Structure of a New Antibiotic, Terrecyclol, from *Aspergillus terreus* Thom

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The structure of a new sesquiterpene antibiotic, terrecyclol (**I**), from *Aspergillus terreus* Thom No. 14 was determined to be **I**. The antimicrobial activity of **I** is as high as that of terrecyclic acid A (**II**), which is a main antimicrobial product of *Asp. terreus* No. 14.

In the previous papers^{1,2)} we have reported the isolation procedure, biological activities and chemical structure of a new sesquiterpene antibiotic terrecyclic acid A (**II**), which was produced from *Aspergillus terreus* Thom No. 14. **II** has the same novel carbon skeleton as does quadron^{3,4)} an antitumor substance produced by *Aspergillus terreus*. In continuation of the work we noticed antimicrobial activity against *Bacillus subtilis* in the neutral fraction of the culture filtrate of the same microorganism.

In this paper we wish to report the purification procedure, biological activity and structure of a new antibiotic, terrecyclol (**I**).

A. terreus No. 14 was cultivated at 30°C for six days on the medium described in EXPERIMENTAL. The culture broth (30 liters) was filtered and the culture filtrate was adsorbed to activated charcoal. The charcoal was extracted with acetone and next with ethyl acetate. The combined extracts were concentrated under reduced pressure and an ethyl acetate soluble neutral fraction was conventionally separated from the residue. The neutral fraction was applied to silica gel column chromatography (Wako gel C-200; solvent system, benzene-ethyl acetate). The antimicrobial activity against *Bacillus subtilis* was shown in the eluate with benzene containing

20% ethyl acetate. This eluate was rechromatographed on a silica gel column with benzene containing 12% ethyl acetate to give a crude oil (80 mg). The active substance (**I**) seemed to be a major component of the oil, but its impurities could not be removed. The crude oil was, however, reduced with sodium borohydride and the crystalline substance (**III**), mp 189~190°C, was obtained in a high yield.

FIG. 1. Structures of **I**, **II**, **III**, **IV** and **V**.

By high resolution mass spectrometry, the molecular formula of **III** was determined to be $C_{15}H_{24}O_2$ (M^+ m/z 236.1796; 236.1776 calcd. for $C_{15}H_{24}O_2$). **III** did not give a positive color reaction to 2,4-dinitrophenylhydrazine, while **I** did, and the IR spectrum of **III** showed no absorption band due to a carbonyl group.

In the 1H -NMR spectrum (in d_5 -pyridine) of **III** there existed two broad singlets at δ 5.90 and δ 6.70 ppm attributable to hydroxyl protons. A broad triplet (1H, $J=8.1$ Hz) at δ 4.65 ppm was due to the $-CH_2CHOH$ moiety, and a double doublet (1H, $J=9.5$, 2.8 Hz) at δ 4.03 ppm and a double doublet (1H, $J=9.5$, 9.5 Hz) at δ 3.77 ppm were assigned to the $-CH-CH_2OH$ moiety. Other signals in the 1H -NMR spectrum of **III** were very similar to those in the 1H -NMR spectrum of terrecyclic acid **A** (**II**). For example, two singlets at δ 5.04 and 5.56 ppm were attributable to exomethylene protons and this assignment was supported by the ^{13}C -NMR spectrum of **III** (δ 104.00 (t) and 163.46 (s) ppm). Two doublet methyl protons at δ 1.06 ppm and 1.09 ppm also existed. Accordingly **I** was presumed to have a hydroxymethyl group instead of a carboxyl group at the C-7 position*¹ of **II**.

II was reduced with sodium borohydride and a crystalline substance (**IV**), mp 187°C, $C_{15}H_{22}O_3$ (M^+ m/z 250), was obtained. The ^{13}C -NMR spectral data of **III** and **IV** are shown in Table I. These two spectra were very similar each other except for a signal at δ 63.01 ppm (t) in **III** and a signal at δ 177.61 ppm (s) in **IV**. Consequently, the above-mentioned presumption was deemed reasonable.

Further, terrecyclic acid **A** (**II**) was reduced with lithium aluminum hydride and a crystalline substance was obtained. This substance, mp 189~190°C, MS M^+ m/z 236, was identified to be **III**. **III** was then oxidized with manganese dioxide to yield an oily substance, which was identified as **I** by comparison with

TABLE I. ^{13}C -NMR SPECTRAL DATA OF **III** (100 MHz, d_5 -PYRIDINE) AND **IV** (25 MHz, d_5 -PYRIDINE)

δ , ppm (multiplicity)	
III	IV
21.44 (t)	23.37 (t)
26.92 (q)	27.32 (q)
28.64 (t)	29.75 (t)
35.02 (q)	35.10 (q)
38.80 (s)	39.31 (s)
39.15 (t)	39.31 (t)
47.92 (d)	48.00 (d)
48.07 (d)	49.17 (d)
49.32 (d)	50.14 (d)
55.91 (t)	55.40 (t)
56.60 (s)	55.99 (s)
63.01 (t)	76.75 (d)
77.23 (d)	104.48 (t)
104.00 (t)	163.57 (s)
163.46 (s)	177.61 (s)

their R_f values on some thin layer chromatogram plates and with their IR spectra (Fig. 2). The structure of **I** was determined as shown in Fig. 1 and **I** was named terrecyclol.

The configuration of terrecyclol (**I**) is the same as that of terrecyclic acid **A** (**II**), because four assymetric centers (C_1 , C_2 , C_8 and C_{11}) seem to be unchanged in the reactions from **II** to **I**. **II** was easily pyrolyzed at 190°C in an oil bath to afford quadrone.²⁾ Accordingly, the stereochemistry of the four assymetric centers of **I**, **II** and quadrone should be the same.

The relative stereochemistry of quadrone was determined by a direct single X-ray analysis.⁴⁾ We tried to ascertain the relative stereochemistry of these novel sesquiterpenes by a Nuclear Overhauser Enhancement experiment. This experiment was conducted with the diazomethane adduct of the methyl ester of **II**, the 400 MHz 1H -NMR spectrum of which was completely analyzed.²⁾ The results are shown in Scheme 1, and the relative stereochemistry of quadrone, which was proposed by Ranieri and Calton,⁴⁾ was supported by the NOE experiment.

*¹ The numbering system of the carbon skeleton was followed after the proposal by Ranieri and Calton⁴⁾ in quadrone.

FIG. 2. IR Spectra of Crude I (A) and I Derived from II (B) (Film).

Proton irradiated	Nuclear Overhauser Enhancement observed on
δ 3.97 (H_c)	H_d, H_g, H_h
δ 2.48 (H_f)	H_m
δ 1.16 (CH_3)	$H_e, H_h, H_j, H_k, H_m, H_n$

SCHEME 1. NOE Experiment of the Diazomethane Adduct of the Methyl Ester of II.

The antimicrobial spectrum of terrecyclol*² is shown in Table II. I showed slightly high activities against gram-positive bacteria compared with II, and I also showed rather weak activities against *P. chrysogenum* and *F. oxysporum*.

Since III and IV have no antimicrobial

activity, the carbonyl group at the C-4 position is necessary for this activity. II was hydrogenated over palladium-charcoal to give a crystalline compound (V), mp 128~129°C, MS M^+ m/z 250. From spectroscopic data, the structure of V was determined as shown in Fig. 1. V also had no antimicrobial activity. In conclusion an α -methylene carbonyl moiety is essential for antimicrobial activity and, as Kupchan *et al.* have stated,⁵⁾ an electrophilic α -methylene ketone might be the carrier of biological activity in the studies of antitumor substances from higher plants.

EXPERIMENTAL

Melting points were determined on a microscope hot plate from Yanagimoto Co. and are uncorrected. The optical rotation was measured with a JASCO DIP-SL polarimeter, and the IR spectra were recorded on a JASCO IRA-2 infrared spectrometer. The 400 MHz ¹H-NMR and 25 MHz ¹³C-NMR spectra were measured with a JNM-FX-400 and a JNM-FX-100 spectrometer, respectively. Mass spectra and high resolution mass spectra were obtained with a Hitachi RMU-6M and a JEOL JMS D-300 mass spectrometer, respectively.

*² Since the impurities could not be removed, terrecyclol artificially derived from terrecyclic acid A was used in the bioassay.

TABLE II. ANTIMICROBIAL SPECTRA OF I, II, III, IV AND V

	MIC (mcg/ml)				
	I	II	III	IV	V
<i>Staphylococcus aureus</i> IFO 3060	25	25	> 500	> 500	> 500
<i>Bacillus subtilis</i> IFO 12210	12	50	> 500	> 500	> 500
<i>Micrococcus roseus</i> IFO 3764	12	25	> 500	500	500
<i>Escherichia coli</i> K-12 IFO 3301	> 200	> 200	> 500	> 500	> 500
<i>Pseudomonas aeruginosa</i> IFO 3923	> 200	200	> 500	> 500	> 500
<i>Serratia marcescens</i> IFO 12648	> 200	> 200	> 500	> 500	> 500
<i>Aspergillus niger</i> IFO 4416	> 200	> 200	> 500	> 500	> 500
<i>Penicillium chrysogenum</i> IFO 4897	25	200	> 500	> 500	> 500
<i>Fusarium oxysporum</i> IFO 5880	25	200	> 500	> 500	> 500
<i>Candida albicans</i>	> 200	150	> 500	> 500	> 500

Cultivation. The medium composition was glucose 30 g, soybean meal 2.5 g, yeast extract 0.5 g, KH_2PO_4 1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1 g, NaCl 0.5 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.5 g, $\text{FeCl}_3 \cdot 2\text{H}_2\text{O}$ 2.0 mg, and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 2.0 mg in 1,000 ml of tap water and it was adjusted to pH 5.5 before sterilization. Fermentation was carried out 30°C for six days on a rotary shaker and CaCO_3 (one gram per one liter) was added at the 2nd day.

Purification of terrecyclol (I). The culture filtrate (30 liters, pH ca. 4.3) was adsorbed on activated charcoal (390 g) and eluted first with acetone and then with ethyl acetate. The combined extracts were concentrated under reduced pressure and the residue was dissolved in water, adjusted to pH 3.0 with dil. hydrochloric acid and extracted with ethyl acetate. The organic layer was washed with sodium bicarbonate solution and the neutral fraction (7 g) was obtained after drying over anhydrous sodium sulfate and removing the solvent. The neutral fraction was applied to a silica gel column (Wako gel C-200, 100 g) and eluted with benzene-ethyl acetate (80:20). After concentrating the active fractions, the concentrate was applied to a silica gel column (Wako gel C-200, 27 g) and eluted with benzene-ethyl acetate (88:12). Concentration of the active fractions gave a crude oil (80 mg). Terrecyclol (I) is a major component of the crude oil, and *R_f* values of I on thin layer chromatogram plates (Merck GF₂₅₄, 0.25 mm thickness) are 0.48 (benzene-MeOH, 4:1) and 0.55 (ethyl acetate). I was positive to 2,4-dinitrophenylhydrazine and was also detected on a Merck GF₂₅₄ silica gel plate under a UV lamp (2536 Å). The IR spectrum of crude I is shown in Fig. 2.

Reduction of I with sodium borohydride. Crude I (20 mg) was treated with sodium borohydride (20 mg) in 2-propanol (5 ml) at room temperature for 40 min and to the reaction mixture was added water (5 ml). After standing overnight at room temperature, the reaction mixture was concentrated *in vacuo* and extracted with ethyl acetate.

The extract was applied to a silica gel column (Wako gel C-200). Crude III was eluted with benzene-ethyl acetate (70:30) and recrystallized from *n*-hexane-ethyl acetate to afford needles (14 mg). mp 189~190°C. $[\alpha]_D^{24} - 146.8^\circ$ ($c = 0.07$, EtOH). MS *m/z* (abundance): 236 (M^+ , 1.6), 221 (0.9), 218 (0.5), 205 (10.9), 179 (100), 161 (8.0), 149 (14.5). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3250 (broad), 2950, 2900, 1470, 1090, 1060, 1035, 890. $^1\text{H-NMR}$ (400 MHz, d_5 -Pyr., ppm): δ 1.06 (3H, s), 1.09 (3H, s), 1.56 (1H, m), 1.64 (1H, m), 1.71 (1H, t), 1.77 (1H, d), 1.88 (1H, d), 1.90 (1H, d), 1.95 (1H, dd), 2.00~2.25 (4H, m), 3.77 (1H, dd), 4.03 (1H, dd), 4.65 (1H, br. t), 5.04 (1H, s), 5.56 (1H, s), 5.90 (1H, br. s), 6.70 (1H, br. s). $^{13}\text{C-NMR}$ (100 MHz, d_5 -Pyr., ppm): Table I.

Reduction of II with sodium borohydride. Terrecyclic acid A (II) (50 mg) was treated with sodium borohydride (20 mg) in 2-propanol (5 ml) at room temperature for 30 min and water (5 ml) was added. After standing for 30 min, the reaction mixture was concentrated *in vacuo* and extracted with ethyl acetate at pH 3. Crude IV was obtained from the extract and was recrystallized from ethyl acetate to afford needles (40 mg). mp 187°C. MS *m/z* (abundance): 250 (M^+ , 1.2), 235 (1.2), 232 (0.4), 193 (100), 149 (9.2), 147 (9.7). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3420, 3360, 2950 (broad), 1730, 1690, 1450, 1320, 1240, 1210, 1180, 1135, 1080, 1060, 1050, 980, 880. $^1\text{H-NMR}$ (400 MHz, d_5 -Pyr., ppm): δ 1.05 (3H, s), 1.13 (3H, s), 1.71 (1H, m), 1.73 (1H, d), 1.79 (1H, br. t), 1.82 (1H, m), 1.96 (1H, d), 2.07 (1H, d), 2.08 (1H, m), 2.18 (1H, ddd), 2.33 (1H, ddd), 3.05 (1H, dd), 3.17 (1H, d), 5.18 (1H, m), 5.22 (1H, br. s), 5.64 (1H, br. s). $^{13}\text{C-NMR}$ (25 MHz, d_5 -Pyr., ppm): Table I.

Reduction of II with lithium aluminum hydride. Terrecyclic acid A (II) (1 g) was treated with lithium aluminum hydride (1 g) in ethyl ether (30 ml). The mixture was stirred at room temperature for 20 hr and to the reaction mixture was added water. The reaction mixture was filtered and the filtrate was evaporated *in vacuo*. The residue was extracted with ethyl acetate, the extract was

applied to a silica gel column (Wako gel C-200, 15 g) and the column was eluted with benzene-ethyl acetate (85:15). Crude crystals obtained were recrystallized to afford colorless needles (137 mg). mp 189.0~190.5°C. $[\alpha]_D^{24} - 134.6^\circ$ ($c=0.16$, EtOH). The IR and mass spectra were identical with those of **III**.

Oxidation of III with manganese dioxide. **III** (91 mg) was treated with activated manganese dioxide (300 mg) in acetone (25 ml) and the mixture was stirred at room temperature for 7.5 hr. The reaction mixture was filtered and the filtrate was evaporated *in vacuo*. The residue was extracted with ethyl acetate, the extract was applied to a silica gel column (Wako gel C-200, 15 g) and the column was eluted with benzene-ethyl acetate (85:15). **I** was obtained as a colorless oil (33 mg). $[\alpha]_D^{24} + 8.5^\circ$ ($c=0.3$, EtOH). MS m/z (abundance): 234 (M^+ , 100), 219 (28), 178 (28), 177 (50), 163 (56), 147 (63), 119 (38). *Rf* values on tlc plates were identical with those of natural **I**. The IR spectrum of this oil is shown in Fig. 2.

Catalytic reduction of II. **II** (100 mg) was dissolved in methanol (10 ml) and hydrogenated over 10% palladium-charcoal catalyst (20 mg) under atmospheric pressure for 10 min. The reaction mixture was filtered, and the catalyst was washed with methanol. The combined filtrates were evaporated *in vacuo* to afford crude **V** (90 mg), which was recrystallized from *n*-hexane-ethyl acetate to give pure **V** as colorless needles. mp 128~129°C. MS m/z (abundance): 250 (M^+ , 8.1), 235 (9.5), 232 (2.0), 193 (100), 163 (10.7), 121 (10.1). IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 3150 (broad), 2940, 1710 (broad), 1450, 1380, 1290, 1240, 1190, 1160, 1060, 1040, 855. $^1\text{H-NMR}$ (400 MHz, CDCl_3 , ppm): δ 1.03 (3H, d), 1.13 (3H, s), 1.16 (3H, s), 1.41 (1H, d), 1.45 (1H, d), 1.81 (1H, m), 1.85~2.00 (4H, m), 2.39 (1H, dd), 2.48 (1H, q),

2.54 (1H, dd), 2.79 (1H, d), 2.79 (1H, dd). In addition to these signals, there existed a broad singlet (1H) at δ 10.1 ppm in the 100 MHz $^1\text{H-NMR}$ spectrum (CDCl_3). $^{13}\text{C-NMR}$ (25 MHz, CDCl_3 , ppm): δ 8.57 (q), 22.17 (t), 27.17 (q), 34.19 (q), 39.55 (s), 40.45 (t), 45.02 (d), 47.68 (t), 48.03 (d), 49.32 (d), 51.60 (d), 54.93 (s), 181.24 (s), 218.83 (s).

Antimicrobial assay. The minimal inhibitory concentration of **I**, **II**, **III**, **IV** and **V** was determined by the agar dilution method using bouillon agar for bacteria and Czapek's agar for fungi and yeast. Observation was made after an 18 hr incubation for bacteria and a 36 hr incubation for fungi and yeast at 30°C.

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