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젖버섯아재비 자실체로부터 분리한 Azulene계 화합물이 Interferon-γ 생성에 미치는 영향

Guang Hua Xu · 김 재 화* · Gao Li · 유 익 동*,†

중국 연변대학교 약학대학, *한국생명공학연구원 화학생물연구센터
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Modulatory Effect of Four Azulene Derivatives from the Fruiting Bodies of *Lactarius hatsudake* on Interferon-γ Production

Guang Hua Xu, Jae Wha Kim*, Gao Li, and Ick Dong Yoo*,†

College of Pharmacy, Yanbian University, Yanji 133000, Jilin, China
*Chemical Biology Research Center, Korea Research Institute of Bioscience and Biotechnology, Yuseong, Daejeon 305-806, Korea
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요 약: 버섯유래 생리활성물질을 탐색하고자, 젖버섯아재비 자실체로부터 각종 컬럼크로마토그래피 및 HPLC 등 기법에 의하여 4종의 azulene계 화합물을 순수히 분리정제 하였다. 분리된 화합물은 각종 물리화학적 특성 및 분광학적 분석 자료에 의하여 1-formyl-4-methyl-7-isopropyl azulene (1), lactaroviolin (2), 4-methyl-7-isopropyl-azulene-1-carboxylic acid (3) 및 1-formyl-4-methyl-7-(1-hydroxy-1-methylethyl) azulene (4)로 동정되었다. 이들 화합물의 인터페론 감마 생성에 미치는 영향을 조사하였다. 화합물 1과 4는 자연살해세포주(NK92 cell)에서 인터페론 감마 생성을 농도 의존적으로 억제하였으며, 400 μM농도에서 각각 101.3 %와 92.7 %, 100 μM농도에서 각각 11.9 %와 24.1 %의 높은 저해활성을 보였으며, 화합물 2와 3은 400 μM농도에서 45.9 %와 18.0 %의 다소 낮은 저해활성을 나타내었다.

Abstract: Investigation of secondary bioactive metabolites from the fruiting bodies of *Lactarius hatsudake* led to the isolation of four azulene derivatives by means of repeated column chromatography and preparative HPLC, and they were identified as 1-formyl-4-methyl-7-isopropyl azulene (1), lactaroviolin (2), 4-methyl-7-isopropyl-azulene-1-carboxylic acid (3), and 1-formyl-4-methyl-7-(1-hydroxy-1-methylethyl) azulene (4) by their physico-chemical properties and spectroscopic analysis. The isolated compounds were evaluated for the effects on modulation of cytokines in natural killer cell line (NK92 cells). Compounds 1 and 4 strongly inhibited IFN-γ production in a dose-dependent manner, corresponding to 101.3 % and 92.7 % inhibition at 400 μM, and 11.9 % and 24.1 % at 100 μM, respectively, whereas compounds 2 and 3 showed weak inhibitory effect on INF-γ production, corresponding to 45.9 % and 18.0 % inhibition at 400 μM.

Keywords: *Lactarius hatsudake, azulene, interferon-γ, natural killer cell*

1. Introduction

The interferons (IFNs) are a large family of cytokine mediators with a variety of biological functions including inhibition of proliferation, induction of differentiation, modulation of immune system, and inhibition of angiogenenesis[1]. Although initially IFNs were discovered through their antiviral activities, they are now known to exert pleiotropic effects on important cellular functions through multiple signaling pathways[2]. The

† 주 저자 (e-mail: idyoo@kribb.re.kr)

IFNs may be classified into two distinct types: type I and type II according to receptor specificity and sequence homology. Type I IFNs are produced in direct response to virus infection and consist of the products of the IFN-α multigene family, which are predominantly synthesized by leukocytes, and the product of the IFN-β gene, which is synthesized by most cell types but particularly by fibroblasts[3]. Type II IFN consists of the product of the IFN-γ gene and, rather than being induced directly by virus infection, is synthesized in response to the recognition of infected cells by activated T lymphocytes and natural killer (NK) cells[4]. IFN-γ, the sole type II IFN, is a dimerized soluble cytokine involved in the regulation of nearly all phases of immune and inflammatory responses, including the activation, growth and differentiation of T-cells, B-cells, macrophages, NK cells and other cell types such as endothelial cells and fibroblasts[5,6]. This interferon was originally called macrophage-activating factor, a term now used to describe a larger family of proteins to which IFN-γ belongs. Aberrant IFN-γ expression is associated with a number of autoinflammatory and autoimmune diseases[7,8].

Mushrooms are nutritionally functional foods and important sources of physiologically beneficial and non-toxic medicines. They have been used in folk medicine throughout the world since ancient times. Many pharmaceutical secondary metabolites have been isolated from medicinal mushrooms. *Lactarius hatsudake* is an edible, slightly bitter mushroom belonging to the genus *Lactarius* (DC.) Gray, family Russulaceae of Basidiomycete, which is widely distributed in China, Korea and Japan. It has long been used as antitumor and antiviral agent in Chinese folk medicine[9]. Previous investigation on bioactive constituents of *L. hatsudake* resulted in the isolation of several ergosterol derivatives[10] and azulene pigments[11]. In the present study, we describe the isolation, structure elucidation and biological activity of four azulene derivatives from the fruiting bodies of *L. hatsudake*.

2. Materials and Methods

2.1. Reagents and Equipments

The UV spectra were recorded on a UV-1601 UV-Visible spectrometer (Shimadzu, Kyoto, Japan). The NMR spectra were measured on Varian Unity Inova 400 spectrometer (Palo Alto, CA, USA), and chemical shifts were reported in ppm downfield from TMS. The ESI-MS spectra were recorded on a Waters Q-TOF Premier mass spectrometer (Waters, Milford, MA, USA). Column chromatography was carried out on silica gel (40 ~ 63 μm, Merck), Lichroprep RP-18 gel (40 ~ 63 μm, Merck, Darmstadt, Germany) and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). TLC was performed on the plates pre-coated with silica gel 60 F_{254} (Merck) and RP-18 F_{254s} (Merck). Preparative HPLC was performed on a YMC-Pack ODS column (20 × 250 mm, flow rate 4 mL/min, detection wave length 254 nm) equipped with a Waters 600 pump, 600 Controller, and 486 Tunable Absorbance Detector (Waters).

2.2. Mushroom Material

The fresh fruiting bodies of *L. hatsudake* (6 kg) were provided by the Korea Agricultural Culture Collection (KACC) of the National Institute of Agricultural Biotechnology (NIAB), Suwon, Republic of Korea, in July 2009.

2.3. Extraction and Isolation

The fresh fruiting bodies of *L. hatsudake* (6 kg) were extracted three times with MeOH at room temperature for three days each. The combined MeOH solution was concentrated under reduced pressure to give a residue (240 g), which was suspended in H_2O and then successively partitioned with *n*-hexane and EtOAc. The hexane layer (17 g) was loaded on a silica gel column and eluted with CH_2Cl_2-MeOH in a gradient mode (20 : 1 → 1) to give nine fractions (LH1-9). The fraction LH2 (1.5 g) was applied to a RP-18 column and eluted with MeOH-H_2O (1 : 1 → 0) to give three fractions (LH21-23), then the fraction LH21 was subjected to Sephadex LH-20 column chromatography and eluted

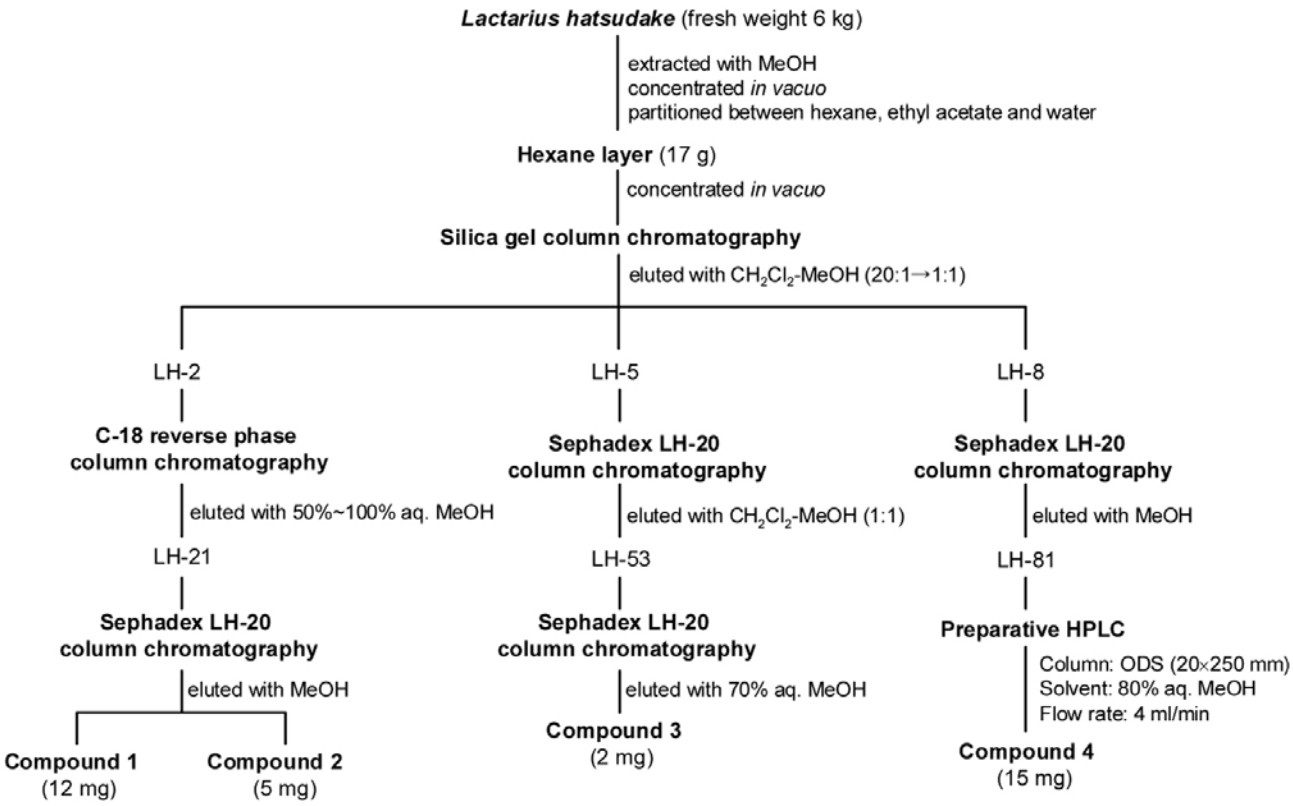

Figure 1. Isolation procedure of compounds **1-4** from *L. hatsudake*.

with MeOH to afford **1** (12 mg) and **2** (5 mg), respectively. The fraction LH5 (1.8 g) was subjected to Sephadex LH-20 column chromatography and eluted with CH_2Cl_2-MeOH (1 : 1) to give four fractions (LH51-54), then the subfraction LH53 was purified by Sephadex LH-20 columneluting with 70 % MeOH to yield **3** (2 mg). The fraction LH8 (300 mg) was subjected to Sephadex LH-20 column chromatography and eluted with MeOH was to give two fractions (LH81 and 82), and the fraction LH81 was further purified by preparative HPLC (MeOH : H_2O = 4 : 1) to afford **4** (15 mg, t_R: 18 min) (Figure 1).

Compound 1 : Violet-red solid. TLC (RP-18, MeOH/H_2O 9:1) : R_f = 0.35. UV (MeOH) λ_{max} nm (log ε): 228 (4.07), 240 (4.05), 311 (4.18), 380 (3.25). ESI-MS *m/z*: 213 $[M+H]^+$. ^{1}H-NMR ($CDCl_3$, 400 MHz) : δ_H 10.30 (1H, s, H-15), 9.71 (1H, d, J = 2.0 Hz, H-8), 8.15 (1H, d, J = 4.4 Hz, H-2), 7.70 (1H, dd, J = 10.8, 2.0 Hz, H-6), 7.48 (1H, d, J = 10.8 Hz, H-5), 6.24 (1H, d, J = 4.4 Hz, H-3), 3.22 (1H, sept, J = 6.8 Hz, H-11), 2.91 (3H, s, H-14), 1.39 (6H, d, J = 6.8 Hz, H-12, 13); ^{13}C-NMR ($CDCl_3$, 100 MHz): δ_C 186.8 (C-15), 150.0 (C-7), 148.1 (C-4), 144.5 (C-10), 141.9 (C-2), 139.9 (C-9), 137.9 (C-8), 137.6 (C-6), 131.5 (C-5), 125.8 (C-1), 115.8 (C-3), 38.8 (C-11), 25.0 (C-14), 24.8 (C-12, 13).

Compound 2 : Brown-red solid. TLC (RP-18, MeOH/H_2O 9 : 1) : R_f = 0.32. UV (MeOH) λ_{max} nm (log ε) : 241 (4.24), 315 (4.25), 392 (3.65). ESI-MS *m/z* : 211 $[M+H]^+$. ^{1}H-NMR ($CDCl_3$, 400 MHz) : δ_H 10.35 (1H, s, H-15), 9.91 (1H, d, J = 2.0 Hz, H-8), 8.19 (1H, d, J = 4.4 Hz, H-2), 7.91 (1H, dd, J = 10.8, 2.0 Hz, H-6), 7.50 (1H, d, J = 10.8 Hz, H-5), 7.32 (1H, d, J = 4.4 Hz, H-3), 5.47 (1H, br s, H-12a), 5.35 (1H, quinet, J = 1.6 Hz, H-12b), 2.96 (3H, s, H-14), 2.35 (3H, s, H-13); ^{13}C-NMR ($CDCl_3$, 100 MHz) : δ_C 186.9 (C-15), 148.9 (C-4), 146.4 (C-11), 144.3 (C-10), 142.9 (C-7), 142.2 (C-2), 139.0 (C-9), 136.7 (C-6, 8), 130.9 (C-5), 126.9 (C-1), 116.9 (C-12), 116.6 (C-3), 25.0 (C-14), 23.2 (C-13).

Compound 3 : Violet solid. TLC (RP-18, MeOH/H_2O 9:1) : R_f = 0.47. UV (MeOH) λ_{max} nm (log ε) : 220 (4.08), 239 (4.10), 296 (4.21), 368 (3.57). ESI-MS *m/z*: 229 $[M+H]^+$. ^{1}H-NMR ($CDCl_3$, 400 MHz) : δ_H 9.79 (1H, br s, H-8), 8.38 (1H, d, J = 4.4 Hz, H-2), 7.71 (1H, d, J = 10.4 Hz, H-6), 7.46 (1H, d, J = 10.4 Hz, H-5), 7.26 (1H, d, J = 4.4 Hz, H-3), 3.25 (1H, sept, J = 6.8 Hz, H-11), 2.96 (3H, s, H-14), 1.43 (6H, d, J = 6.8 Hz, H-12, 13); ^{13}C-NMR ($CDCl_3$, 100 MHz) : δ_C 170.8 (C-15), 148.0 (C-7), 147.1 (C-4), 143.5 (C-10), 140.9 (C-9), 140.7 (C-2), 137.9 (C-8), 137.2 (C-6), 130.8 (C-5), 115.8 (C-1), 114.4 (C-3), 38.8 (C-11), 24.9 (C-14), 24.7 (C-12, 13).

Compound 4 : Red solid. TLC (RP-18, MeOH/H_2O 9:1) : R_f = 0.59. UV (MeOH) λ_{max} nm (log ε): 228 (4.02), 312 (4.25), 381 (3.84). ESI-MS *m/z* : 251 $[M+Na]^+$. ^{1}H-NMR ($CDCl_3$, 400 MHz) : δ_H 10.33 (1H, s, H-15), 10.01 (1H, d, J = 2.4 Hz, H-8), 8.22 (1H, d, J = 11.2, 2.4 Hz, H-6), 8.18 (1H, d, J = 4.4 Hz, H-2), 7.55 (1H, d, J = 11.2 Hz, H-5), 7.30 (1H, d, J = 4.4 Hz, H-3), 2.95 (3H, s, H-14), 1.79 (6H, s, H-12, 13); ^{13}C-NMR ($CDCl_3$, 100 MHz) : δ_C 187.1 (C-15), 149.5 (C-7), 149.0 (C-4), 144.4 (C-10), 141.9 (C-2), 139.3 (C-9), 135.6 (C-6,8), 131.1 (C-5), 126.4 (C-1), 116.2 (C-3), 74.5 (C-11), 32.8 (C-12, 13), 25.0 (C-14).

2.4. Cell Culture

The IL-2-dependent NK cell line NK92 (human NK lymphoma) was obtained from the American Type Culture Collection (ATCC). NK92 cells were maintained in α-MEM (Life Technologies, Karlsruhe, Germany) containing 20 % FCS (HyClone, Logan, UT), 2 mM L-glutamate, 100 μg/mL penicillin, 100 μg/mL streptomycin (Life Technologies, Paisley, UK) and supplemented with 100 U/mL IL-2 (Chiron, Emeryville, CA, USA). NK92 cell culture was performed at 37 ℃ in 5 % CO_2 humidified atmosphere.

2.5. ELISA for Analysis IFN-γ Production

Quantification of human IFN-γ was performed as protocol description using commercially available mAb pairs (Endogen, Woburn, MA, USA). Cell-free supernatants were collected after 18 h of incubation at 37 ℃. For the detection of IFN-γ, ELISA kits from Endogen were used. Results are shown as the means of triplicate wells ± SEM.

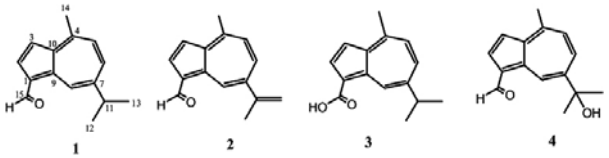

Figure 2. Structures of the compounds isolated from *L. hatsudake*.

3. Results and Discussion

3.1. Structure Determination of Isolated Compounds

The MeOH extract of the fruiting bodies of *L. hatsudake* was partitioned successively with *n*-hexane, EtOAc, *n*-BuOH and H_2O. Repeated chromatographic purification of the *n*-hexane soluble fraction led to the isolation of four compounds (Figure 2), and they were identified as 1-formyl-4-methyl-7-isopropyl azulene (**1**), lactaroviolin (**2**), 4-methyl-7-isopropyl-azulene-1-carboxylic acid (**3**), and 1-formyl-4-methyl-7-(1-hydroxy-1-methylethyl) azulene (**4**) by comparison of their spectral data with those reported in the literature.

Compound **1** was obtained as a violet-red solid. Its molecular formula, $C_{15}H_{16}O$, was determined by ESI-MS, ^{1}H-NMR, ^{13}C-NMR and DEPT spectral data. The UV absorptions at 228, 240, 311 and 380 nm suggested an azulene skeleton. The ^{1}H-NMR spectrum of **1** displayed one aldehyde proton at δ_H 10.30 (1H, s, H-15), two AB type signals at δ_H 8.15 and 6.24 (each 1H, d, J = 4.4 Hz, H-2 and 3), three ABX type signals at δ_H 9.71 (1H, d, J = 2.0 Hz, H-8), 7.70 (1H, dd, J = 10.8, 2.0 Hz, H-6) and 7.48 (1H, d, J = 10.8 Hz, H-5), a methyl group at δ_H 2.91 (3H, s, H-14), and an isopropyl group at δ_H 3.22 (1H, sept, J = 6.8 Hz, H-11) and 1.39 (6H, d, J = 6.8 Hz, H-12, 13). The ^{13}C-NMR spectrum of **1** exhibited total fifteen carbon signals including one carbonyl group at δ_C 186.8, five quaternary aromatic carbons at δ_C 150.0, 148.1, 144.5, 139.9 and 125.8, five olefinic methines at δ_C 140.7, 137.9, 137.2, 130.8 and 114.4, one aliphatic methine at δ_C 38.8, and three methyl groups at δ_C 25.0 and 24.8. Therefore, compound **1** was identified as 1-formyl-

4-methyl-7-isopropyl azulene by comparison its NMR spectral data with those reported in the literature[12].

Compound **2** was obtained as a brown-red solid. The UV spectrum of **2** suggested that it was also an azulene derivative. It revealed an $[M+H]^+$ ion peak at *m/z* 211 in the ESI-MS spectrum, indicating the molecular weight of **2** is 210. The ^{1}H- and ^{13}C-NMR data of **2** was quite similar to that of **1** except for the moiety at C-7. The ^{1}H-NMR spectrum exhibited a set of double bond signals at δ_H 5.47 (1H, br s, H-12a) and 5.35 (1H, quinet, J = 1.6 Hz, H-12b) and a quaternary methyl group at δ_H 2.35 (3H, s, H-13), indicating the presence of an isopropenyl group. The HMBC correlations of H-12 with C-7 at δ_C 142.9, C-11 at δ_C 146.4 and C-13 at δ_C 23.2, and H-13 with C-7, C-11 and C-12 at δ_C 116.9 fixed its position. On the basis of above evidences, the structure of **2** was determined as lactaroviolin[12,13], and complete assignments of ^{13}C-NMR resonances were achieved for the first time by a combination of 1D and 2D NMR techniques.

Compound **3** was obtained as a violet solid, and its molecular formula, $C_{15}H_{16}O_2$, was determined by ESI-MS, ^{1}H-NMR, ^{13}C-NMR and DEPT spectral data. The UV spectrum of **3** showed characteristic absorption bands of azulene derivatives at 220, 239, 296 and 368 nm. The ^{1}H- and ^{13}C-NMR spectra of **3** were closely comparable to those of **1**, with the notable presence of a distinctive resonance at δ_C 170.8 instead of the aldehyde group. Thus, the structure of compound **3** was elucidated as 4-methyl-7-isopropyl-azulene-1-carboxylic acid, and confirmed by comparison of its spectral data with those reported in the literature[14].

Compound **4** was obtained as a red solid. Its UV spectrum showed absorption at 228, 312 and 381 nm, indicating that compound **4** was also an azulene derivative. The molecular formula of **4** was established as $C_{15}H_{16}O_2$ on the basis of ESI-MS, ^{1}H-NMR, ^{13}C-NMR and DEPT spectral data. Comparison of the ^{1}H- and ^{13}C-NMR spectral data of **4** with those of **1** revealed that they were closely related in structure except for the moiety at C-7. The downfield shift of two methyl groups at δ_H 1.79 (6H, s, H-12, 13) and one quaternary aliphatic carbon at δ_C 74.5 suggested the

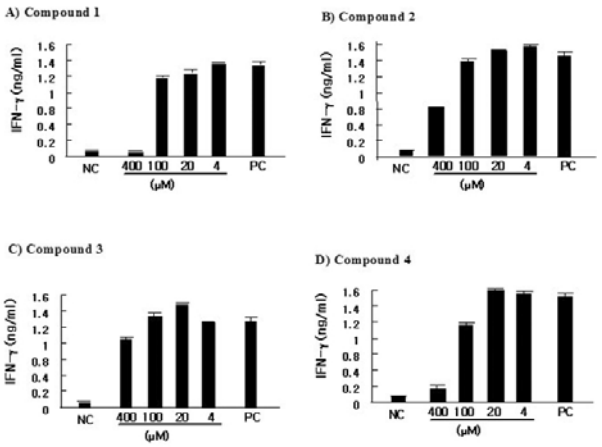

Figure 3. The results of ELISA for IFN-γ evaluation in compounds **1-4** treated NK92 cells. NK92 cells were cultured in DMEM medium containing 20 % FCS and IL-2. For evaluation of the released IFN-γ, the washed 5.0 × 105 cell/ml was distributed into 48 well culture plates. The modulation of IFN-γ was assayed by ELISA method with culture supernatant in compounds treated cells. As negative control (NC) and positive control (PC) was used released IFN-γ in the culture supernatant originated from culture medium only and culture medium containing 100 ng of phorbol 12-myristate 13-acetate (PMA) treated cell, respectively.

presence of a hydroxyl-substituted isopropyl group. Therefore, the structure of compound **4** was elucidated as 1-formyl-4-methyl-7-(1-hydroxy-1-methylethyl) azulene and confirmed by comparison of its spectral data with those reported in the literature[12].

3.2. Modulation Capacity of INF-γ Production in NK cell

The isolated compounds were evaluated for their effects on the modulation of INF-γ production in NK92 cells. As shown in Figure 3, all of the compounds exhibited IFN-γ production inhibitory activity. Compound **1** strongly inhibited IFN-γ production in NK 92 cells in a dose-dependent manner, corresponding to 101.3 % inhibition at 400 μM and 11.9 % at 100 μM, respectively. Compound **4** also exhibited a dose-dependent effect, with 92.7 % inhibition at 400 μM and 24.1 % at 100 μM. However, compounds **2** and **3** showed weak inhibitory effect on INF-γ production, corresponding to 45.9 % and 18.0 % inhibition at 400 μM. The potency of inhibitory activity was in the order of compound **1**

≥ **4** 〉 **2** 〉 **3**. These results suggested that the aldehyde group at C-1 position of azulene moiety seemed to play an important role in the inhibitory effect on INF-γ production, and the isopropyl group at C-7 also influenced potency.

The WST-1 cell proliferation assay showed that no cytotoxicity was observed in NK92 cells up to a concentration of 400 μM (data is not shown).

To the best of our knowledge, this is the first study to demonstrate that the azulene derivatives exhibit INF-γ production inhibitory effect.

4. Conclusion

In the present study, four azulene derivatives were isolated from the MeOH extract of the fruiting bodies of *L. hatsudake*, and their chemical structures were determined by spectroscopic method. The isolated compounds were evaluated for their modulation capacity of INF-γ production in NK92 cells. Compounds **1** and **4** showed potent inhibitory effect on IFN-γ production with 101.3 % and 92.7 % inhibition at 400 μM.

Acknowledgements

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