COMMUNICATIONS TO THE EDITOR

# Terprenins, Novel Immunosuppressants Produced by *Aspergillus candidus*

Sir:

During a screening program to find novel immunosuppressants from microbial fermentation products, we isolated terprenins[1] and terphenyllins[2~4] (Fig. 1 and 2) from the fermentation broth of *Aspergillus candidus* RF-5672 (FERM BP-5882), which was isolated from a soil sample collected on Shodo Island, Kagawa Prefecture, Japan. In this communication, we report the fermentation, isolation, structure elucidation and biological properties of these terprenins.

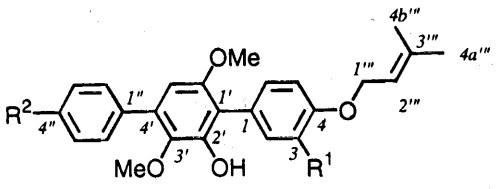
The activities of the terprenins found were evaluated with respect to proliferation of mouse spleen lymphocytes which had been stimulated with concanavalin A (Con A) and lipopolysaccharide (LPS). Spleen cells ($5 \times 10^5$) taken from BDF1 mouse (Shizuoka Laboratory Animal Center, Hamamatsu, Japan), used as a responder, were mixed with stimulators Con A (5 mcg/ml) or LPS (10 mcg/ml). The responder cells were cultured with RPMI-1640 medium (Gibco) containing 10% fetal calf serum in 96-well micro-titer plates. Each well contained the responder cells, the stimulator (Con A or LPS) and test samples at a final volume of 0.2 ml. The plates were cultured at 37°C for 48 hours in 5% $CO_2$ in 100% humidified air. The inhibitory activities were determined by measuring the incorporation of [$^3$H]-thymidine into the cultured cells by liquid scintillation counting. The labeled reagent was pulsed 6 hours before the cell harvest.

A loopful of slant culture of strain *Aspergillus candidus* RF-5672 was inoculated into 500-ml Erlenemyer flasks containing 100 ml of seed medium consisting of 5.0% glucose, 5.0% corn steep liquor and 0.2% $CaCO_3$ in tap water, with the pH adjusted to 7.0, and cultured on a rotary shaker (220 rpm) at 25°C for 4 days. For the production of terprenins, 4-ml aliquots of each culture were transferred into twenty 500-ml Erlenmyer flasks, each containing 100 ml of production medium consisting of 2.0% glycerine, 2.0% sucrose, 0.3% beef extract (Difco) and 0.2% yeast extract (Difco) in tap water, adjusted to pH 7.0 and cultured on a rotary shaker (180 rpm) at 23°C for 12 days.

The whole broth was subjected to filtration. The mycelial cake was extracted twice with acetone (500 ml), which was then removed from the extract by evaporation. The mycelium extract and the filtrate were combined and then extracted twice with ethyl acetate (500 ml) after adjusting the pH to 6.0 with HCl. The organic layer was washed with water and evaporated to dryness under reduced pressure, giving an oily extract (7.85 g). The terprenins and terphenyllins were separated and purified by repeated silica gel column chromatography and reverse phase column chromatography from the extract according to Fig. 3.

The structures of terprenin (**1**), 3-methoxyterprenin (**2**) and 4″-deoxyterprenin (**3**) were elucidated by $^1$H, $^{13}$C NMR and mass spectroscopy and confirmed by X-ray crystallographic analysis. The physico-chemical properties of the molecules are summarized in Table 1. The molecular formula was established as $C_{25}H_{26}O_6$ for **1**, $C_{26}H_{28}O_6$ for **2** and $C_{25}H_{26}O_5$ for **3** by HRSI-MS spectra and $^{13}$C NMR spectra. In the UV spectra, for

Fig. 1. Structures of terprenins.

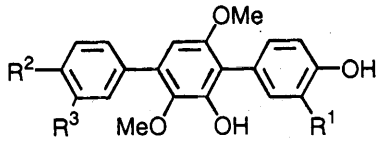



Terprenin (1) : $R^1$ = OH, $R^2$ = OH

3-Methoxyterprenin (2) : $R^1$ = $OCH_3$, $R^2$ = OH

4″-Deoxyterprenin (3) : $R^1$ = OH, $R^2$ = H

Fig. 2. Structures of terphenyllins.



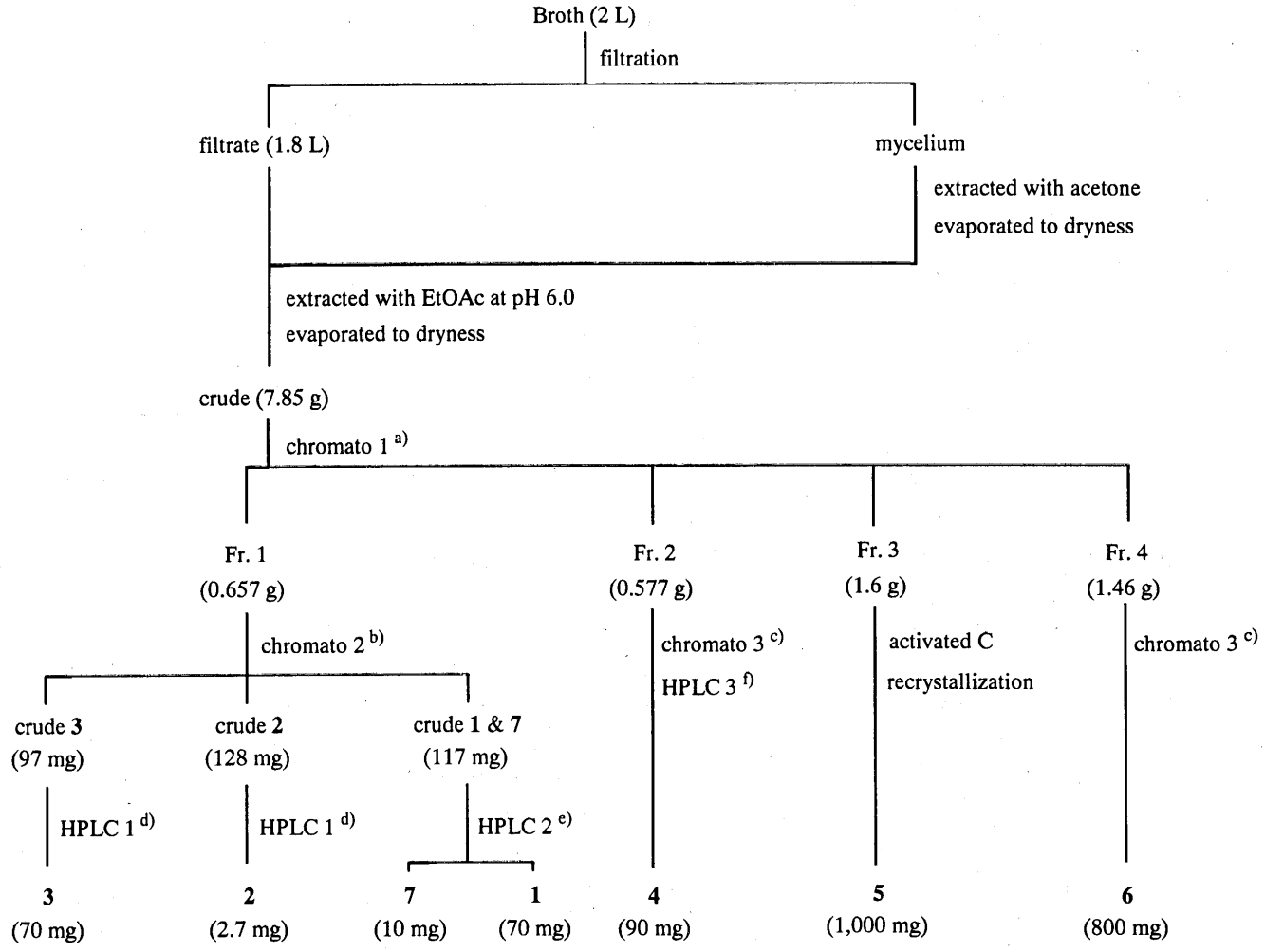

Terphenyllin (4) : $R^1$ = $R^3$ = H, $R^2$ = OH

3-Hydroxyterphenyllin (5) : $R^1$ = $R^2$ = OH, $R^3$ = H

3,3″-Dihydroxyterphenyllin (6) : $R^1$ = $R^2$ = $R^3$ = OH

4″-Deoxyterphenyllin (7) : $R^1$ = $R^2$ = $R^3$ = H

Fig. 3. Isolation and purification of terprenins and terphenyllins.




a) chromato 1 ($SiO_2$, 240 ml, $CHCl_3$, $CHCl_3$ : MeOH = 20 : 1~20 : 10)

b) chromato 2 ($SiO_2$, 110 ml, toluene : MeCN = 85 : 15)

c) chromato 3 ($SiO_2$, 90 ml, toluene : MeCN = 80 : 20)

d) HPLC 1 (YMC GEL ODS-AM 120-S50, MeCN : $H_2O$ = 7 : 3)

e) HPLC 2 (YMC GEL ODS-AM 120-S50, MeCN : $H_2O$ = 1 : 1)

f) HPLC 3 (YMC GEL ODS-AM 120-S50, MeCN : $H_2O$ = 4 : 6)

example in terprenin (**1**), $\lambda_{max}$ at 277 nm in the neutral solution shifted to longer wavelengths (+20 nm) in alkaline solution. This shift suggested that terprenins have a phenolic moiety in their structures. The $^1H$ and $^{13}C$ NMR spectral data of **1**, **2** and **3** are summarized in Table 2. NMR analysis indicated terprenin (**1**) has an *O*-prenyl group [$\delta_H$ 1.77, 1.79 (each 3H), 4.63 (2H), 5.52 (1H) and $\delta_C$ 18.31 (q), 26.00 (q), 66.18 (t), 121.44 (d), 137.50 (s)], two methoxyl groups [$\delta_H$ 3.37, 3.73 (each 3H) and $\delta_C$ 56.04 (q), 60.67 (q)], three phenolic hydroxyl groups [$\delta_H$ 7.62, 7.78, 8.64] and three aromatic rings [an ABX-type spin system on ring A ($\delta_H$ 6.83, 6.96, 6.92), a one proton system on ring B ($\delta_H$ 6.49) and an $A_2B_2$-type spin system on ring C {$\delta_H$ 6.94 (2H), 7.54 (2H)}]. Long-range $^1H$-$^{13}C$ correlations observed in the heteronuclear multiple-bond correlation (HMBC) spectrum of terprenin (**1**) suggested the proposed structure shown in Fig. 4.

3-Methoxyterprenin (**2**) was similar to terprenin (**1**), but the phenolic OH of the ring A was replaced by OMe [$\delta_H$ 3.80 (3H), $\delta_C$ 56.13 (q)]. 4″-Deoxyterprenin (**3**) was also similar to terprenin (**1**), but the phenolic OH of ring

Table 1. Physico-chemical properties of terprenins.

| | Terprenin (1) | 3-Methoxyterprenin (2) | 4"-Deoxyterprenin (3) |
|---|---|---|---|
| Appearance | colorless prisms | white powder | white powder |
| MP ℃ | 155.5~156 | — | — |
| Molecular formula | $C_{25}H_{26}O_6$ | $C_{26}H_{28}O_6$ | $C_{25}H_{26}O_5$ |
| HRSI-MS | | | |
| calcd | 422.1728 | 436.1884 | 406.1779 |
| obsd $(M)^+$ | 422.1730 | 436.1880 | 406.1780 |
| UV $\lambda_{max}$ nm (ε) | | | |
| in MeOH | 230 (sh), 277 (25,700) | 230 (sh), 278 (25,300) | 225 (sh), 274 (17,600) |
| in 0.1N NaOH-MeOH | 235 (sh), 297 (26,200) | 235 (sh), 295 (25,100) | 225 (sh), 255 (sh), 295 (26,200) |
| in 0.1N HCl-MeOH | 230 (sh), 276 (24,500) | 230 (sh), 278 (24,500) | 225 (sh), 273 (18,000) |
| HPLC (min.)*) | 5.6 | 7.4 | 13.5 |

*) Column, YMC-Pack ODS-AM, AM-302 (4.6 i.d. x 150 mm); flow rate, 1 ml/min.; detection, UV at 280 nm; solvent, $CH_3CN : H_2O = 55 : 45$

Fig. 4. Long-range $^1H$-$^{13}C$ correlation of terprenin (1) by HMBC experiment.

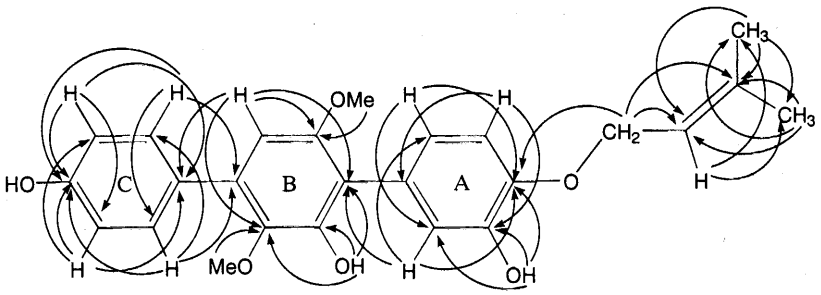



Fig. 5. The perspective view of terprenin (1) by X-ray crystallographic analysis.

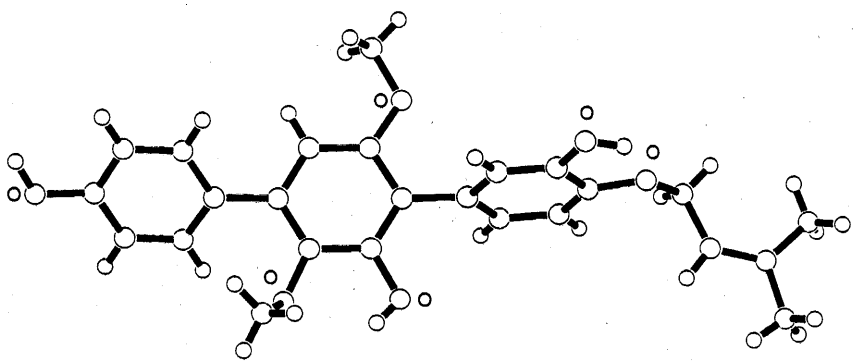


Table 2. $^{1}H$ and $^{13}C$ NMR spectral data of terprenins (600 MHz in acetone-$d_6$).

| | Terprenin (1) | | 3-Methoxyterprenin (2) | | 4"-Deoxyterprenin (3) | |
|---|---|---|---|---|---|---|
| Position | $\delta_C$ (ppm) | $\delta_H$ (ppm, J in Hz) | $\delta_C$ (ppm) | $\delta_H$ (ppm, J in Hz) | $\delta_C$ (ppm) | $\delta_H$ (ppm, J in Hz) |
| 1 | 127.91 (s) | | 127.73 (s) | | 127.96 (s) | |
| 2 | 118.97 (d) | 6.92 (d, 2.2) | 116.42 (d) | 6.99 (d, 2.0) | 119.07 (d) | 6.93(d, 2.0) |
| 3 | 146.79 (s) | | 149.98 (s) | | 147.07 (s) | |
| 3-OH | | 7.62 (br. s) | | | | 7.44 (s) |
| 3-OMe | | | 56.13 (q) | 3.80 (s) | | |
| 4 | 146.23 (s) | | 148.37 (s) | | 146.50 (s) | |
| 5 | 112.75 (d) | 6.96 (d, 8.2) | 113.80 (d) | 6.97 (d, 8.3) | 113.10 (d) | 6.97 (d, 8.2) |
| 6 | 123.15 (d) | 6.83 (dd, 8.2 & 2.2) | 124.37 (d) | 6.93 (dd, 8.3 & 2.0) | 123.28 (d) | 6.84 (dd, 8.2 & 2.0) |
| 1' | 117.61(s) | | 117.70 (s) | | 118.63 (s) | |
| 2' | 149.24 (s) | | 149.16 (s) | | 149.40 (s) | |
| 2'-OH | | 7.78 (s) | | 7.83 (s) | | 7.65 (s) |
| 3' | 140.04 (s) | | 140.17 (s) | | 140.46 (s) | |
| 3'-OMe | 60.67 (q) | 3.37 (s) | 60.65 (q) | 3.38 (s) | 61.04 (q) | 3.38 (s) |
| 4' | 133.54 (s) | | 133.66 (s) | | 133.85 (s) | |
| 5' | 103.85 (d) | 6.49 (s) | 104.17 (d) | 6.50 (s) | 104.45 (d) | 6.52 (s) |
| 6' | 154.51 (s) | | 154.52 (s) | | 154.78 (s) | |
| 6'-OMe | 56.04 (q) | 3.73 (s) | 56.16 (q) | 3.74 (s) | 56.26 (q) | 3.73 (s) |
| 1" | 130.48 (s) | | 130.50 (s) | | 139.65 (s) | |
| 2" | 130.85 (d) | 7.54 (m) | 130.79 (d) | 7.54 (m) | 129.84 (d) | 7.66 (m) |
| 3" | 116.06 (d) | 6.94 (m) | 116.06 (d) | 6.95 (m) | 129.35 (d) | 7.46 (m) |
| 4" | 157.79 (s) | | 157.80 (s) | | 128.25 (d) | 7.35 (m) |
| 4"-OH | | 8.64 (br. s) | | 8.65 (s) | | |
| 5" | 116.06 (d) | 6.94 (m) | 116.06 (d) | 6.95 (m) | 129.35 (d) | 7.46 (m) |
| 6" | 130.85 (d) | 7.54 (m) | 130.79 (d) | 7.54 (m) | 129.84 (d) | 7.66 (m) |
| 1'" | 66.18 (t) | 4.63 (br. d, 6.6) | 66.18 (t) | 4.59 (d-like, 6.7) | 66.44 (t) | 4.63 (m) |
| 2'" | 121.44 (d) | 5.52 (m) | 121.63 (d) | 5.53 (m) | 121.56 (d) | 5.53 (m) |
| 3'" | 137.50 (s) | | 137.40 (s) | | 137.70 (s) | |
| 4a'" | 26.00 (q) | 1.79 (m) | 25.84 (q) | 1.79 (s-like) | 26.00 (q) | 1.78 (s-like) |
| 4b'" | 18.31 (q) | 1.77 (m) | 18.19 (q) | 1.77 (s-like) | 18.34 (q) | 1.77 (s-like) |

C was replaced by a proton [$\delta_H$ 7.35 (1H), $\delta_C$ 128.25 (d)]. These structures (**2** and **3**) were confirmed by HMBC and NOESY experiments.

The proposed structure of terprenin (**1**) was confirmed also by X-ray crystallographic analysis (Fig. 5). Crystals suitable for X-ray analysis were grown from solvent mixtures of *n*-hexane and ethyl acetate. The crystal data are as follows: monoclinic, space group P21/n, a = 12.097(1) Å, b = 13.840(2) Å, c = 15.534(1) Å, $\beta$ = 107.07(1)°, V = 2486.1(4) Å$^3$, Z = 4.

Terprenins possessed very strong proliferation against mouse spleen lymphocytes stimulated with Con A and LPS. The $IC_{50}$ values of terprenin (**1**), 3-methoxyterprenin (**2**) and 4"-deoxyterprenin (**3**) were calculated

Table 3. Effect of terprenins against Con A-induced proliferation.

| | Terprenin (1) | | 3-Methoxyterprenin (2) | | 4"-Deoxyterprenin (3) | |
|---|---|---|---|---|---|---|
| | Radioactivity | Inhibition | Radioactivity | Inhibition | Radioactivity | Inhibition |
| (ng / ml) | cpm±SD | % | cpm±SD | % | cpm±SD | % |
| - Con A | 3,440±568 | 100 | 3,440±568 | 100 | 3,440±568 | 100 |
| 0 | 277,061±7,118 | 0 | 277,061±7,118 | 0 | 277,061±7,118 | 0 |
| 0.25 | 292,470±542 | -5.6 | 285,408±7,252 | -3.1 | 281,904±6,522 | -1.8 |
| 0.98 | 210,046±3,288 | 24.5 | 266,173±6,208 | 4.0 | 281,371±10,119 | -1.6 |
| 3.91 | 56,871±1,554 | 80.5 | 101,504±1,326 | 64.2 | 191,575±6,969 | 30.9 |
| 15.60 | 11,366±372 | 97.1 | 20,510±287 | 93.8 | 41,660±531 | 86.0 |
| 62.50 | 6,411±246 | 98.9 | 7,755±624 | 98.4 | 11,793±235 | 96.9 |
| 250.00 | 6,404±403 | 98.9 | 7,522±485 | 98.5 | 8,233±254 | 98.2 |
| $IC_{50}$(ng / ml) | **1.2** | | **2.0** | | **5.6** | |

Table 4. Effect of terprenins against LPS-induced proliferation.

| | Terprenin (1) | | 3-Methoxyterprenin (2) | | 4"-Deoxyterprenin (3) | |
|---|---|---|---|---|---|---|
| | Radioactivity | Inhibition | Radioactivity | Inhibition | Radioactivity | Inhibition |
| (ng / ml) | cpm±SD | % | cpm±SD | % | cpm±SD | % |
| - LPS | 2,939±167 | 100 | 2,939±167 | 100 | 2,939±167 | 100 |
| 0 | 153,851±5,649 | 0 | 153,851±5,649 | 0 | 153,851±5,649 | 0 |
| 0.98 | 153,396±6,123 | 0.3 | 184,366±10,625 | -20.2 | 208,023±8,941 | -35.9 |
| 3.91 | 88,405±10,394 | 43.4 | 128,436±4,167 | 16.8 | 132,834±5,106 | 13.9 |
| 15.60 | 32,548±315 | 80.4 | 46,765±2,209 | 71.0 | 78,686±4,135 | 49.8 |
| 62.50 | 16,070±944 | 91.3 | 22,961±1,187 | 86.7 | 39,824±651 | 75.6 |
| 250.00 | 15,046±344 | 92.0 | 14,962±866 | 92.0 | 22,312±1,122 | 87.2 |
| $IC_{50}$(ng / ml) | **4.5** | | **8.0** | | **15.6** | |

as 1.2, 2.0 and 5.6 ng/ml against Con A-induced proliferation and 4.5, 8.0 and 15.6 ng/ml against LPS-induced proliferation. These values are summarized in Table 3 and 4. Terprenins had no antimicrobial activity against bacteria and fungi.

To further clarify the biological properties, we established the route of chemical conversion to terprenin (**1**) from 3-hydroxyterphenyllin (**5**). The route included a prenylation step (prenyl bromide, $K_2CO_3$ and acetone) and a separation step (ODS column, MeCN : $H_2O$ = 1 : 1) which separated the 4-*O*-prenylated product (=terprenin) and the 3-*O*-prenylated by-product. M. OHTANI, K. KAWADA and their co-workers at Shionogi Research Laboratories have recently finished the first total synthesis of terprenin[5].

We isolated three new *O*-prenylated *para*-terphenyl compounds, terprenin (**1**), 3-methoxyterprenin (**2**) and 4″-deoxyterprenin (**3**), which possess strong immunosuppressive activities *in vitro*. No such activity was found for four known *para*-terphenyl compounds, terphenyllin (**4**), 3-hydroxyterphenyllin (**5**), 3,3″-dihydroxyterphenyllin (**6**) and 4″-deoxyterphenyllin (**7**).

The action mechanisms of the active terprenins are now under study.

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