Identification of Immunosuppressive Components of a Mushroom, *Lactarius flavidulus*

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Three metabolites having a suppressive effect on proliferation of mouse lymphocytes stimulated with mitogens such as concanavalin A (Con A) and lipopolysaccharide (LPS) were isolated from a mushroom, *Lactarius flavidulus*, and identified as geranylphenols, flavidulols A, B and C, respectively, which had previously been isolated from the same mushroom.

The IC_{50} values of flavidulols A, B and C were found to be 8.9, 4.9 and 36.3 μg/ml against Con A-induced proliferation of mouse lymphocytes, and 6.7, 3.9 and 28.3 μg/ml against LPS-induced proliferation, respectively.

Keywords *Lactarius flavidulus*; mushroom metabolite; immunosuppressant; Basidiomycetes; geranylphenol; flavidulol

In our screening program on biologically active principles of mushrooms (Basidiomycetes), nine new neurotoxic glycosides from *Hebeloma vinosophyllum*[1] and three triterpene esters having papaverine-like relaxation activity from *Hebeloma spoliatum*[2] have so far been isolated. Now, we have found that the methanolic extract of *Lactarius flavidulus* (Japanese name: kihatsutake) appreciably suppressed proliferation of mouse lymphocytes stimulated with mitogens, concanavalin A (Con A) and lipopolysaccharide (LPS). Three active principles from the mushroom, tentatively named LF-1, -2 and -3, were identified as flavidulols A, B and C, which had previously been isolated from the same mushroom.

Experimental

The melting points were measured on a Yanagimoto micro melting point apparatus (hot stage type) and are uncorrected. The optical rotations were measured with a JASCO DIP-140 digital polarimeter. The IR spectra were recorded with Hitachi EPI-G3 and IR 260-10 spectrometers, the electron impact MS (EI-MS) with a Hitachi M-60 spectrometer, the high-resolution EI-MS (HREI-MS), the FAB-MS and the high-resolution FAB-MS (HRFAB-MS) with a JEOL JMS-HX110 spectrometer, the ^{1}H-NMR spectra with a JEOL JNM-GX270, -GSX400, -GSX500 or -A500 spectrometer at 270, 400, 500 or 500 MHz, and the ^{13}C-NMR spectra with a JEOL JNM-GSX270, -GSX400, -GSX500 or -A500 spectrometer at 67.8, 100.4, 125.65 or 125.65 MHz. Chemical shifts are expressed in δ (ppm) values from tetramethylsilane (TMS) as an internal standard.

Evaluation of Effects of Samples on Mitogen-Induced and Mitogen-Free Proliferations of Mouse Spleen Lymphocytes[3] Urografin 60% (Schering) (20.0 ml) was added to 60.0 ml of a solution of Ficoll-400 (Sigma) (9.1 g) in water (125 ml), to prepare Ficoll-Urografin. Three male BALB/c mice (7—11 weeks old, purchased from Nippon SLC Co. Ltd., Hamamatsu, Japan) were killed and the spleens were teased in a polystyrene Petri dish containing 5 ml of RPMI-1640 medium (Gibco). The product was filtered through a silicone gauze to remove clumps and prepare a suspension. The suspension (6 ml) was gently piled on a layer of Ficoll-Urografin (6 ml) in a polypropylene centrifuge tube, and centrifuged at 1500 rpm for 20 min at 25 °C to remove erythrocytes. The supernatant was centrifuged at 1000 rpm for 5 min at 4 °C to separate lymphocytes. After centrifugal washing with 10 ml of RPMI-1640 medium at 1000 rpm for 5 min at 4 °C 3 times, the lymphocytes were suspended in 5 ml of RPMI-1640 medium. A small quantity of the suspension (100 μl) was diluted with RPMI-1640 medium to count the number of lymphocytes in the suspension with a hemocytometer (Kayagaki), based on trypan blue exclusion (cell viability: greater than 98%). To 87.0 ml of RPMI-1640 medium, 10.0 ml of fetal bovine serum (FBS) (Gibco) which was incubated at 56°C for 30 min in advance, 2.0 ml of penicillin-streptomycin (5000 IU/ml and 5000 μg/ml, Flow Laboratories) and 1.0 ml of 3.0% glutamine (Nissui) in RPMI-1640 medium were added to prepare FBS/RPMI-medium. The remainder of the suspension was diluted with FBS/RPMI-medium to prepare lymphocyte suspension at a concentration of 8.0×10^6 cells/ml. Con A (Sigma) and LPS (Difco, from *E. coli*) were dissolved in FBS/RPMI-medium to prepare the mitogen solutions. As the stimulative concentration of Con A or LPS, 70% of the optimum stimulative concentration of Con A or LPS, which was calculated from a dose–response curve between concentration of Con A or LPS and proliferative response of the lymphocytes, was employed. Each sample was dissolved in 10.0 ml of 1.0% EtOH in FBS/RPMI-medium to prepare sample solution. The sample solution (100 μl) was incubated with 50 μl of the cell suspension and 50 μl of mitogen solution (in the case of mitogen-induced proliferation) or 50 μl of FBS/RPMI-medium (in the case of mitogen-free proliferation) in a 96-well microtiter plate (Inter Med, Nunclon) at 37 °C in a 5% CO_2 humidified atmosphere for 48 h using a CO_2 incubator (Sanyo, MCO-96). Then, 20 μl of the [methyl-^{3}H]thymidine solution, which was prepared by dilution of 1.0 ml of an aqueous solution of [methyl-^{3}H]thymidine (37.0 MBq/ml, Amersham) with 79.0 ml of saline, was added to the culture (9.25 kBq/well), and the mixture was further incubated for 22 h. The culture was harvested on a glass microfiber filter disk with a semi-automatic multiple cell harvester (Labo Science, Labo-Mash). The filter disk was steeped in 7.0 ml of a scintillation cocktail (Nacalai Tesque, Clearsol I). The radioactivity was measured with a liquid scintillation counter (Beckman, LS 1800 or LS 5800). The effect of each sample on mitogen-induced and mitogen-free proliferations of the lymphocytes was evaluated in triplicate, and expressed as a percentage of [methyl-^{3}H]-thymidine incorporated in the incubation with the sample to that without the sample (control).

Isolation of LF-1 (1), -2 (2) and -3 (3) The fruit-bodies of *L. flavidulus* were collected at Mt. Kiyosumi, Chiba, Japan in October, 1989 and 1990. The dried fruit-bodies (330.0 g) were cut into small pieces and soaked in MeOH (2.5 l) with occasional stirring for 24 h at room temperature 6 times to afford a methanolic extract (83.7 g). A portion of the extract (20.0 g) was dissolved in MeOH (10 ml) and suspended in water (200 ml). The suspension was extracted with *n*-hexane (200 ml) 5 times. The *n*-hexane-soluble portion (7.56 g) was chromatographed on a silica gel column (47 i.d. × 265 mm) with *n*-hexane–C_6H_6 (2 : 1, v/v) to give fractions I (0.14 g) and II (0.64 g), with *n*-hexane–C_6H_6 (1 : 2) to give fraction III (0.06 g), with C_6H_6 and C_6H_6–acetone (1 : 1) to give fraction IV (1.99 g), and with acetone to give fraction V (3.97 g). Fraction II was separated further on a flash-chromatographic silica gel column (13 i.d. × 200 mm) with *n*-hexane–C_6H_6 (2 : 1) and (1 : 2, v/v) using a pump (NRK UP2), and successively on a medium-pressure liquid chromatographic (MPLC) silica gel column (22 i.d. × 100 mm) with *n*-hexane–AcOEt (30 : 1) at a flow rate of 5.2 ml/min to afford a solid, which was recrystallized from *n*-hexane to afford **3** (24 mg). Fraction IV was separated further on a flash-chromatographic silica gel column with *n*-hexane–C_6H_6 (1 : 1) and on the MPLC silica gel column with *n*-hexane–AcOEt (15 : 1) at a flow rate of 5.4 ml/min twice successively to afford **1** (40 mg). Fraction V was separated further on a chromatographic silica gel column with *n*-hexane–C_6H_6 (1 : 2), on a flash-chromatographic silica gel column with *n*-hexane–C_6H_6 (2 : 1) twice and on the MPLC silica gel column with *n*-hexane–AcOEt (15 : 1) at a flow rate of 5.0 ml/min twice successively to afford **2** (21 mg). The yields of **1**, **2** and **3** from the dried fruit-bodies were 0.27, 0.011 and 0.013%,

respectively.

LF-1 (**1**): Colorless oil. HRFAB-MS (matrix: *m*-nitrobenzyl alcohol (NBA)) positive ion *m/z* Calcd for $C_{17}H_{22}O_2$ [$(M+H)^+$]: 259.1698. Found: 259.1682. $IR_{max}^{CHCl_3}$ cm^{-1}: 3590 (O–H), 2990, 2930, 2860 (C–H), 1600, 1470, 1435 (C=C), 1250, 1075, 1040 (C–O).

LF-2 (**2**): Pale brown oil. This compound was identical with an authentic sample of flavidulol B[4] in terms of ^{1}H-NMR spectrum ($CDCl_3$) and TLC behavior (plate: Merck Kieselgel 60F254, solvent system: C_6H_6, *n*-hexane–AcOEt (4:1, v/v), spray reagent: 10% H_2SO_4).

LF-3 (**3**): Colorless needles, mp 160.5—165 °C (from *n*-hexane–acetone). HREI-MS *m/z* Calcd for $C_{34}H_{42}O_4$ (M^+): 514.3083. Found: 514.3081. EI-MS *m/z* (%): 514 (M^+, 100). $IR_{max}^{CHCl_3}$ cm^{-1}: 3540, 3000, 2930, 2860, 1605, 1455, 1430, 1265, 1105, 1075, 835. This compound was identical with an authentic sample of flavidulol C[4] on the basis of IR ($CHCl_3$), ^{1}H- and ^{13}C-NMR ($CDCl_3$) spectral comparisons, and TLC behavior (plate: Merck Kieselgel 60F254, solvent system: C_6H_6, *n*-hexane–AcOEt (10:1, v/v), spray reagent: 10% H_2SO_4).

Methylation of LF-1 (1) A solution of **1** (40 mg) in dry tetrahydrofuran (THF) (1.0 ml) was added dropwise to a suspension of NaH (50% dispersion in mineral oil, 16.5 mg) in dry THF (1.0 ml) at 0 °C, and the mixture was stirred at room temperature for 30 min. The suspension was treated with methyl iodide (19.2 μl) and stirred at room temperature for 1 h under argon gas. Ice-water was added and the whole was extracted with AcOEt. Evaporation of the AcOEt layer *in vacuo* gave an oily residue which was chromatographed twice on slica gel columns to afford the methylether of **1** (**4**) (11 mg) (9 mg of **1** was recovered), colorless oil. HRFAB-MS (matrix: NBA) *m/z* Calcd for $C_{18}H_{24}O_2$ (M^+): 272.1776. Found: 272.1772. $IR_{max}^{CHCl_3}$ cm^{-1}: 2990, 2930, 2850, 2830, 1590, 1460, 1435, 1240, 1090.

Acetylation of LF-1 (1) A solution of **1** (40 mg) in Ac_2O (0.2 ml) and pyridine (0.3 ml) was allowed to stand overnight at room temperature under argon gas, then ice-water was added and the whole was extracted with Et_2O. Evaporation of the Et_2O layer gave a solid residue, which was chromatographed on a silica gel column to afford the acetate of **1** (**5**) (38 mg), colorless needles (after recrystallization from *n*-hexane). This compound was identical with an authentic sample of flavidulol A acetate[4] on the basis of mixed melting point determination (112—112.5 °C), IR ($CHCl_3$), ^{1}H- and ^{13}C-NMR ($CDCl_3$) spectral comparisons and TLC behavior (plate: Merck Kieselgel 60F254, solvent: *n*-hexane–AcOEt (4:1, v/v), spray reagent: 10% H_2SO_4).

Catalytic Hydrogenation of LF-1 (1) A suspension of PtO_2 (40 mg) in EtOH (1.0 ml) was stirred under H_2 gas at room temperature for 15 min. A solution of **1** (40 mg) in EtOH (1.0 ml) was added to the suspension, and the reaction mixture was stirred under H_2 gas at room temperature for 30 min. After removal of the catalyst, evaporation of the solvent gave a product mixture, which was chromatographed on an MPLC silica gel column with *n*-hexane–acetone (15:1, v/v) at a flow rate of 5.4 ml/min to afford tetrahydro LF-1 (**7**) (2.6 mg) and dihydro LF-1 (**6**) (6.4 mg). Compound **6**: colorless fine needles, mp 128—129 °C (from *n*-hexane). HREI-MS *m/z* Calcd for $C_{17}H_{24}O_2$ (M^+): 260.1776. Found: 260.1773. EI-MS *m/z* (%): 260 (M^+, 100). Compound **7**: colorless needles, mp 124—125.5 °C (from *n*-hexane), $[\alpha]_D^{20}$ ±0.0° (*c*=1.1, $CHCl_3$). HREI-MS *m/z* Calcd for $C_{17}H_{26}O_2$ (M^+): 262.1933. Found: 262.1929. EI-MS *m/z* (%): 262 (M^+, 100).

Preparation of LF-1A1 (8) and LF-1A2 (9) from LF-1 (1) A solution of **1** (113 mg) in MeOH (10.0 ml) was acidified with 2 N HCl (2.0 ml), added dropwise under ice-cooling, and the reaction mixture was stirred at room temperature. After 2.5 and 6.4 h, 2.0 and 1.0 ml of 2 N HCl were added, respectively, and the mixture was stirred continuously at room temperature. After 7.3 h, the mixture was diluted with water (25 ml) and extracted with AcOEt (30 ml) 3 times. Evaporation of the AcOEt layer gave a product mixture (121 mg), which was separated on a flash-chromatoraphic silica gel column (11 i.d. × 160 mm) with C_6H_6–AcOEt (15:1 and 4:1, v/v) to afford two fractions. The fraction eluted earlier was crystallized from *n*-hexane, and then recrystallized from aqueous MeOH to afford **8** (25 mg). From the mother liquor, 6 mg of **8** was further obtained through MPLC octadecylsilica gel column (22 i.d. × 100 mm) chromatography with MeOH–H_2O (5:1, v/v) at a flow rate of 5.0 ml/min. The fraction eluted later was chromatographed on an MPLC silica gel column (22 i.d. × 100 mm) with *n*-hexane–AcOEt (2:1, v/v) at a flow rate of 5.1 ml/min to give **9** (18 mg). Compound **8**: colorless plates, mp 156—157 °C, $[\alpha]_D^{21}$ ±0.0° (*c*=1.0, $CHCl_3$). HREI-MS *m/z* Calcd for $C_{18}H_{26}O_3$ (M^+): 290.1882. Found: 290.1874. EI-MS *m/z* (%): 290 (M^+, 100). $IR_{max}^{CHCl_3}$ cm^{-1}: 3580, 2990, 2930, 1465, 1440, 1255, 1085. Compound **9**: colorless needles, mp 190—192 °C. HREI-MS *m/z* Calcd for $C_{17}H_{24}O_3$ (M^+): 276.1726. Found: 276.1730. EI-MS *m/z* (%): 276 (M^+, 100), IR_{max}^{KBr} cm^{-1}: 3430, 3260, 2930, 2860, 1620, 1470, 1260, 1080, 800, 730.

Results and Discussion

The methanolic extract of *L. flavidulus* suppressed the proliferation (blastogenesis) of mouse spleen lymphocytes stimulated with Con A (T-cells), or with LPS (B-cells). The *n*-hexane-soluble portion from the methanolic extract also suppressed Con A- and LPS-induced proliferations of the lymphocytes. However, the water-soluble portion did not suppress either proliferation at concentrations up to 50.0 μg/ml. Thus, the *n*-hexane-soluble portion was divided into five fractions, I—V, by column chromatography. Fraction IV was further fractionated by flash column chromatography and MPLC to afford an immunosuppressive component tentatively named LF-1 (**1**). Fractions V and II were also fractionated in a similar way to afford two other immunosuppressive components tentatively named LF-2 (**2**) and -3 (**3**), respectively.

LF-1 (**1**), was obtained as a colorless oil, $C_{17}H_{22}O_2$; its ^{1}H-NMR data are given in Table I and ^{13}C-NMR data in Table II. On methylation, **1** afforded a monomethylether (**4**), $C_{18}H_{24}O_2$. These spectral and chemical data of **1** were quite similar to those of flavidulol A, which has previously been isolated from the same mushroom as an anti-microbial principle, together with two related compounds, flavidulols B and C.[4] On acetylation, **1** afforded a monoacetate (**5**), colorless needles, mp 112—112.5 °C, $C_{19}H_{24}O_3$, which was identical with authentic flavidulol A acetate.[4] Accordingly, LF-1 was identified as flavidulol A (**1**). Configurations of the double bonds at positions 2 and 6 in **1** were formerly assigned to be (*E*) by Nozoe *et al.*[4] A nuclear Overhauser effect (NOE) experiment on **1**, however, revealed difference NOEs from H_3-9 (δ 1.60) to H-3 (5.11), and from H-7 (5.26) to Ha-5 (1.74) of 10.0 and 2.9%, respectively, suggesting that the configuration of the double bond at position 2 in **1** should be revised from (*E*) to (*Z*), as shown in Chart 1.[5]

LF-2 (**2**) afforded ^{1}H-NMR signals (see Table I) quite similar to those of flavidulol B described in the literature.[4] In fact, LF-2 was proved to be identical with flavidulol B (**2**)[4] by direct comparison of its ^{1}H-NMR spectrum and TLC behavior with those of an authentic sample. Flavidulol B may be an artifact derived from **1** by Cope-type rearrangement.[4]

LF-3 (**3**) gave a similar ^{1}H-NMR spectrum to that of **1**. In the ^{13}C-NMR spectrum of **3**, all signals are quite similar to the corresponding signals of **1** except that the signal of C-3′ was observed to be quaternary and shifted to δ 119.91 (+7.36) (see Table II). These data suggested that **3** may be flavidulol C, which is a 3′,3′-dimer of **1**.[4] LF-3 was proved to be identical with authentic flavidulol C (**3**)[4] by direct comparison of IR and ^{1}H-NMR spectra and TLC behavior. The configuration of the double bond at position 2 in **3** should also be revised to (*Z*), as in **1**,[5] because of the similarity of the ^{1}H-NMR spectrum of **3** to that of **1** (see Table I).

On catalytic hydrogenation, **1** gave two products, a dihydro derivative (**6**) and a tetrahydro derivative (**7**). In the ^{1}H-NMR spectrum of **6**, the signals at δ 1.78 (3H) and 5.26 (1H) disappeared and the signals of a secondary methyl at δ 0.82 (3H, d, *J*=6.6 Hz) and an aliphatic methylene and a methine newly appeared (see Table I). In

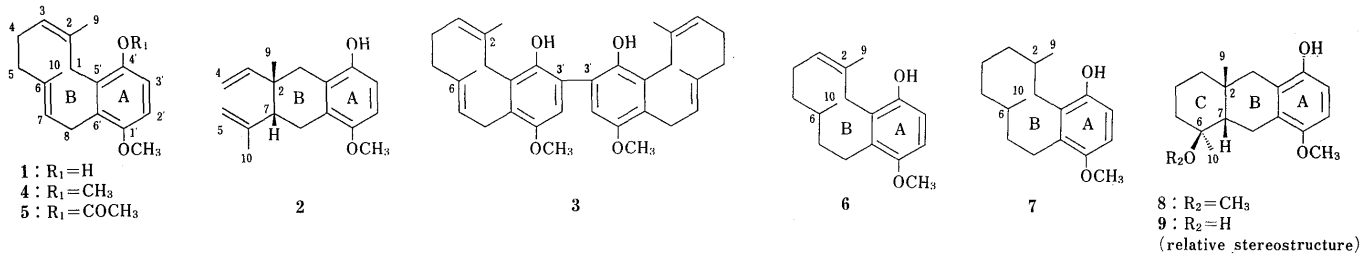

Chart 1

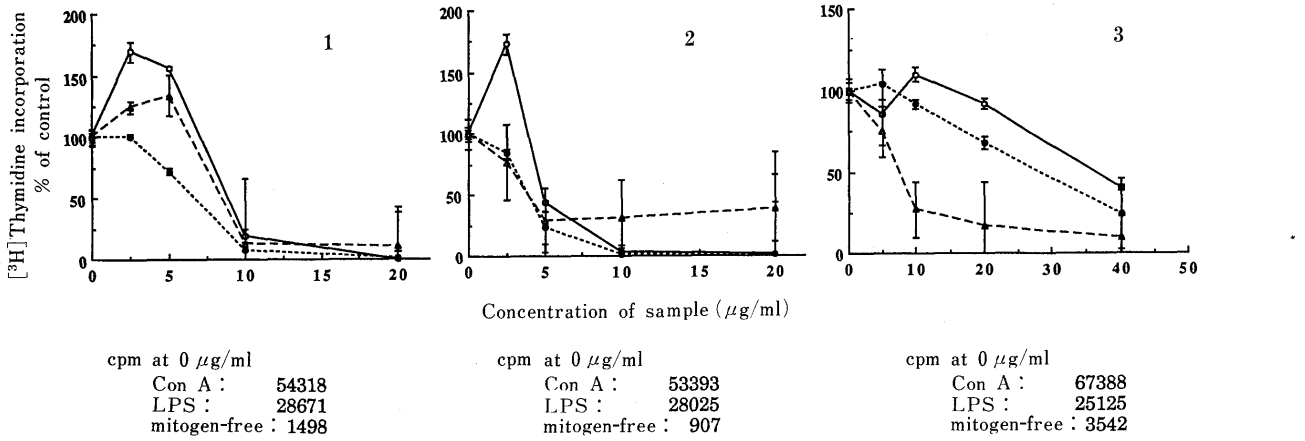

Fig. 1. Effects of LF-1 (**1**), -2 (**2**) and -3 (**3**) on Mitogen-Induced and Mitogen-Free Proliferations of Mouse Spleen Lymphocytes

—○—, against Con A-induced proliferation (T cell); ---●---, against LPS-induced proliferation (B cell); ---▲---, against mitogen-free proliferation. Each point represents the mean ± S.E. of 3 experiments.

a tricyclic system (composed of A, B and C rings) as expressed by **8** in Chart 1. A difference NOE experiment on **8** showed NOEs from H_3-9 to Ha-1, from H_3-9 to H-7, from H_3-10 to Hb-1, and from CH_3O-6 to H-7 of 4.9, 7.1, 5.1 and 3.2%, respectively. These NOE data suggested that the juncture between the B and C rings in **8** may be *cis* and the relative configurations at positions 2, 6 and 7 in **8** are as shown in Chart 1. Comparison of the ^{1}H-NMR spectrum of **9** with that of **8** indicated that all of the signals of **9** are quite similar to the corresponding signals of **8** except that the signal of CH_3O-6 at δ 3.19 (3H, s) is replaced with that of a hydroxyl at δ 3.13 (1H, s). The signal of CH_3O-6 at δ 47.92 is absent in the ^{13}C-NMR spectrum of **9**. Thus, the structure of LF-1A2 was deduced to be **9**, as shown in Chart 1. It was supposed that proton-catalyzed bond formation between C-2 and -7 followed by introduction of a methoxyl or hydroxyl anion at cationic C-6 in **1** afforded **8** or **9**.

LF-1 showed suppressive effects of −69.7, −55.8, 80.7 and 99.7% on Con A-induced and −1.2, 28.2, 93.3 and 99.2% on LPS-induced proliferations of lymphocytes at the concentrations of 2.5, 5.0, 10.0 and 20.0 μg/ml, respectively. LF-2 also showed suppressive effects of −72.9, 56.3, 96.5 and 98.9% on Con A-induced and 16.0, 77.1, 98.6 and 99.0% on LPS-induced proliferations at the same concentrations as in the case of **1**, respectively. Meanwhile, LF-3 showed suppressive effects of 14.2, −9.7, 8.2 and 59.8% on Con A-induced and −4.0, 8.4, 31.9 and 76.0% on LPS-induced proliferations at the concentrations of 5.0, 10.0, 20.0 and 40.0 μg/ml, respectively (see Fig. 1). The IC_{50} values of **1**, **2** and **3** were calculated to be 8.9, 4.9 and 36.3 μg/ml against Con A-induced and 6.7, 3.9 and 28.3 μg/ml against LPS-induced proliferations of mouse spleen lymphocytes, respectively.

The IC_{50} values of **4**—**9** against Con A- and LPS-induced proliferations of mouse spleen lymphocytes were calculated to be 14.9, 11.2, 12.4, 4.1, 17.3, >20.0 μg/ml against Con A-induced and 8.4, 7.5, 10.0, 3.2, 14.5, >20.0 μg/ml against LPS-induced proliferations, respectively. The fact that **4** and **5** were still active suggested that the presence of the free phenolic hydroxyl at position 4′ is not indispensable for immunosuppressive activity. The fact that **6** and **7** were also active and **7** was about two times more active than **1** suggested that the presence of the double bonds in the ten-membered ring is also not indispensable. Low activities of **8** and **9** might be due to their structures, which are composed of three conjugated rings. The compounds (**8** and **9**) having two conjugated six-membered rings instead of the ten-membered ring no longer exhibit high immunosuppressive activity. Compounds **1**, **2** and **3** also showed considerable suppressive activity toward lymphocytes at the concentration of 10.0 μg/ml even under mitogen-free conditions (see Fig. 1), though the average number of lymphocytes employed for the assay was only 5.4% of that in the mitogen-induced conditions. However, this compound showed no suppressive activity against human KB cells at a concentration of 10.0 μg/ml. This fact suggested that **1** has a significant specificity of suppressive activity against living cells.

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