Isolation and Characterization of Immunosuppressive Components of Three Mushrooms, *Pisolithus tinctorius*, *Microporus flabelliformis* and *Lenzites betulina*

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Two components having an immunosuppressive activity were isolated together with non-active pisolactone from *Pisolithus tinctorius*, and they were deduced to be 24-methyllanosta-8,24(28)-diene-3β,22ξ-diol and a mixture of two new compounds, (22*S*,24*R*)-24-methyllanosta-8-en-22,28-epoxy-3β,28α-diol and (22*S*,24*S*)-24-methyllanosta-8-en-22,28-epoxy-3β,28β-diol, respectively. Among them, pisolactone and 24-methyllanosta-8,24(28)-diene-3β,22ξ-diol have previously been isolated from the same mushroom. Ergosterol peroxide and 9(11)-dehydroergosterol peroxide were also isolated as active components from this mushroom in small amounts, and from *Microporus flabelliformis* and *Lenzites betulina*. The IC_{50} values of these components were evaluated against proliferation of mouse spleen lymphocytes stimulated with concanavalin A and lipopolysaccharide.

Keywords mushroom metabolite; immunosuppressant; Basidiomycetes; *Pisolithus tinctorius*; *Microporus flabelliformis*; *Lenzites betulina*

In our screening program on biologically active principles of mushrooms (Basidiomycetes), nine new neurotoxic glycosides from *Hebeloma vinosophyllum*,[1a] three triterpene esters having papaverine-like relaxation activity from *Hebeloma spoliatum*[1b] and three immunosuppressive geranylphenols from *Lactarius flavidulus*[1c] have so far been isolated. Now, it has been found that the methanolic extracts of *Pisolithus tinctorius*, *Microporus flabelliformis* and *Lenzites betulina* (Japanese name: kotsubutake, uchiwatake and kaigaratake, respectively) appreciably suppressed proliferation of mouse spleen lymphocytes stimulated with mitogens, concanavalin A (Con A) and lipopolysaccharide (LPS). From *P. tinctorius*, 24-methyllanosta-8,24(28)-diene-3β,22ξ-diol (**1**)[2] and a mixture of (22*S*,24*R*)-24-methyllanosta-8-en-22,28-epoxy-3β,28α-diol and (22*S*,24*S*)-24-methyllanosta-8-en-22,28-epoxy-3β,28β-diol (**3**) were isolated as immunosuppressive components, together with non-active pisolactone (**2**).[3] Small amounts of ergosterol peroxide (**4**)[4] and 9(11)-dehydroergosterol peroxide (**5**)[4] were also isolated from this mushroom as active components. These sterol peroxides were also isolated from *M. flabelliformis* and *L. betulina* as immunosuppressive principles.

Experimental

The IR and the UV spectra were recorded with Hitachi IR 260-10 and Hitachi U-3200 spectrometers, the electron impact MS (EI-MS), the high-resolution EI-MS (HREI-MS) and the high-resolution FAB-MS (HRFAB-MS) spectra with a JEOL JMS-HX110, and the ^{1}H-NMR and the ^{13}C-NMR spectra with a JEOL JNM-A500 or -GSX500 at 500 and 125.65 MHz, respectively. The TLC analyses were run under four different conditions (plate, solvent, spray reagent), a) Merck HPTLC Kieselgel 60F254S, *n*-hexane–AcOEt (1 : 1, v/v), 10% H_2SO_4, b) Merck HPTLC RP18F254S, MeOH, 5% phosphomolybdate in EtOH, c) Merck HPTLC RP18F254S, CH_3CN–acetone (2 : 1), 5% phosphomolybdate in EtOH, and d) Merck HPTLC RP18WF254S, EtOH–H_2O (3 : 1), 10% H_2SO_4. Other experimental conditions, including those for evaluation of the effects of samples on proliferation of mouse spleen lymphocytes, were as described in our preceding report.[1c]

Isolation of Components 1—5 from *P. tinctorius* The dried fruit-bodies of the mushroom (533.0 g) (collected in Aichi, Japan) were soaked in MeOH (1.6 l) to afford a methanolic extract (76.0 g), which was then divided into C_6H_6-soluble (4.0 g) and -insoluble portions (63.1 g). The C_6H_6-soluble portion was chromatographed on a silica gel flash-column to give fractions I—VIII. Fraction IV (0.30 g) was further chromatographed on a silica gel flash-column with C_6H_6–AcOEt (8 : 1, v/v) and on a medium-pressure liquid chromatographic (MPLC) silica gel column (22 mm i.d. × 100 mm) with C_6H_6–AcOEt (7 : 1) at a flow rate of 5.4 ml/min to afford components **2** (26 mg) and **1** (57 mg). Fraction V (0.12 g) was further chromatographed on a silica gel flash-column and an MPLC octadecylsilica gel (ODS) column (22 mm i.d. × 100 mm) with MeOH at a flow rate of 3.0 ml/min to give **2** (12 mg), **1** (26 mg) and **3** (11 mg). Fraction VI (0.14 g) was further chromatographed in a similar way to fraction V to give **3** (17 mg), **5** (1.5 mg) and **4** (1.5 mg).

Component **1**: Colorless fine needles, mp 157—158 °C (from MeOH), $[\alpha]_D^{27}$ +56.9° (c=0.20, $CHCl_3$). Component **2**: Colorless fine needles, mp 283—285 °C (from MeOH), $[\alpha]_D^{27}$ +62.6° (c=0.42, $CHCl_3$). Component **3**: Colorless amorphous powder. HRFAB-MS m/z Calcd for $C_{31}H_{52}O_3$ (M^+): 472.3916. Found: 472.3918. This component was considered to be a mixture of two compounds (ratio=*ca.* 2 : 1) from the NMR data, as described in Results and Discussion. Separation of the two compounds was achieved on the MPLC ODS column (t_R, 14.5, 16.8 min), but evaporation of the solvent *in vacuo* afforded again a mixture of the same two compounds (ratio=*ca.* 2 : 1). Component **4**: Colorless needles, mp 187—188 °C (from MeOH), $[\alpha]_D^{16}$ −32.4° (c= 1.10, $CHCl_3$). Component **5**: Colorless needles, mp 169—171 °C (from $CHCl_3$–MeOH), $[\alpha]_D^{18}$ +72.9° (c=1.00, $CHCl_3$).

Isolation of Components 4 and 5 from *M. flabelliformis* The dried fruit-bodies of the mushroom (514 g) (collected in Chiba, Japan) were extracted in the same way as above to afford a methanolic extract (17.2 g), which was then partitioned into AcOEt (4.8 g) and aqueous layers. The AcOEt layer was chromatographed on a silica gel column, on a silica gel flash-column, and finally on an MPLC ODS column to give two components (5.5, 40 mg), which were identical with components **5** and **4**, respectively, in terms of TLC behavior under conditions a—d.

Isolation of Components 4 and 5 from *L. betulina* The dried fruit-bodies of the mushroom (150 g) (collected in Chiba, Japan) were extracted in the same way as above to afford a methanolic extract (1.89 g), which was then divided into acetone-soluble (0.62 g) and -insoluble portions (0.99 g). The acetone-soluble portion was chromatographed in the same way as described for the AcOEt layer of *M. flabelliformis* to give two components (1.7, 9.1 mg), which were identical with components **5** and **4**, respectively, in terms of ^{1}H- and ^{13}C-NMR spectra ($CDCl_3$) and TLC behavior under conditions a—d.

Results and Discussion

The methanolic extract of *P. tinctorius* suppressed by 33 and 53% the proliferation (blastogenesis) of mouse

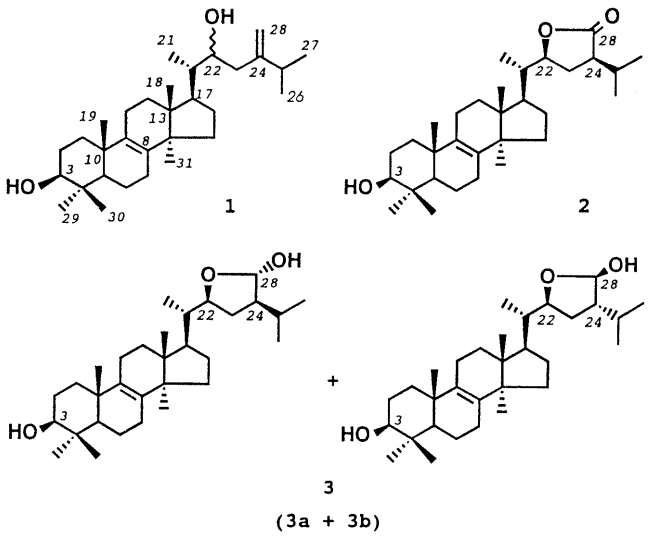

Chart 1

spleen lymphocytes stimulated with Con A (T-cells) and LPS (B-cells) at 100.0 μg/ml, respectively. The C_6H_6-soluble portion from the methanolic extract suppressed by 68 and 83% the Con A- and LPS-induced proliferations of the lymphocytes, respectively, but the C_6H_6-insoluble portion suppressed them by less than 30% at 100.0 μg/ml. Repeated chromatography of the C_6H_6-soluble portion afforded components **1**—**5**. Among them, two immunosuppressive components, **1** and **3**, were concluded to be 24-methyllanosta-8,24(28)-diene-3β,22ξ-diol (**1**)[2] and a mixture of two new compounds (**3**) on the basis of their physicochemical and spectral data, respectively. Component **2** was shown to be identical with pisolactone [(22*S*)-24-methyllanosta-8-en-22,28-epoxy-3β-ol-28-one] (**2**).[3] Components **1** and **2** have previously been isolated together from the same mushroom by Lobo *et al.*[3] (see Chart 1). The small amount of **1** obtained precluded any investigation of the configuration at position 22 of **1**. Component **4** was proved to be identical with ergosterol peroxide[4] in direct comparison with an authentic specimen. Comparison of the ^{1}H- and ^{13}C-NMR spectral data of **5** with those of **4** indicated that **5** is identical with 9(11)-dehydroergosterol peroxide. This conclusion was supported by HRFAB-MS, ^{1}H- and ^{13}C-NMR spectra.

Comparison of the ^{1}H- and ^{13}C-NMR spectra of **3** with those of **2** suggested that **3** is a mixture of two unknown dihydro derivatives of **2**, namely, **3a** and **3b** (ratio = *ca.* 2:1), indicating that the –O–C(=O)– group at position 28 in **2** (δ 178.45 s) is replaced by a –O–CH(–OH)– group in **3a** (δ 102.21 d, 5.17 d) and **3b** (δ 97.77 d, 5.27 d). These facts suggested that **3a** and **3b** are isomeric at position 28. This view was supported by a comparison of signals in the ^{1}H- and ^{13}C-NMR spectra of **3a** with those of **3b** (see Table I). The coupling constant of H-28 of **3a** (J = 3.4 Hz) is similar to that of **3b** (J = 3.9 Hz), indicating that the dihedral angles between H-24 and -28 in **3a** and **3b** could be both ca. 120°, based on the Karplus equation,[5] and H-24 and -28 are mutually *trans* in both **3a** and **3b**. Nuclear Overhauser effect (NOE) between H-24 and -28 was not observed in **3a** and **3b**, supporting the *trans* relationship of H-24 and H-28 in both **3a** and **3b**. The signals of H-22 of **3a** at δ 4.28 (ddd, J_1 = 10.9, J_2 = 5.1, J_3 = 2.0 Hz) and **3b** at δ 4.13 (ddd, J_1 = 10.9, J_2 = 5.1, J_3 = 2.0 Hz) are quite similar to that of **2** at δ 4.47 (ddd, J_1 = 10.7, J_2 = 6.1, J_3 = 1.1 Hz), suggesting that the configurations at position 22 of both **3a** and **3b** are the same as that of **2** (22*S* configuration). Accordingly, **3a** and **3b** were deduced to be (22*S*,24*R*)-24-methyllanosta-8-en-22,28-epoxy-3β,28α-diol and (22*S*,24*S*)-24-methyllanosta-8-en-22,28-epoxy-3β,28β-diol, respectively (see Chart 1). Compounds **3a** and **3b** are supposed to be non-enzymatically formed from **2** through its enolic form at position 28.

TABLE I. ^{13}C-NMR and ^{1}H-NMR Data for Component **3** (**3a** and **3b**)

Position	3a		3b	
	^{13}C-NMR	^{1}H-NMR	^{13}C-NMR	^{1}H-NMR
1	35.58 t	1.23 m, 1.74 m	35.58 t	1.23 m, 1.74 m
2	27.85 t	1.55 m, 1.68 m	27.85 t	1.55 m, 1.68 m
3	78.99 d	3.24 dd (11.6, 4.5)	78.99 d	3.24 dd (11.6, 4.5)
4	38.89 s		38.89 s	
5	50.40 d	1.05 dd (12.5, 2.0)	50.40 d	1.05 dd (12.5, 2.0)
6	18.27 t	1.52 m, 1.68 m	18.27 t	1.52 m, 1.68 m
7	26.49 t	2.04 2H, m	26.49 t	2.04 2H, m
8	134.35 s[a]		134.35 s[b]	
9	134.47 s[a]		134.47 s[b]	
10	37.02 s		37.02 s	
11	21.02 t	2.02 2H, m	21.02 t	2.02 2H, m
12	30.92 t	1.21 m, 1.75 m	30.93 t	1.21 m, 1.75 m
13	44.63 s		44.61 s	
14	49.72 s		49.77 s	
15	30.97 t	1.70 m, 1.80 m	30.89 t	1.70 m, 1.80 m
16	27.87 t	1.38 m, 2.03 m	27.92 t	1.54 m, 2.01 m
17	47.88 d	1.82 m	48.00 d	1.86 m
18	15.55 q	0.70 3H, s	15.58 q	0.70 3H, s
19	19.16 q	0.98 3H, s	19.16 q	0.98 3H, s
20	39.05 d	1.46 m	39.35 d	1.57 m
21	12.98 q	0.91 3H, d (6.4)	13.01 q	0.93 3H, d (6.4)
22	80.52 d	4.28 ddd (10.9, 5.1, 2.0)	83.01 d	4.13 ddd (10.9, 5.1, 2.0)
23	33.40 t	1.42 m, 1.84 m	31.29 t	1.22 m, 1.76 m
24	54.63 d	1.78 m	52.94 d	1.72 m
25	30.58 d	1.60 m	27.81 d	1.67 m
26	20.76 q[c]	0.91 3H, d (6.4)[d]	21.48 q[e]	0.99 3H, d (6.8)[f]
27	21.41 q[c]	0.99 3H, d (6.8)[d]	22.06 q[e]	0.95 3H, d (6.8)[f]
28	102.21 d	5.17 d (3.4)	97.77 d	5.27 d (3.9)
29	15.43 q	0.81 3H, s	15.43 q	0.81 3H, s
30	27.97 q	1.00 3H, s	27.97 q	1.00 3H, s
31	24.40 q	0.90 3H, s	24.36 q	0.90 3H, s

δ (ppm) from TMS in $CDCl_3$ (coupling constants (Hz) in parentheses). *a*—*f*) Assignments may be interchanged.

The methanolic extracts of *M. flabelliformis* and *L. betulina* suppressed by 89 and 45% the Con A-induced proliferation and by 98 and 39% the LPS-induced proliferation of mouse spleen lymphocytes, respectively. Successive fractionations of the extract of *M. flabelliformis* afforded two components, ergosterol peroxide (**4**) and 9(11)-dehydroergosterol peroxide (**5**), and those of the extract of *L. betulina* afforded the same sterol peroxides as immunosuppressive principles. Although ergosterol peroxide has already been isolated from many fungi and mushrooms[6] and 9(11)-dehydroergosterol peroxide has so far been isolated together with ergosterol peroxide from several fungi and mushrooms such as *Rhizoctonia repens*[4] and *Scleroderma aurantium*,[7] the isolation of both sterol peroxides together from *P. tinctorius*, *M. flabelliformis* and *L. betulina* is the first such example, to

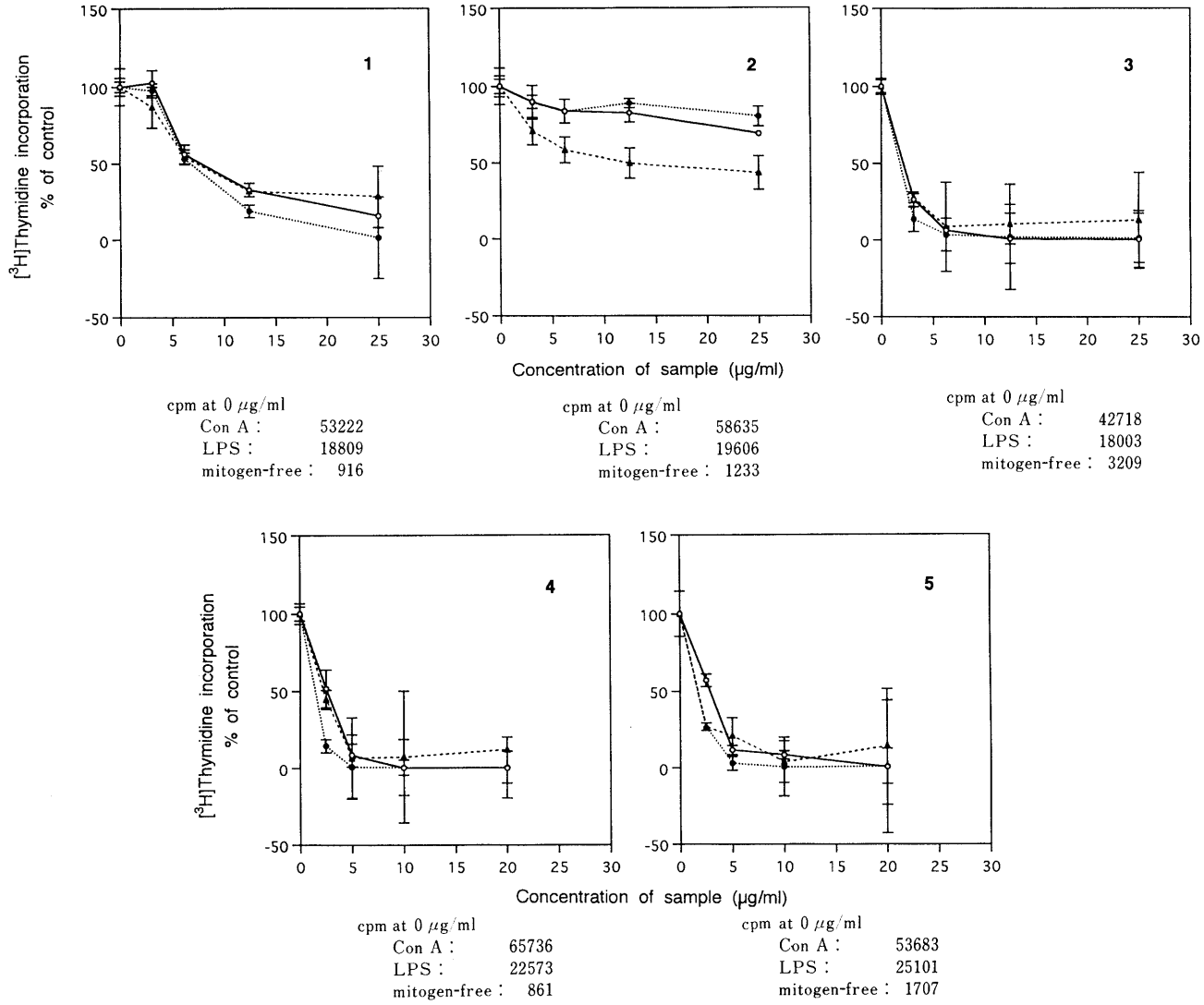

Fig. 1. Effects of Components **1**, **2**, **3**, **4** and **5** on Mitogen-Induced and Mitogen-Free Proliferations of Mouse Spleen Lymphocytes

—○—, 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.

the best of our knowledge.

The IC_{50} values of **1** and **3** were calculated to be 8.1 and 2.3 μg/ml against the Con A-induced proliferation and 7.1 and 1.9 μg/ml against the LPS-induced proliferation of mouse spleen lymphocytes, respectively (see Fig. 1). Pisolactone (**2**) suppressed by only 30.9% the Con A-induced proliferation and by 19.8% the LPS-induced proliferation even at 25 μg/ml. These facts suggested that the presence of the five-membered ring containing the 22,28-epoxy-28-hydroxyl moiety increases the immunosuppressive potency, but replacement of the hydroxyl at position 28 with a carbonyl decreases the potency. The IC_{50} values of ergosterol peroxide (**4**) and 9(11)-dehydroergosterol peroxide (**5**) were calculated to be 2.6 and 2.8 μg/ml against the Con A-induced and 1.5 and 1.6 μg/ml against the LPS-induced proliferations, respectively (see Fig. 1). Ergosterol peroxide has recently been isolated from a mushroom, *Tricholoma populinum*, as its immunosuppressive principle by Lindequist *et al.*[8] Our result shows that 9(11)-dehydroergosterol peroxide has immunosuppressive activity, like ergosterol peroxide.

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