 (d, 14.4) 3.77 (dd, 14.4, 8.7)	3.49 (d, 14.6) 3.74 (dd, 14.6, 8.7)	3.49 (d, 14.5) 3.75 (dd, 14.5, 8.5)	3.48 (d,14.7) 3.73 (dd, 14.7, 8.6)	3.48 (d, 14.4) 3.73 (dd, 14.4, 8.7)
$N^+(CH_3)_3$	3.12 (s)	3.07 (s)	3.04 (s)	3.06 (s)	3.06 (s)	3.05 (s)	3.05 (s)	3.05 (s)
2′		2.02 (s)	2.35 (q, 7.7)	2.67 (m)	2.39 (m) 2.51 (m)	2.51 (m)	2.48 (d, 6.4)	1.61 (dddd, 6.5, 6.5, 6.4, 6.7)
3′			0.95 (t, 7.7)	3.59 (d, 6.1)	4.12 (m)	4.00 (m)	1.18 (s)	0.88 (m)
4′					1.10 (d, 6.4)	1.05 (d, 6.4)		
2′-CH_3				1.00 (d, 7.0)		1.00 (t, 7.1)		
3′-CH_3							1.18 (s)	

Table 2
^{13}C NMR spectroscopic data for **1**–**8** (in D_2O)

Position	1	2	3	4	5	6	7	8
1	178.9	177.2	177.2	177.9	177.1	176.9	176.2	177.2
2	43.8	40.7	40.9	40.8	40.9	45.5	40.6	41.0
3	64.9	67.4	67.4	67.4	67.6	66.3	67.4	67.4
4	71.0	68.7	69.0	68.7	69.0	67.5	68.6	68.9
$N^+(CH_3)_3$	54.9	54.3	54.5	54.3	54.5	53.1	54.2	54.5
1′		173.3	176.7	176.8	173.4	175.0	172.4	177.0
2′		21.2	28.2	42.6	44.0	40.0	47.6	13.5
3′			8.7	63.9	65.2	67.4	70.3	9.4
4′					22.7	18.7	28.4[a]	9.5
2′-CH_3				12.9		9.6		
3′-CH_3							29.0[a]	

[a] Interchangeable.



1 R = H
2 CH_3CO-
3 CH_3CH_2CO-
4 (*R*)-$HOCH_2(CH_3)CHCO$-
5 (*R*)-$CH_3(HO)CHCH_2CO$-
6 $CH_3(HO)CH(CH_3)CHCO$-
7 $CH_3(CH_3)(HO)CCH_2CO$-
8 

Compound **5** was isolated as a clear amorphous solid and its FABMS showed a molecular ion peak at *m*/*z* 248 ($[M+H]^+$). Its molecular formula, $C_{11}H_{21}NO_5$, as determined by HRFABMS, indicated the presence of two degrees of unsaturation in the molecule. The ^{1}H and ^{13}C NMR spectra of **5**, along with DEPT and HMQC analyses, suggested that this compound was also an ester of (*R*)-carnitine (Tables 1 and 2). The COSY and HMBC spectra indicated a contiguous sequence of coupled signals of the acid part of the ester, CH_3–CH–CH_2–COO– [δ_H 1.10 (3H, d, $J = 6.4$), δ_C 22.7; 4.12 (1H, m), 65.2; 2.39 (1H, m), 2.51 (1H,m), 44.0; 173.4]. This sequence and the molecular formula suggested that this compound was a 3-hydroxybutanoate ester of (*R*)-carnitine. Its absolute configuration was determined by preparation of both diastereomers. The ^{1}H and ^{13}C NMR spectroscopic data, and $[\alpha]_D$ of (*R*)-3-hydroxybutanoyl-(*R*)-carnitine were completely identical with those of **5**.

Compounds **1**–**8** were evaluated in a hyaluronan-degradation promoting assay. Among them, (*R*)-3-hydroxy-2-methylpropanoyl-(*R*)-carnitine (**4**) only promoted the degradation; 24% and 28% of HA was degraded at 0.4 and 0.8 mM, respectively.

3. Experimental

3.1. General

The ^{1}H NMR spectra (one- and two-dimensional) were recorded on a JEOL lambda-500 spectrometer at 500 MHz, while ^{13}C NMR spectra were recorded on the same instrument at 125 MHz. The FABMS and HR-FABMS spectra were recorded on a JEOL DX-303HF. A JASCO grating infrared spectrophotometer was used to obtain the IR spectra. The $[\alpha]_D$ spectrum was measured by using a JASCO DIP-1000 spectropolarimeter. MPLC was carried out using a YAMAZEN MPLC-system (Japan) and an UltraPack ODS-S50D column (50 × 300 mm, YAMAZEN, Japan). HPLC separations were performed with a JASCO Gulliver system. YMC-Pack polyamine II columns (20 × 250 mm, YMC, Japan) or Wakosil II columns (20 × 250 mm, Wako pure chemicals, Japan) were used for HPLC. Silica gel plate (Merck F_{254}) and silica gel 60 N (Merck 100–200 mesh) were used for analytical TLC and for flash CC, respectively. Sepharose CL-2B was purchased from Pharmacia Fine Chemicals (Sweden). Methyl (*R* or *S*)-3-hydroxybutanoate and methyl (*R* or *S*)-3-hydroxy-2-methylpropanoate were products of Sigma-Aldrich, USA. Hyaluronan variants extracted from pigskin ($4–6 \times 10^4$ Da) and human umbilical cord ($8–12 \times 10^5$ Da) were obtained from Seikagaku Corporation (Japan).

3.2. Fungus materials

Mature fruiting bodies of *Suillus laricinus* (Berk. in Hook.) O. Kuntze were collected at Narusawa village, Yamanashi Prefecture in Japan and identified by the one of the authors (H. K.). A voucher specimen of the organism (SL-02-09) is located in Shizuoka University.

3.3. Extraction and isolation

Fresh fruiting bodies of *S. laricinus* (17.1 kg) were extracted with EtOH (10 l, 4 times) and then acetone (5 l). The extracts were combined, concentrated under reduced pressure, and partitioned between $CHCl_3$ and H_2O. The H_2O layer was further partitioned between EtOAc and H_2O. The residue (253.4 g) obtained after removing H_2O was fractionated by silica gel flash CC ($CHCl_3$/MeOH/H_2O = 5.5/4.0/0.5, 4.5/4.5/1.0, 3.5/5.5/1.0, 0/8.0/2.0, 0/5.0/5.0, each 3 L) to obtain 13 fractions. Fraction 10 (6.5 g) was further separated by reversed-phase MPLC (1% aqueous MeOH) and 14 fractions were obtained. Fraction 10-1 (349.1 mg) was separated by polyamine HPLC (1% aqueous MeOH) to afford compounds **1** (3.9 mg), **2** (6.2 mg), **3** (2.6 mg), **4** (1.3 mg), **5** (1.5 mg), **6** (2.3 mg), **7** (1.2 mg), and **8** (1.0 mg).

Compound **4**: $[\alpha]_D^{25}$ −25 (H_2O, *c* 0.11). IR (neat) ν_{max} cm^{-1}: 3342, 1740; FAB-MS (matrix, glycerol) *m/z* 248 $(M+H)^+$; HR-FAB-MS *m/z* 248.1499 [calcd for $C_{11}H_{22}NO_5$ $(M+H)^+$, 248.1492].

Compound **5**: $[\alpha]_D^{25}$ −28 (H_2O, *c* 0.55). IR (neat) ν_{max} cm^{-1}: 3347, 1735; FABMS (matrix, glycerol) *m/z* 248 $(M+H)^+$; HRFABMS *m/z* 248.1490 [calcd for $C_{11}H_{22}NO_5$ $(M+H)^+$, 248.1492].

3.4. Preparation of *4*, *5* and their diastereomers

(*R*)-3-Hydroxybutanoic acid (20.8 mg, 0.20 mmol) derived from its methyl ester by hydrolysis with 1 M KOH was dissolved in DMSO (0.2 ml) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (31.0 mg, 0.20 mmol) was added to the solution. The mixture was stirred at room temperature for 1 h and (*R*)-carnitine (32.2 mg, 0.20 mmol) was added to the mixture. The reaction mixture was stirred at 80 °C for 24 h and lyophilized. The dried reaction mixture was separated by reversed-phase HPLC (1% aqueous MeOH), giving (*R*)-3′-hydroxybutanoyl-(*R*)-carnitine (7.2 mg, 0.029 mmol).

(*S*)-3′-Hydroxybutanoyl-(*R*)-carnitine (5.0 mg), (*R*)-3′-hydroxy-2′-methylpropanoyl-(*R*)-carnitine (2.3 mg), and (*S*)-3′-hydroxy-2′-methylpropanoyl-(*R*)-carnitine (2.8 mg) were also prepared by the same method.

(*R*)-3′-Hydroxybutanoyl-(*R*)-carnitine; $[\alpha]_D^{25}$ + 29 (H_2O, *c* 0.20).

(*S*)-3′-Hydroxybutanoyl-(*R*)-carnitine; $[\alpha]_D^{25}$ − 5.1 (H_2O, *c* 0.20). ^{1}H NMR (D_2O): δ 1.08(3H, d, J = 6.4), 2.37(1H, m), 2.40(1H, m), 2.50(1H, m), 2.52(1H, m), 3.04(9H, s), 3.48(1H, d, J = 14.3), 3.73(1H, dd, J = 14.3, 8.7), 4.11(1H, m), 5.48(1H, m).

(*R*)-3′-Hydroxy-2′-methylpropanoyl-(*R*)-carnitine; $[\alpha]_D^{25}$ − 26 (H_2O, *c* 0.10).

(*S*)-3′-Hydroxy-2′-methylpropanoyl-(*R*)-carnitine; $[\alpha]_D^{25}$ + 0.68 (H_2O, *c* 0.10). ^{1}H NMR (D_2O): δ 0.99(3H, d, J = 7.0), 2.37(1H, dd, J = 15.6,7.8), 2.49(1H, dd, 15.6, 5.5), 2.62(1H, m), 3.04(9H, s), 3.48(1H, d, J = 14.3), 3.56(1H, dd, J = 11.0, 7.0), 3.61(1H, dd, J = 14.3,8.9), 3.75(1H, dd, J = 11.0, 3.9), 5.49(m).

3.5. Bioassay

3.5.1. Cell lines and culture procedure

Detroit 551 human skin fibroblasts purchased from the American Type Culture Collection (USA) were grown to confluence on 12-well plates in Eagle's minimal medium (ICN Biomedicals Ltd., USA) supplemented with non-essential amino acids, 1 mM sodium pyruvate, and 10% (v/v) FBS (JRH Pharmaceutical Co., USA). Cultures were grown at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

3.5.2. Preparation of [^{3}H]HA

[^{3}H]HA was prepared by the method described by Underhill and Tool with some modification. (Underhill and Toole, 1979) Briefly, confluent Detroit 551 fibroblasts were incubated with 10μCi/ml of [^{3}H]glucosamine (American Radiolabeled Chemicals Inc., USA) at 37 °C. The conditioned medium was pooled, digested with pronase

(0.3 mg/ml) (Calbiochem, USA) at 37 °C for 24 h, and precipitated with four volumes of absolute EtOH (−20 °C). After suspending the resulting pellets with distilled H_2O and then centrifuging them (12,000 × g, 20 min), the supernatants were incubated at room temperature in 0.1% cetylpyridinium chloride and 0.4 M NaCl, centrifuged again (12,000 × g, 20 min), precipitated with four volumes of absolute EtOH (−20 °C), resolubilized, and applied to a Sepharose CL-2B column (1.0 × 60 cm) in 0.5 M NaCl. The void fractions were collected, precipitated with four volumes of absolute EtOH (−20 °C), and finally resolubilized with 5 mM phosphate buffer (pH 7.5). The uronic acid concentration was determined by the method of Rai et al. (Rai et al., 2001). The specific activity of isolated [^{3}H]HA (>10^6 Da) was 1.3×10^5 dpm/μg (2.2 kBq/μg). The isolated [^{3}H]HA was completely degraded by *Streptomyces* hyaluronidase (Seikagaku Kogyo Co., Japan), indicating that this material was pure.

3.5.3. Assay for [^{3}H]HA depolymerization

At confluence, the medium was changed to Eagle's minimal medium supplemented with non-essential amino acids and 1 mM sodium pyruvate without 10% (v/v) FBS. After one-day culture without FBS, the fibroblasts were incubated with [^{3}H]HA and each sample at various concentrations in FBS-free medium as mentioned above. The concentration of added [^{3}H]HA was 0.4 μg/mL. After incubation with [^{3}H]HA (3 days), the medium was harvested, boiled, and applied to a Sepharose CL-2B column (1.0 cm × 60 cm) eluted with 0.5% NaCl. The flow rate was 0.6 ml/min. The activity of each fraction (2.4 ml) was measured by a liquid scintillation counter (Aloka LSC-1000, Japan). The total radioactivities recovered in the medium were matched with that of cell-free culture and degraded completely by *Streptomyces* hyaluronidase. To determine activities of cellular HA depolymerization, the total radioactivities of high-molecular-mass fractions (fractions 1–8) were measured. The activities were given by as follows:

$$\text{Depolymerizing activity(dpm/well)} = \text{Cell Free}^{*} - \text{Cultured}^{**}$$

(Cell Free* is the total of the radioactivities of fractions 1–8 after culture without cells, and Cultured** is the total of the radioactivities of fractions 1–8 of the CL-2B column after culture).

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