Pergamon

SCIENCE DIRECT

Bioorganic & Medicinal Chemistry 11 (2003) 2511–2518

BIOORGANIC & MEDICINAL CHEMISTRY

Cancer Preventive Potential of Trichothecenes from *Trichothecium roseum*

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Received 14 February 2003; accepted 17 March 2003

Abstract—Bioassay-guided separation of extracts from the culture broth and mycelium of the fungus *Trichothecium roseum*, aiming at the discovery for cancer preventive agents, resulted in the isolation of three new trichothecene sesquiterpenes, trichothecinols A–C (**1**–**3**) together with three known analogues, trichothecin (**4**), trichodermol (**5**) and trichothecolone (**6**). Compounds **1**–**6** exhibited remarkably potent inhibition against Epstein-Barr virus early antigen (EBV-EA) activation induced by the tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Further compound **1** strongly inhibited TPA-induced tumor promotion on mouse skin initiated with 7,12-dimethylbenz[*a*]anthracene (DMBA) in two-stage carcinogenesis tests. These results suggest that compound **1** might be a valuable lead for further evaluation as a cancer preventive agent. In addition to their cancer preventive activity, compound **2** was found to show modest antifungal activity against *Crypotcoccus albidus* and *Saccharomyces cerevisiae*.

Introduction

Trichothecene mycotoxins produced by a lot of species of imperfect fungi such as *Fusarium*, *Trichothecium* and *Myrothecium* are a family of closely related sesquiterpenoids[1] that have been responsible for outbreaks of disease in human and farm animals due to the spoilage of cereal crops and other agricultural products.[2] Toxic manifestations caused by these toxins include cardiovascular lesions, gastrointestinal disorders, hypotension, anemia and lymphoid necrosis.[3] Accordingly, trichothecenes are of great importance in mycotoxicoses similarly to aflatoxins, potent carcinogenic *Aspergillus* metabolites causing acute and chronic lesions.[4] Trichothecenes exhibit a wide array of biological activities such as antibiotic, antibacterial and antiviral activity and insecticidal and phytotoxic behavior.[5] In addition, many of them have cytotoxic and antitumor activity.[6] At the cellular level, trichothecenes inhibit protein synthesis in eukaryotic cell lines, resulting in inhibition of DNA synthesis.[7]

As part of our ongoing search for bioactive compounds from medicinal plants and fungal metabolites,[8] we have investigated a combined extract from the mycelium and culture broth of *Trichothecium roseum*. Bioassay-guided fractionation of the extract, utilizing a short-term in vitro assay to monitor Epstein–Barr virus early antigen (EBV-EA) activation, resulted in the isolation of three novel 12,13-epoxytrichothecenes, trichothecinols A–C (**1**–**3**)[9] together with three known analogues, trichothecin (**4**),[10] trichodermol (**5**) and trichothecolone (**6**).[11] Compounds **1**–**6** strongly inhibited EBV-EA activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells, the EBV genome-carrying human lymphoblastoid cells.[12] Furthermore, the most active **1** also suppressed TPA-induced tumor promotion on mouse skin initiated with 7,12-dimethylbenz[*a*]anthracene (DMBA) in mouse skin two-stage carcinogenesis experiments.[13] Therefore, trichothecinol A (**1**) was suggested to be a promising lead for further evaluation as a cancer preventive agent.[14] In addition, the isolated compounds were evaluated for their ability to inhibit the growth of several bacteria and fungi. In this work, we report the structure elucidation of new compounds **1**–**3** together with the biological activity of compounds **1**–**6**.

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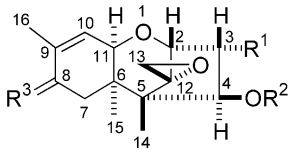
doi:10.1016/S0968-0896(03)00215-3



	R^1	R^2	R^3
1	OH	COCH=CHCH$_3$(*cis*)	O
2	H	COCH=CHCH$_3$(*cis*)	α-OH, β-H
3	OH	COCH=CHCH$_3$(*cis*)	α-OH, β-H
4	H	COCH=CHCH$_3$(*cis*)	O
5	H	H	α-H, β-H
6	H	H	O
7	H	COCH=CHCH$_3$(*cis*)	α-H, β-OH
8	OH	COCH=CHCH$_3$(*cis*)	α-H, β-OH

Results and Discussion

Isolation of trichothecenes from *T. roseum*

T. roseum (TMI-32358), supplied from The Tottori Mycological Institute (Tottori, Japan), was fermented in a potato dextrose culture medium containing yeast extract at 28 °C for 7 days. A combined EtOAc extract of the lyophilized mycelium and culture broth was purified by chromatographic methods including silica gel column chromatography, reversed-phase HPLC and preparative TLC to yield three novel trichothecenes, trichothecinols A (**1**), B (**2**) and C (**3**) together with the known compounds, trichothecin (**4**), trichodermol (**5**) and trichothecolone (**6**).

Structures of trichothecinols A (1), B (2) and C (3)

Each molecular formula of **4–6** obtained from their high-resolution chemical ionization mass spectrometry (HRCIMS) was found to coincide with those of three known metabolites of *T. roseum*. Comparison of their NMR and IR data with the reported values[10,11] allowed us to identify **4–6**, in turn, as trichothecin, trichodermol and trichothecolone.

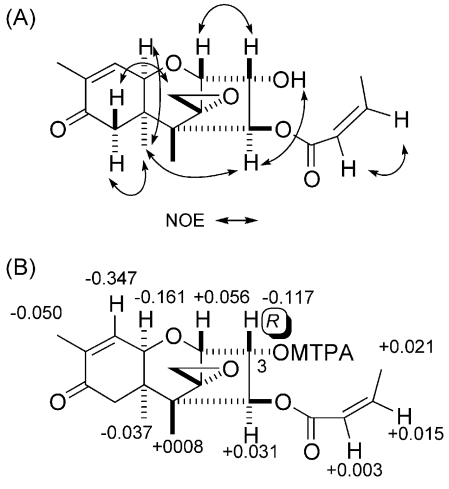


Figure 1. Structure of trichothecinol A (**1**): (a) selected NOE correlations for **1**; (b) chemical shift differences, Δδ (δ*S*–δ*R*), for the (*S*)- and (*R*)-MTPA ester derived from **1**.

The molecular formula of **1** was determined as $C_{19}H_{24}O_6$ by HRCIMS. Its ^{1}H NMR, ^{13}C NMR and IR spectra were very similar to those of **4**, indicating that **1** also belongs to a family of trichothecenes with a conjugated ketone and an isocrotonyl ester. The presence of the two functional groups in **1** was confirmed by its IR absorption bands at 1720 and 1680 cm^{-1} and NMR data (Fig. 1 and Experimental). Comparison of the molecular formula of **1** with that of **4** ($C_{19}H_{24}O_5$) suggested that the structure of **1** is a hydroxy form of **4**. The presence of a hydroxyl group was confirmed by IR (3450 cm^{-1}) and the disappearance of a doublet signal (δ 3.50) in the ^{1}H NMR spectrum of **1** treated by D_2O. Furthermore, the oxygenated methine carbon signal was observed at δ 78.77 in the ^{13}C NMR spectrum of **1** though no methylene carbon signal corresponding to C-3 (δ 37.00) of **4** was recognized. The ^{1}H NMR spectrum of **1** also exhibited the presence of the oxygenated methine proton (δ 4.29) instead of the methylene protons (δ 2.12 and 2.30) at C-3 in **4**. Thus, it was supported that the hydroxyl group of 1 attaches to C-3 in **4**. Detailed analysis of two-dimensional homo- and heteronuclear *J*-connectivities, and intramolecular NOE correlations allowed us to assign the relative structure of **1** as 3α-hydroxytrichothecin (Fig. 1A). For determination of the absolute configuration of **1**, the modified Mosher's method[15] was applied. A systematic arrangement of positive and negative values for the (*R*)- and

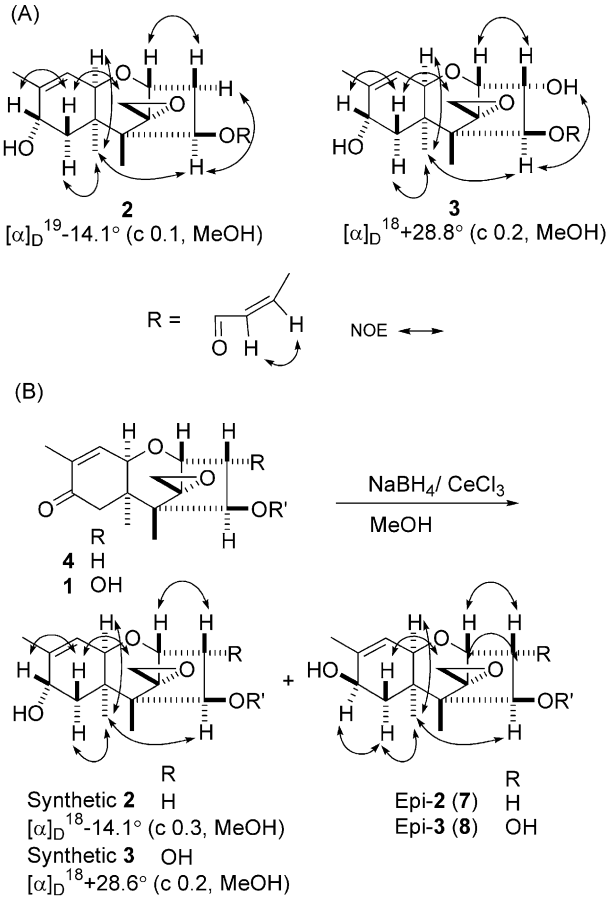


Figure 2. Structures of trichothecinols B (**2**) and C (**3**): (a) selected NOE correlations for **2** and **3**; (b) reduction of the ketone in **4** and **1**. R′ represents an isocrotonyl group.

(*S*)-MTPA esters derived from **1** was observed on the right and left sides of the MTPA plane, implying that **1** has the (*R*)-configuration at C-3 (Fig. 1B). This result demonstrated that the absolute framework of **1** is the same as that of **4**. Therefore, the complete structure of **1** was determined as (+)-12,13-epoxy-3α,4β-dihydroxytrichothec-9-en-8-one 4-isocrotonate, to which the trivial name trichothecinol A has been accorded.

The molecular weight ($C_{19}H_{26}O_5$) of **2** by HRCIMS was two a.m.u higher than that of **4**. The ^{13}C NMR spectrum of **2** showed an additional methine carbon signal at δ 67.83 instead of the carbonyl signal due to the conjugated ketone of **4**. Furthermore, the presence of a hydroxyl group in **2** was confirmed by its IR absorption at 3450 cm^{-1}. Thus, the structure of **2** was deduced to coincide with one of two hydroxyl forms that would be generated by reduction of the C-8 ketone of **4**. In fact, two-dimensional *J*-connectivities and intramolecular NOE correlations of **2** demonstrated that this compound possesses a α-oriented hydroxyl group at C-8 with the same relative stereochemistry as **4** except for C-8 (Fig. 2A). The structure of **3** was also predicted to accord with one of two hydroxyl forms that would be generated by reduction of the C-8 ketone of **1** based on its molecular weight ($C_{19}H_{26}O_6$) that is two a.m.u higher than that of **1** and the appearance of an additional methine carbon signal at δ 67.73 instead of the carbonyl signal due to a conjugated ketone. Subsequent detailed analysis of its two dimensional NMR data unambiguously framed the relative structure of **3**, in which a hydroxyl group is located at C-8 with the α-orientation (Fig. 2A). Determination of the absolute configuration of **2** and **3** was achieved by chemical correlation with **4** and **1**, respectively. Reduction of the C-8 ketone of **4** by $NaBH_4$ in the presence of $CeCl_3{\cdot}7H_2O$ afforded a mixture of two separable epimers, semisynthetic **2** and its *epi*-form **7**, in which the orientations of the 8-hydroxyl groups were determined as α and β, respectively, by virtue of diagnostic NOE correlations (Fig. 2B). The NMR data of the minor product (semisynthetic **2**) were identical with those of **2**. In addition, its specific rotation [$[\alpha]_D^{18}$ −14.1° (*c* 0.3, MeOH)] and retention time on HPLC were also in agreement with those of **2** (see Experimental). Therefore, the absolute structure of **2** was established as (−)-12,13 - epoxy - 4β,8α - dihydroxytrichothec-9-ene 4-isocrotonate, to which the trivial name trichothecinol B has been accorded. Similarly, the absolute structure of **3** was determined as (+)-12,13-epoxy-3α,4β,8α-trihydroxytrichothec-9-ene 4-isocrotonate (trichothecinol C) by reducing **1** to the corresponding diols, semisynthetic **3** with $[\alpha]_D^{18}$ + 28.6° (*c* 0.2, MeOH) and its *epi*-form **8** (Fig. 2B).

Inhibitory effect of trichothecenes 1–8 on EBV-EA activation

In vitro cancer preventive activity of natural trichothecenes and analogues **7** and **8** was evaluated for their ability to inhibit TPA-induced EBV-EA activation in Raji cells in comparison with that of curcumin,[16] a positive control substance that has been reported as a potent cancer preventive agent (Table 1). Trichothecenes **1–8** were all found to strongly inhibit TPA-induced EBV-EA activation. Among them, compounds **1**, **3**, **4** and **8** were amazingly potent inhibitors with IC_{50} values (mol ratio/32 pmol TPA) of 0.51, 0.56, 0.92 and 0.76, respectively. In particular, compounds **1** and **3** were 600-fold more potent than curcumin. To the best of our knowledge, no other cancer preventive agents that have been evaluated in this in vitro assay are more effective than these four trichothecenes. Although the remaining trichothecens **2**, **4–6** and **7** were much less potent than the former analogues, they still maintained 10-times more potent than curcumin. On the other hand, they did not show significant cytotoxicity against Raji cells at all and preserved the high viability (70%) even at 1000 mol ratio/TPA.

Potent inhibitors **1** and **4** both possess a conjugate ketone in the A ring and an isocrotonyl group in the C ring, suggesting that a concomitant presence of these two functional groups is one of important structural requirements for increasing activity. In fact, inhibitory activity of **2** and **6**, which are lacking one of the conjugate ketone and ester moieties in **4**, is drastically reduced. Further, the inhibitory activity of **2** and **6** was comparable with that of **5** which is devoid of both functional groups. This result also supports that both

Table 1. Inhibitory effects of trichothecenes **1–8** on TPA

Compd	EBV-EA-positive cells[a] (% viability)[b] Compound concentration (mol/ratio/32 pmol TPA)						
	1000	500	100	10	1.0	0.1	IC_{50}[c]
1	0.0±0.0 (70)	0.0±0.0	0.0±0.0	0.0±0.3	31.6±1.9	80.2±1.2	0.51
2	0.0±0.0 (70)	0.0±0.0	0.0±0.8	62.9±2.2	82.4±1.3	100±0.8	25.7
3	0.0±0.0 (70)	0.0±0.0	0.0±0.0	0.0±0.3	32.5±1.8	81.0±1.3	0.56
4	0.0±0.0 (70)	0.0±0.0	0.0±0.3	22.7±1.7	42.0±2.1	87.4±1.1	0.92
5	0.0±0.0 (70)	0.0±0.4	32.7±1.4	67.1±2.3	80.0±1.5	100±0.5	30.2
6	0.0±0.0 (70)	0.0±0.0	0.0±0.4	72.8±1.9	92.0±1.1	100±0.3	31.0
7	0.0±0.0 (70)	0.0±0.0	0.0±0.8	82.0±1.7	94.0±1.2	100±0.3	34.5
8	0.0±0.0 (70)	0.0±0.0	0.0±0.0	0.0±0.5	39.0±1.7	81.0±0.9	0.76
Curcumin[d]	8.9±1.3 (60)	40.3±2.2	74.5±2.4	95.8±1.3	100±1.1	100±0.0	345

[a]Values represent percentages relative to the positive control value. TPA (32 pmol = 20 ng/mL) = 100%. Data are expressed as mean ± SD.
[b]Values in parentheses represent viability percentages of Raji cells; unless otherwise stated, the viability percentage of Raji cells were more than 80%.
[c]IC_{50} values represent mol ration to TPA that inhibits 50% of positive control (100%) activated with 32 pmol TPA.
[d]Positive control substance.

functional groups play a crucial role in enhancing inhibitory activity. On the other hand, inhibitor **3**, which is the 3-hydroxy form of weakly active **2**, exhibited higher inhibitory activity than **4** despite of the absence of a conjugate ketone in the A ring. This result indicates that a combination of the hydroxyl group at C-3 and the isocrotonyl ester in the C ring is another structural requisite for increasing the inhibitory activity. Moreover, inhibitory activity of **7** and **8** slightly decreased compared with that of their epi-analogues **2** and **3**. This result indicates that the orientation of the hydroxyl group at C-8 also makes a small contribution toward increasing the inhibitory activity. It is, however, noted that these functional groups are not essential for the activity.

In vivo two-stage carcinogenesis test on mouse skin papillomas promoted by TPA

On the basis of the above in vitro results, it seems that the most active **1** could be a valuable lead for further evaluation as a cancer preventive agent. Thus, we investigated inhibitory effect of **1** on mouse skin tumor promotion in two-stage carcinogenesis experiments in which DMBA is used as an initiator and TPA as a promoter. Figure 3A shows the incidence (%) of papilloma-bearing mice during 20 weeks of promotion. In group I, in which only TPA was applied to mouse skin initiated by DMBA (positive control), the first tumors were observed at 6 weeks with about 33% incidence. The rate of papilloma-bearing mice reached 100% in additional 4 weeks. On the other hand, that in group II which was treated with **1** before application of TPA was approximately 7% at 10 weeks, 46% at 15 weeks and 83% even at 20 weeks, respectively. The inhibitory activity of **1** was also observed as the average numbers of papillomas per mouse during 20 weeks of promotion (Fig. 3B). Twenty weeks of promotion allowed group I to form 10 papillomas per mouse, while group II possessed only 4.5 papillomas per mouse. These results demonstrate that **1** possesses inhibitory activity against DMBA and TPA-induced two-stage skin carcinogenesis. Since compound **1** is also one of trichothecene mycotoxins, a possibility that oral administration of **1** may causes various acute and chronic lesions to animals cannot be ruled out.[4] Furthermore, some of these mycotoxins are also severe skin irritants,[17] which cause inflammation and scabbing. Thus, the discovery of cancer preventive effect as a new biological activity of trichothecenes would not directly lead to the development of trichothecene-based cancer preventive agents. Recently, we have completed the stereoselective synthesis of a trichothecene analogue toward structure–activity relationship studies.[18] Therefore, further synthetic efforts based on the present results are more likely to provide informative and useful structural requirements for the design and synthesis of potent new cancer preventive agents.

Antimicrobial activity

Trichothecenes have been shown to have inhibitory activity against various microorganisms.[5,19] In particular, compound **4** is a well-known inhibitor against fungi such as *Candida albicans*, *Cryptcoccus albidus* and *Saccharomyces cerevisiae*.[19a] Thus, antifungal activity of new trichothecenes was examined along with that of the known compounds (Table 2). Among the trichothecenes excluding **4**, only **2** possessed modest antifungal activity against *S. cerevisiae*, while **5** and **7** exhibited weak activity only against *C. albidus* and *S. cerevisiae*. Contrary to our expectation, the remaining trichothecens did not show antifungal activity at all at concentrations up to 100 μg/mL. It is of interest that drastically decreased antifungal activity of **1** and **3** is probably due to the presence of a hydroxyl group at C-3 by comparison with that of **2** and **4**. On the other hand, the trichothecenes examined displayed no antibacterial activity against common Gram-positive and Gram-negative bacteria at concentrations up to 100 μg/mL.

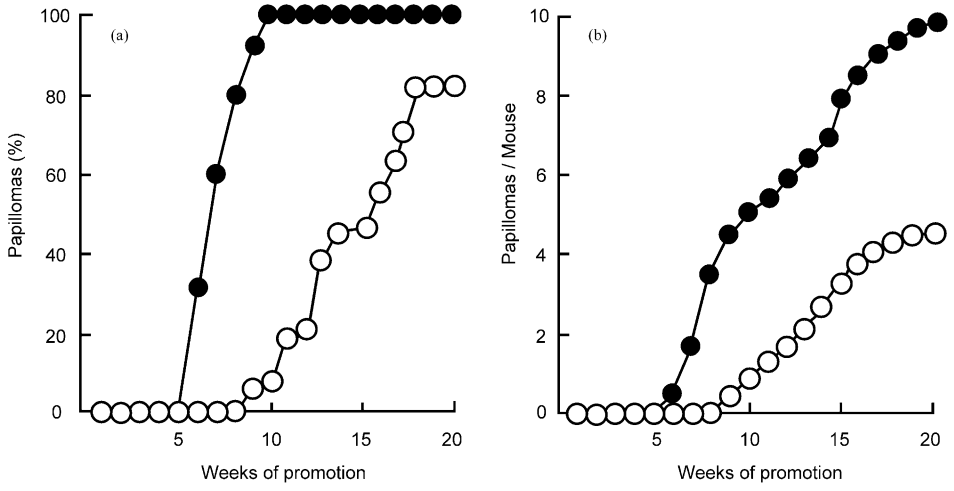


Figure 3. Inhibitory effect of trichothecinol A (**1**) on mouse skin carcinogenesis induced by DMBA and TPA: ●: control DMBA (390 nmol) and TPA (1.7 nmol) (group I); ○: DMBA (390 nmol), TPA (1.7 nmol) and **1** (85 nmol) (group II). (a) Percentage of mice with papillomas. (b) Average number of papillomas per mouse. At 20 weeks of promotion, the number of papillomas per mouse differed significantly ($p < 0.05$) between group I and group II (10.0 ± 2.5 for group I and 4.5 ± 1.2 for group II, respectively).

Table 2. Antimicrobial activity[a] of trichothecenes **1**–**8**

	1	**2**	**3**	**4**[b]	**5**[b]	**6**[c]	**7**	**8**
Bacillus subtilis ATCC9361	>100	>100	>100	>100	>100	>100	>100	>100
Staphylococcus aureus IFO12732	>100	>100	>100	>100	>100	>100	>100	>100
Escherichia coli IFO15034	>100	>100	>100	>100	>100	>100	>100	>100
Pseudomonas aeruginosa IFO3445	>100	>100	>100	>100	>100	>100	>100	>100
Candida albicans IFO1060	>100	50	>100	6.25	>100	>100	>100	>100
Cryptococcus albidus IFO0378	>100	25	>100	3.13	50	>100	50	>100
Saccharomyces cerevisiae IFO10114	>100	6.25	>100	0.78	50	>100	50	>100
Aspergillus fumigatus IFO4400	>100	>100	>100	50	>100	>100	>100	>100
Penicillium expansum IFO8800	>100	>100	>100	50	>100	>100	>100	>100
Paecilomyces variotti IFO4855	>100	>100	>100	50	>100	>100	>100	>100

[a]Values represent minimum growth inhibitory concentration, MIC (μg/mL).
[b]The antifungal activity of **4** and **5** has been reported also in ref 19a.
[c]No detail report on the antifunfal activity of **6** has been published.

Conclusion

Trichothecene mycotoxins have been found to inhibit TPA-induced EBV-EA activation in Raji cells for the first time. Six trichothecene isolated from *T. roseum* in this study and two derivatives all showed potent inhibitory activity in comparison with the positive control curcumin. A small library consisting of eight trichothecenes suggested that the isocrotonyl ester at C-4 is a key functional group that plays a critical role in increasing inhibitory activity. In addition, trichothecinol A strongly inhibited TPA-induced tumor promotion in mouse skin two-stage carcinogenesis experiments. These results reflect chemopreventive potential of trichothecenes. Further modification toward structure–activity relationships is now in progress to better understand structural requirements for cancer preventive activity.

Experimental

Optical rotations were measured with a JASCO DIP-370 digital polarimeter. IR and UV spectra were recorded on Shimadzu IR-435 and UV 2200 spectrophotometers. All NMR experiments were performed on Bruker AC-300 and JEOL JNM-LA500 spectrometers. Samples were dissolved in $CDCl_3$ containing TMS as internal standard. HPLC was performed on Shimadzu LC-6A and 8A systems. CIMS was carried out on a JEOL JMS-HX/HX110A mass spectrometer. Analytical and preparartive TLC were performed on silica gel 60 F_{254} Merck plates. Column chromatography was performed on silica gel 60 (70–230 mesh, Nacalai tesque).

Microorganism

A strain of *T. roseum* (TMI-32358) was kindly provided from The Tottori Mycological Institute (Tottori, Japan) and subcultured on potato dextrose agar (Difco) slants at 28 °C. An agar plug containing mycelium was transferred to potato dextrose broth (pH 4.5) (Difco) containing 0.5% yeast extract (Nacalai tesque) in 500-mL Sakaguchi flasks and incubated at 28 °C for 7 days on a rotary shaker. The bacteria and fungi tested for antimicrobial activity were purchased from the ATCC and IFO. Before assay, bacterial cells incubated for 18 h at 37 °C in Mueller–Hinton broth were diluted with the same medium to 10^6/mL cell concentration and harvest fungal conidia grown on potato dextrose agar slants were dispersed into 0.1% Tween 80 solution at 10^5/mL conidia concentration.

Extraction, isolation and derivatization

The lyophilized mycelium (10 g) from a 4.5-L medium was extracted with $CHCl_3$–MeOH (2:1, 500 mL) at room temperature for 4 days. After filtration, the solvent was removed under vacuum to yield 2.8 g of a brownish residue. Partition of the residue between EtOAc and H_2O gave 1.0 g of a trichothecene-containing EtOAc extract. The culture broth was extracted with EtOAc to afford a trichothecene-containing mixture (0.4 g). The combined extract (6.5 g) of the mycelium and broth after repeated cultivation was separated into 10 fractions by silica gel column chromatography with a gradient of $CHCl_3$–MeOH (10:0→9:1→6:4). Fraction 5 (2.5 g) was further purified by silica gel column chromatography with a gradient of hexane–Et_2O (1:2→1:4). Subfraction 6 (120 mg) of the nine fractions obtained was chromatographed on silica gel with $CHCl_3$–MeOH (99:1) to give **1** (87.4 mg). Subfraction 7 (177 mg) was subjected to column chromatography on silica gel with $CHCl_3$–MeOH (9:1) to give a mixture containing **2** and **5**. The mixture was further purified by reversed-phase HPLC [Cosmosil 5Ph (20 mm i.d.×250, Nacalai tesque), CH_3CN–H_2O (3:7), 9 mL/min, 30 °C] to afford **2** (7.2 mg) and **5** (11 mg). Subfraction 8 (345 mg) was successively purified by silica gel column chromatography using $CHCl_3$–MeOH (19:1), reversed-phase HPLC with CH_3CN–H_2O (3:7) and preparative TLC [silica gel, $CHCl_3$–MeOH (19:1)] to yield 3 (5.5 mg) and **6** (6.2 mg). Subfraction 3 (568 mg) was chromatographed on silica gel column with benzene–EtOAc (19:1→9:1) to give **4** (400 mg).

Trichothecinol A (1). A white solid; $[\alpha]_D^{18}$ +81.5° (*c* 0.7, MeOH); UV (MeOH) λ_{max} 218 nm ($\varepsilon = 1.4\times10^4$); IR ($CHCl_3$) ν_{max} 3450, 1720, 1680 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$), δ 0.77 (s, 5-Me), 1.05 (d, $J = 1.2$ Hz, 6-Me), 1.84 (dd, $J = 0.8$, 1.4 Hz, 9-Me), 2.17 (dd, $J = 1.8$, 7.3 Hz, 3′-Me), 2.31 (dd, $J = 1.6$, 15.2 Hz, H-7α), 2.81 (d, $J = 3.9$ Hz, H-13 *pro-R*), 2.95 (dd, $J = 1.2$, 15.2 Hz, H-7β), 3.08 (d, $J = 3.9$ Hz, H-13 *pro-S*), 3.50 (d, $J = 2.6$ Hz, 3-OH), 3.78 (d, $J = 5.0$ Hz, H-2), 4.29 (ddd, $J = 2.6$,

3.0, 5.0 Hz, H-3β), 4.41 (dd, $J=0.8$, 5.8 Hz, H-11), 4.99 (d, $J=3.0$ Hz, H-4), 5.88 (dq, $J=1.8$, 11.5 Hz, H-2′), 6.45 (dq, $J=7.3$, 11.5 Hz, H-3′), 6.56 (dq, $J=1.4$, 5.8 Hz, H-10); ^{13}C NMR (75 MHz, $CDCl_3$), δ 5.90 (q, C-14), 15.33 (q, C-16), 15.57 (q, C-4′), 18.40 (q, C-15), 42.03 (t, C-7), 44.39 (s, C-6), 46.63 (t, C-13), 48.87 (s, C-5), 65.60 (s, C-12), 70.97 (d, C-11), 78.77 (d, C-3), 79.30 (d, C-2), 83.17 (d, C-4), 119.89 (d, C-2′), 137.24 (d, C-10), 137.77 (s, C-9), 147.05 (d, C-3′), 167.81 (s, C-1′), 198.46 (s, C-8); HRCIMS m/z 349.1656 (MH^+ calcd for $C_{19}H_{25}O_6$, 349.1651).

(*R*)- and (*S*)-MTPA esters of 1. To stirred solutions of two 5.0-mg aliquots of **1** in CH_2Cl_2 (0.5 mL) were added successively (*S*)-(+)- and (*R*)-(−)-MTPA chloride (5 μL each), 4-dimethylaminopyridine (2 mg each) and triethylamine (2 μL each) at room temperature. Usual workup and purification by preparative TLC to give the (*R*)- and (*S*)-MTPA esters of **1**.

Trichothecinol B (2). A white solid; $[\alpha]_D^{19}$ −14.1° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 210 nm ($\varepsilon=1.5\times10^4$); IR (neat) ν_{max} 3450, 1710 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$), δ 0.76 (s, 5-Me), 1.14 (s, 6-Me), 1.73 (d, $J=14.2$ Hz, H-7 α), 1.87 (s, 9-Me), 2.04 (ddd, $J=3.6$, 5.2, 15.5 Hz, H-3β), 2.16 (dd, $J=1.8$, 7.2 Hz, 3′-Me), 2.25 (dd, $J=5.8$, 14.2 Hz, H-7β), 2.58 (dd, $J=7.8$, 15.5 Hz, H-3 α), 2.85 (d, $J=4.0$ Hz, H-13 *pro*-*R*), 3.10 (d, $J=4.0$ Hz, H-13 *pro*-*S*), 3.71 (d, $J=5.7$ Hz, H-11),3.81 (d, $J=5.2$ Hz, H-2), 4.15 (d, $J=5.8$ Hz, H-8β), 5.61 (dd, $J=3.6$, 7.8 Hz, H-4), 5.65 (d, $J=5.7$ Hz, H-10), 5.83 (dq, $J=1.8$, 11.5 Hz, H-2′), 6.35 (dq, $J=7.2$, 11.5 Hz, H-3′); ^{13}C NMR (75 MHz, $CDCl_3$), δ 5.99 (q, C-14), 15.46 (q, C-4′), 19.06 (q, C-15), 20.52 (q, C-16), 33.61 (t, C-7), 36.91 (t, C-3), 39.89 (s, C-6), 47.90 (t, C-13), 48.98 (s, C-5), 65.51 (s, C-12), 67.83 (d, C-8), 70.18 (d, C-11), 73.91 (d, C-4), 79.18 (d, C-2), 120.60 (d, C-2′), 121.41 (d, C-10), 139.42 (s, C-9), 145.63 (d, C-3′), 166.31 (s, C-1′); HRCIMS m/z 335.1859 (MH^+ calcd for $C_{19}H_{27}O_5$, 335.1858).

Trichothecinol C (3). A white solid; $[\alpha]_D^{18}$ +28.8° (*c* 0.2, MeOH); UV (MeOH) λ_{max} 211 nm ($\varepsilon=1.2\times10^4$); IR ($CHCl_3$) ν_{max} 3420, 1705 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$), δ 0.81 (s, 5-Me), 1.10 (s, 6-Me), 1.73 (d, $J=14.2$ Hz, H-7α), 1.88 (s, 9-Me), 2.17 (dd, $J=1.8$, 7.3 Hz, 3′-Me), 2.30 (dd, $J=4.9$, 14.2 Hz, H-7β), 2.81 (d, $J=4.0$, Hz, H-13 *pro*-*R*), 3.05 (d, $J=4.0$ Hz, H-13 *pro*-*S*), 3.36 (d, $J=2.7$ Hz, 3-OH), 3.70 (d, $J=4.9$ Hz, H-2), 4.11 (dd, $J=5.8$ Hz, H-11), 4.13 (d, $J=4.9$ Hz, H-8β), 4.22 (ddd, $J=2.7$, 3.0, 4.9 Hz, H-3β), 5.00 (d, $J=3.0$ Hz, H-4), 5.68 (d, $J=5.8$ Hz, H-10), 5.88 (dq, $J=1.8$, 11.5 Hz, H-2′), 6.42 (dq, $J=7.3$, 11.5 Hz, H-3′); ^{13}C NMR (75 MHz, $CDCl_3$), δ 6.20 (q, C-14), 15.58 (q, C-4′), 19.04 (q, C-15), 20.54 (q, C-16), 33.55 (t, C-7), 40.72 (s, C-6), 47.14 (t, C-13), 48.99 (s, C-5), 64.57 (s, C-12), 67.73 (d, C-8), 71.14 (d, C-11), 78.81 (d, C-3), 79.03 (d, C-2), 83.82 (d, C-4), 120.15 (d, C-2′), 121.58 (d, C-10), 138.94 (s, C-9), 146.66 (d, C-3′), 168.02 (s, C-1′); HRCIMS m/z 351.1805 (MH^+ calcd for $C_{19}H_{27}O_6$, 351.1807).

***epi*-Trichothecinol B (7) (*epi*-2).** To a solution of compound **4** (19.4 mg, 0.06 mmol) in anhydrous MeOH (0.5 mL) were added successively $CeCl_3\cdot7H_2O$ (21.7 mg, 1 equiv) and $NaBH_4$ (2.2 mg, 1 equiv), followed by stirring at room temperature for 10 min. The reaction was quenched with H_2O and extracted with $CHCl_3$. The $CHCl_3$ layer was dried over Na_2SO_4 and concentrated. The residue was subject to preparative TLC on silica gel with benzene–EtOAc (2:1) to give 5 mg of semisynthetic **2** with $[\alpha]_D^{18}$ −14.1° (*c* 0.3, MeOH) and the corresponding *epi*-form 7 (12.9 mg) as a white solid; $[\alpha]_D^{15}$ +58.5 (*c* 0.51, MeOH); IR ($CHCl_3$) ν_{max} 3450, 1710 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$), δ 0.74 (s, 3H, H-5Me), 1.02 (s, 3H, 6-Me), 1.33 (d, 1H, $J=8.6$ Hz, H-7), 1.83 (d, 3H, $J=1.2$ Hz, 9-Me), 1.88 (m, 1H, H-7), 2.04 (ddd, 1H, $J=15.6$, 5.2, 3.7 Hz, H-3), 2.16 (dq, 3H, $J=7.4$, 1.9 Hz, 3′-Me), 2.55 (dd, 1H, $J=15.6$, 8.0 Hz, H-3), 3.13 (d, 1H, $J=4.0$ Hz, H-13 *proR*), 3.66 (d, 1H, $J=5.4$ Hz, H-11), 3.86 (d, 1H, $J=5.2$ Hz, H-2), 3.86 (d, 1H, $J=4.0$ Hz, H-13 *proS*), 4.07 (dd, 1H, $J=8.0$, 3.7 Hz, H-4), 5.50 (dq, 1H, $J=5.5$, 1.2 Hz, H-10), 5.83 (dq, 1H, $J=11.6$, 1.9 Hz, H-2′), 5.89 dd, 1H, $J=7.9$, 4.0 Hz, H-8), 6.36 (dq, 1H, $J=11.6$, 7.4 Hz, H-3′); ^{13}C NMR (125 MHz, $CDCl_3$), δ, 6.0 (q, C-14), 16.9 (q, C-15), 18.8 (q, C-16), 35.4 (t, C-7), 36.8 (t, C-3), 42.4 (s, C-6), 47.8 (t, C-13), 49.1 (s, C-5), 65.3 (s, C-12), 69.1 (d, C-8), 70.3 (d, C-11), 73.8 (d, C-4), 79.4 (d, C-2), 120.5 (d, C-2), 121.2 (d, C-10), 142.2 (d, C-9), 145.8 (d, C-3′), 166.4 (s, C-1′); HRCIMS m/z 335.1859 (MH^+ calcd for $C_{19}H_{27}O_5$, 335.1858).

***epi*-Trichothecinol C (8) (*epi*-3).** Natural **1** (16.3 mg, 0.049 mmol) was treated as described above and the residue was purified by preparative TLC [silica gel, $CHCl_3$–MeOH (49:1)] to yield 2.6 mg of semisynthetic 3 with $[\alpha]_D^{18}$ +28.6° (*c* 0.2, MeOH) and the corresponding *epi*-form **8** (8.2 mg) as a white solid; $[\alpha]_D^{15}$ +98.7 (*c* 0.19, MeOH); IR ($CHCl_3$) ν_{max} 3400, 1710 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$), δ 0.78 (s, 3H, H-5Me), 0.98 (s, 3H, 6-Me), 1.40 (d, 1H, $J=7.9$ Hz, H-7), 1.84 (d, 3H, $J=1.2$ Hz, 9-Me), 1.92 (m, 1H, H-7), 2.17 (dd, 3H, $J=7.3$, 1.9 Hz, 3′-Me), 2.82 (d, 1H, $J=4.0$ Hz, H-13 *proS*), 3.07 (d, 1H, $J=4.0$ Hz, H-13 *proR*), 3.32 (d, 1H, $J=2.8$ Hz, 3-OH), 3.73 (d, 1H, $J=5.2$ Hz, H-2), 4.10–4.06 (m, 2H, H-8 and 11), 4.22 (ddd, 1H, $J=5.2$, 3.1, 2.8 Hz, H-3), 4.99 (d, 1H, $J=3.1$ Hz, H-4), 5.61 (dq, 1H, $J=5.5$, 1.2 Hz, H-10), 5.89 (dq, 1H, $J=11.6$, 1.9 Hz, H-2′), 6.43 (dq, 1H, $J=11.6$, 7.3 Hz, H-3′); ^{13}C NMR (125 MHz, $CDCl_3$), δ 6.1 (q, C-14), 16.9 (q, C-15), 18.8 (q, C-16), 35.1 (t, C-7), 43.1 (s, C-6), 47.0 (t, C-13), 49.0 (s, C-5), 64.4 (s, C-12), 69.0 (d, C-8), 71.3 (d, C-11), 78.6 (d, C-3), 79.2 (d, C-2), 83.6 (d, C-4), 120.1 (d, C-2), 121.3 (d, C-10), 141.7 (d, C-9), 146.8 (d, C-3′), 167.9 (s, C-1′); HRCIMS m/z 351.1805 (MH^+ calcd for $C_{19}H_{27}O_6$, 351.1807).

HPLC analysis of trichothecenes

The retention times of semisynthetic **2**, the *epi*-form **7** and natural **2** on HPLC were 37.4, 43.6 and 37.6 min, respectively [Cosmosil 5Ph (8 mm i.d. ×250, Nacalai tesque), CH_3CN–H_2O (3:7), 1 mL/min, 30 °C]. Under the same HPLC conditions, semisynthetic **3**, the *epi*-form **8** and natural **3** were eluted with a retention time of 21.7, 25.4 and 21.6 min, respectively.

Animals

Six-week-old pathogen-free female ICR mice were obtained from SLC Co, Ltd. (Shizuoka, Japan) and maintained in groups of five animals per polycarbonated cage in a temperature-controlled room at 24±2 °C. Animals were fed a MP solid diet from Oriental Yeast Ltd. (Chiba, Japan) and water ad libitum.

In vitro EBV-EA activation assay

The inhibition of EBV-EA activation was assayed using the same method as described previously.[12a] The cells were incubated for 48 h at 37 °C in a medium containing *n*-butyric acid (4 mM), TPA (32 pmol) and various amounts of the test compounds. Smears were made from the cell suspension and the EBV-EA inducing cells were stained by means of an indirect immunofluorescence technique.[20] In each assay, at least 500 cells were counted and the number of stained cells (positive cells) among them was recorded. Triplicate assays were performed for each data point. The EBV-EA inhibitory activity of the test compound was compared with that of the control experiment (100%) with *n*-butyric acid plus TPA. In the experiments, the EBV-EA activities were ordinarily abound 40% and these values were taken as the positive control (100%). The viability of cells was assayed against treated cells by the trypan-blue staining method. For the determination of cytotoxicity, the cell viability was required to be more than 60%.[21] Student's *t*-test was used for all statistical analyses.

In vivo two-stage carcinogenesis test on mouse skin papillomas promoted by TPA

Mice were divided into two experimental groups of 15 mice each and housed in a controlled environment. The back of each mouse was shaved with surgical clippers one day before initiation. The mice were initiated with 7,12-dimethylbenz[*a*]anthracene (DMBA, 390 nmol) in 0.1 mL of acetone by topical treatment. For group I (positive control group), they were promoted twice a week by application of TPA (1.7 nmol) in 0.1 mL of acetone 1 week after initiation. Group II received a topical application of compound **1** (85 nmol) 1 h before each TPA treatment. The incidence and numbers of papillomas were detected weekly for 20 weeks.[12b] Student's *t*-test was used for all statistical analyses.

Antimicrobial activity

MICs of trichothecenes **1**–**8** were determined by slightly modified NCCLS M27-P broth dilution method.[22] A 100-μL aliquot of Mueller–Hinton broth containing 10^6/mL bacterial cells was added to 2 mL of the same medium containing trichothecenes **1**–**8** at a concentration range of 0.1–100 μg/mL, followed by incubation for 24 h at 37 °C. Similaly, fungal conidia suspended in 100 μL of 0.1% Tween 80 solution (10^5/mL conidia concentration) were added to 2 mL of potato dextrose broth containing trichothecenes **1**–**8** at a concentration range of 0.1–100 μg/mL and incubated for 3 days at 25 °C.

Acknowledgements

The authors thank The Tottori Mycological Institute (Tottori, Japan) for generously providing the *Trichothecium roseum* strain (TMI-32358). This study was financially supported in part by Grants-in-Aid from the Japan Society for the Promotion of Science.

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