A NEW SERIES OF NATURAL ANTIFUNGALS THAT INHIBIT P450 LANOSTEROL C-14 DEMETHYLASE

I. TAXONOMY, FERMENTATION, ISOLATION AND STRUCTURAL ELUCIDATION

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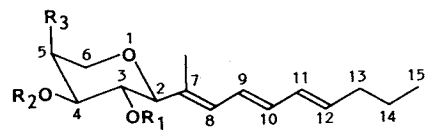
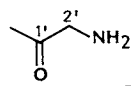
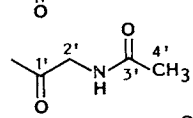
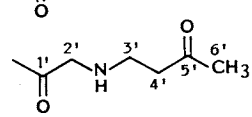
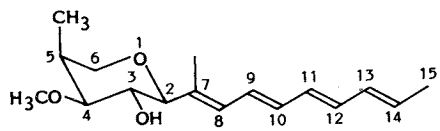
A new series of antifungal antibiotics, Ro 09-1470 and its 6 congeners were isolated from the fermentation broth of *Penicillium* sp. NR6564. Their structures were determined as tetrahydropyran derivatives with an alkenyl side chain on the basis of their spectroscopic and physico-chemical properties. Among these compounds, Ro 09-1470 and Ro 09-1545 possessing a glycyl or an *N*-substituted glycyl ester residue had high antifungal activity. Ro 09-1469, one of the congeners, was found in the fermentation broth of several strains of *Aspergillus sclerotiorum* Huber.

During our microbial screening aimed at finding new antifungals, we discovered a series of compounds that inhibited the fungal P450 lanosterol C-14 demethylase from the fermentation broth of *Penicillium* sp. NR6564. The natural antifungals showing this type of mode of action have hitherto been unknown. Among the compounds we isolated, five compounds were novel and the rest two were each identified as restricticin

Fig. 1. Structures of Ro 09-1470 and its 6 congeners.



	R_1	R_2	R_3
Ro 09-1469 (**1**)	–H	$-CH_3$	$-CH_3$
Ro 09-1470 (**2**)		$-CH_3$	$-CH_3$
Ro 09-1543 (**3**)		$-CH_3$	$-CH_3$
Ro 09-1545 (**4**)		$-CH_3$	$-CH_3$
Ro 09-1547 (**5**)	–H	–H	$-CH_3$
Ro 09-1549 (**6**)	–H	$-CH_3$	–H



Ro 09-1544 (7)

and restrictinol[1]. This paper deals with the taxonomy of the producing microorganism, fermentation, isolation and structural elucidation of the new antifungal antibiotics. We also describe the production of one of the congeners by *Aspergillus sclerotiorum* Huber in this paper. The biological activity and mode of action will be reported in the paper which follows this one[2].

Materials and Methods

General

UV and IR spectra were recorded on a Kontron Uvikon 860 UV spectrometer and on a Hitachi 270-30 infrared spectrophotometer, respectively. Mass spectra were obtained with a Jeol JMS-DX300 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Jeol JNM-GSX400 NMR spectrometer at 400 and 100 MHz, respectively, with TMS as an internal standard. Optical rotation was measured on a Jasco DIP-140 digital polarimeter.

Microorganism and Taxonomic Study

For taxonomic characterization, the producing fungus NR6564 was examined according to PITT[3]. *Aspergillus sclerotiorum* strains with an IFO accession number were purchased from the Institute for Fermentation, Osaka.

Fermentation

For production, the conidial suspension from a well-grown agar slant of *Penicillium* sp. NR6564 was inoculated into a 500-ml baffled Erlenmeyer flask containing 100 ml of a medium consisting of 2% glucose, 3% glycerol, 0.5% Polypeptone, 0.2% yeast extract, 0.3% NaCl, and 1% $CaCO_3$. The inoculated flask was shaken on a rotary shaker at 190 rpm at 27°C for 3 days. Six hunderd milliliters of the seed culture was then transferred to a 50-liter jar fermenter containing 30 liters of the same medium. Fermentation was conducted at 27°C at 300 rpm with an airflow rate of 30 liters/minute.

The potency of the active compounds during fermentation was monitored by the *in vitro* antifungal activity against *Candida albicans* 652. Packed cell volumes were determined by centrifugation of a 10 ml cultured broth in a conical tube at 3,500 rpm for 5 minutes. Glucose concentration was monitored by using a glucose analyzer (Berckman, II).

Antifungal Activity

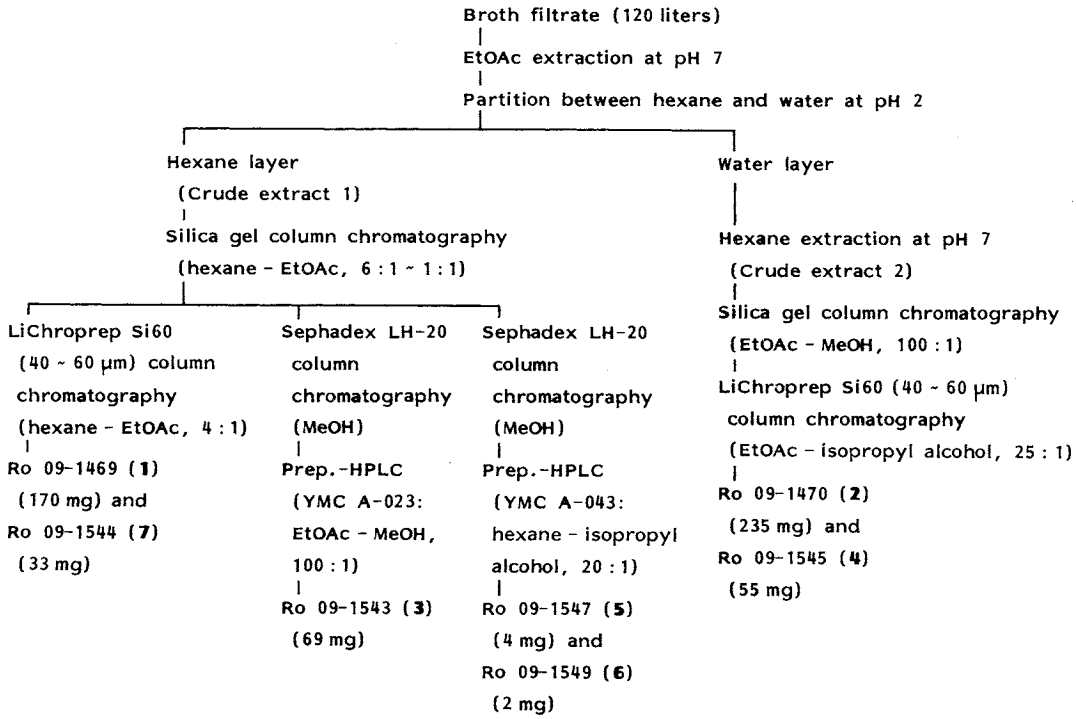
Detection of the antifungal activity was examined with protoplast of *Candida albicans* 652 in YNB broth using a 96-well microtiter plate. This strain was provided by Hoffmann-La Roche, Basle. Protoplast was prepared according to YAMAGUCHI *et al.*[4]. The *in vitro* antifungal activity (IC_{50}) was measured against *Saccharomyces cerevisiae* ATCC 9763 by the semi-solid agar dilution method with 96-well microtiter plate.

Isolation

The whole scheme for isolation is summarized in Fig. 2. Harvested broth (130 liters) was separated into mycelium and filtrate (120 liters) by filtration. The filtrate was extracted with EtOAc (100 liters) at pH 7. The organic layer was concentrated to *ca.* 100 ml under reduced pressure. The concentrate was partitioned between hexane (1,200 ml) and water (1,200 ml) at pH 2. The hexane layer was evaporated under reduced pressure to give a yellow oil (crude extract 1, 27.4 g). The water layer was adjusted to pH 7 with 1 N NaOH and extracted with hexane (900 ml × 2). The organic layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure to give a yellow oil (crude extract 2, 6.3 g).

Crude extract 1 (27.4 g) was applied to a column of silica gel (925 g; Wakogel C-200, Wako) and active principles were eluted with hexane-EtOAc (6:1) and then hexane-EtOAc (1:1). Three active fractions were obtained. The first active fraction (420 mg) was applied to a column of LiChroprep Si60 (Merck), and eluted with hexane-EtOAc (4:1) to give Ro 09-1469 (**1**) (170 mg) and Ro 09-1544 (**7**) (33 mg) as colorless oils. The second active fraction (357 mg) was applied to a column of Sephadex LH-20 (1 liter; Pharmacia Fine Chemicals) and eluted with MeOH. The active fraction was further purified by preparative

Fig. 2. Isolation procedure of **1**, **2**, **3**, **4**, **5**, **6** and **7**.



HPLC (Column: YMC-Pack A-023, silica gel, 250 × 10 mm I.D., Eluent: EtOAc - MeOH (100 : 1)) to give Ro 09-1543 (**3**) (69 mg) as a colorless oil. The third active fraction (81 mg) was also applied to a column of Sephadex LH-20 (1 liter) and eluted with MeOH. The active fraction was then purified by preparative HPLC (Column: YMC-Pack A-043, silica gel, 250 × 20 mm I.D., Eluent: hexane - isopropyl alcohol (20 : 1)) to give Ro 09-1547 (**5**) (4 mg) and Ro 09-1549 (**6**) (2 mg) as colorless oils.

Crude extract 2 (6.3 g) was applied to a column of silica gel (150 g; Wakogel C-200), and eluted with EtOAc - MeOH (100 : 1). The active fraction was then applied to a column of LiChroprep Si60 and eluted with EtOAc - isopropyl alcohol (25 : 1) to give Ro 09-1470 (**2**) (235 mg) and Ro 09-1545 (**4**) (55 mg) as colorless oils. The compounds we isolated were extremely unstable. To avoid rapid degradation, all the concentrates and purified samples were kept in an atmosphere of argon.

HPLC Analysis

The samples for HPLC analysis were prepared as follows. The method for culturing *Aspergillus sclerotiorum* IFO 5863, 7542, 8863 and 9198 was the same as that for strain NR6564. About 60 ml of the broth filtrate was extracted twice with hexane. The hexane layer was washed with water, then applied to a SEP-PAK silica cartridge (Waters), and finally eluted with EtOAc - MeOH (25 : 1). The eluate was concentrated under reduced pressure, and dissolved in 1 ml of MeOH. This MeOH solution was analyzed by HPLC. The HPLC analysis was performed on YMC-Pack A-003 (silica gel, 250 × 4.6 mm I.D.) at a flow rate of 2 ml/minute with hexane - isopropyl alcohol (20 : 1). The detection was carried out with the Waters 990J photodiode array detector. By this system, Ro 09-1469 was eluted after 3.86 minutes.

Results

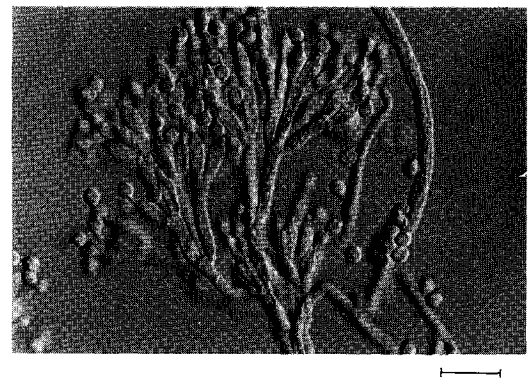
Taxonomy of the Producing Microorganisms

The fungal strain NR6564 was isolated from a soil sample collected in Hong Kong. It grew rapidly, at 25°C to reach 39 mm ~ 43 mm in diameter; growth at 37°C was weak; growth on 25% glycerol - nitrate

agar (G25N) was also poor. The conidia did not germinate at 5°C. It formed characteristic pink mycelium on Czapek yeast extract agar (CYA) and malt extract agar (MEA) and bright greenish yellow mycelium on MEA. Dark red soluble pigment was produced in CYA. The conidial area was light grayish green to blue green. Penicilli were typically biverticillate and divergent, but occasionally irregular. Phialides were ampulliform-acerose. These cultural and morphological properties (Fig. 3) suggest that the strain should be included in the subgenus *Furcatum* Pitt of the genus *Penicillium* Link: Fries. This strain also showed some affinity to the subgenus *Biverticillium* Dierckx. However, the above properties did not agree with those of any known species in the genus. The definite nomenclature of the producing organism will be reported elsewhere. In addition, we found that *Aspergillus sclerotiorum* Huber, IFO 5863, IFO 7542, IFO 8863 and IFO 9198 produced Ro 09-1469 (**1**). The broths of these strains showed high antifungal activity as well.

Fig. 3. *Penicillium* sp. NR6564.

Standard bar: 10 μm.



Fermentation

Fig. 4 shows the average time course of four fermentations. The amount of Ro 09-1470 (**2**) in the

Fig. 4. Fermentation profile of NR6564.

● Ro 09-1470 (**2**) potency, △ packed cell volume, □ residual glucose, ■ pH, ▲ dissolved oxygen (DO), ○ expired CO_2.

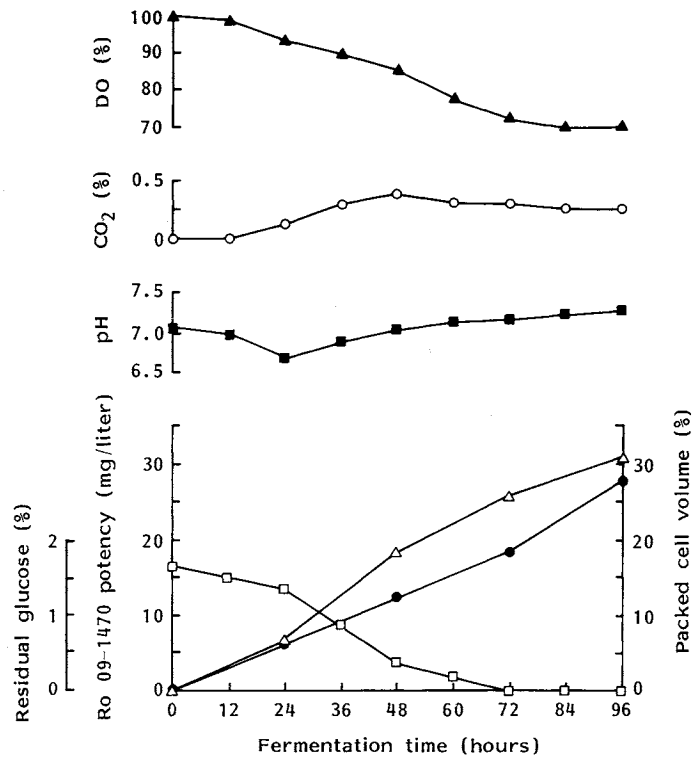


Table 1. Physico-chemical properties of **1**, **2**, **3**, **4**, **5**, **6** and **7**.

	Ro 09-1469 (**1**)	Ro 09-1470 (**2**)	Ro 09-1543 (**3**)	Ro 09-1545 (**4**)	Ro 09-1547 (**5**)	Ro 09-1549 (**6**)	Ro 09-1544 (**7**)
Appearance	Colorless oil	Colorless oil	Colorless oil	Colorless oil	Colorless oil	Colorless oil	Colorless oil
EI-MS (*m/z*)	280 $(M)^+$, 262 $(M-18)^+$, 171 $(M-109)^+$	337 $(M)^+$, 262 $(M-75)^+$	379 $(M)^+$, 279 $(M-100)^+$, 262 $(M-117)^+$	407 $(M)^+$, 337 $(M-70)^+$, 262 $(M-145)^+$	266 $(M)^+$, 248 $(M-18)^+$	266 $(M)^+$, 248 $(M-18)^+$	278 $(M)^+$, 260 $(M-18)^+$
HREI-MS (*m/z*) Found:	280.2033	337.22518	379.23646	407.26623	266.18879	266.18743	278.18749
Calcd:	280.2037	337.22513	379.23568	407.26696	266.18805	266.18805	278.18545
Molecular formula	$C_{17}H_{28}O_3$	$C_{19}H_{31}NO_4$	$C_{21}H_{33}NO_5$	$C_{23}H_{37}NO_5$	$C_{16}H_{26}O_3$	$O_{16}H_{26}O_3$	$C_{17}H_{26}O_3$
UV λ_{max}^{MeOH} nm (log ε)	264 (4.57), 274 (4.67), 285 (4.59)	264 (4.58), 274 (4.69), 285 (4.58)	265, 275, 286	264 (4.03), 274 (4.16), 286 (4.05)	266 (4.32), 275 (4.43), 286 (4.33)	266 (4.05), 275 (4.15), 286 (4.03)	260 (sh, 3.91), 291 (4.09), 304 (4.23), 319 (4.19)
IR ν_{max} (neat) cm^{-1}	3468 (OH)	3392 (NH), 1748 (ester)	3352 (NH), 1746 (ester), 1670 (amide), 1548 (amide)	3350 (NH), 1746 (ester), 1716 (ketone)	3412 (OH)	3480 (OH)	3475 (OH)
$[\alpha]_D^{20}$	+51° (*c* 0.2, MeOH)	+100° (*c* 0.2, MeOH)	+59° (*c* 0.16, $CHCl_3$)	+94° (*c* 0.2, MeOH)	+109° (*c* 0.1, MeOH)	+23° (*c* 0.1, MeOH)	+18° (*c* 0.3, MeOH)
Color reaction Ninhydrin	−	+	−	+	−	−	−

Table 2. ^{1}H NMR spectral data for **1**, **2**, **3**, **4**, **5**, **6** and **7** in $CDCl_3$.

	Ro 09-1469 (**1**)	Ro 09-1470 (**2**)	Ro 09-1543 (**3**)	Ro 09-1545 (**4**)
Position				
2	3.48 (1H, d, J=9 Hz)	3.55 (1H, d, J=10 Hz)	3.54 (1H, d, J=9 Hz)	3.55 (1H, d, J=10 Hz)
3	3.62 (1H, t, J=9 Hz)	5.04 (1H, t, J=10 Hz)	5.03 (1H, t, J=9 Hz)	5.05 (1H, t, J=10 Hz)
4	3.27 (1H, dd, J=5, 9 Hz)	3.41 (1H, dd, J=5, 10 Hz)	3.38 (1H, dd, J=5, 9 Hz)	3.38 (1H, dd, J=6, 10 Hz)
5	2.22 (1H, m)	2.27 (1H, m)	2.27 (1H, m)	2.26 (1H, m)
6a	3.61 (1H, dd, J=2.5, 12 Hz)	3.58 (1H, dd, J=2, 12 Hz)	3.58 (1H, dd, J=2, 12 Hz)	3.58 (1H, dd, J=2, 12 Hz)
6b	3.81 (1H, dd, J=2, 12 Hz)	3.82 (1H, dd, J=2, 12 Hz)	3.83 (1H, dd, J=2, 12 Hz)	3.82 (1H, dd, J=2, 12 Hz)
8	6.14 (1H, dq, J=11, 1 Hz)	5.97 (1H, br d, J=11 Hz)	5.95 (1H, br d, J=11 Hz)	5.95 (1H, dq, J=11, 1 Hz)
9	6.33 (1H, dd, J=11, 14.5 Hz)	6.25 (1H, dd, J=11, 14 Hz)	6.25 (1H, dd, J=11, 15 Hz)	6.25 (1H, dd, J=11, 15 Hz)
10	6.21 (1H, dd, J=11, 14.5 Hz)	6.12 (1H, dd, J=11, 14 Hz)	6.15 (1H, dd, J=11, 15 Hz)	6.13 (1H, dd, J=11, 15 Hz)
11	6.10 (1H, ddt, J=11, 15.1 Hz)	6.07 (1H, ddt, J=11, 14, 1 Hz)	6.06 (1H, ddt, J=11, 15, 1 Hz)	6.07 (1H, ddt, J=11, 15, 1 Hz)
12	5.71 (1H, dt, J=15, 7.5 Hz)	5.72 (1H, dt, J=14, 7 Hz)	5.73 (1H, dt, J=15, 7 Hz)	5.69 (1H, dt, J=15, 7.5 Hz)
13	2.08 (2H, dq, J=1, 7.5 Hz)	2.07 (2H, br q, J=7 Hz)	2.07 (2H, br q, J=7 Hz)	2.06 (2H, dq, J=1, 7.5 Hz)
14	1.42 (2H, sextet, J=7.5 Hz)	1.41 (2H, sextet, J=7 Hz)	1.41 (2H, sextet, J=7.5 Hz)	1.41 (2H, sextet, J=7.5 Hz)
15	0.90 (3H, t, J=7.5 Hz)	0.90 (3H, t, J=7 Hz)	0.90 (2H, t, J=7.5 Hz)	0.90 (3H, t, J=7.5 Hz)
2'a		3.50 (1H, d, J=18 Hz)	3.81 (1H, dd, J=5, 18 Hz)	3.23 (1H, d, J=18 Hz)
2'b		3.77 (1H, d, J=18 Hz)	4.12 (1H, dd, J=5, 18 Hz)	3.35 (1H, d, J=18 Hz)
3'a				2.73 (1H, dt, J=12, 6 Hz)
3'b				2.77 (1H, dt, J=12, 6 Hz)
4'			1.98 (3H, s)	2.55 (2H, t, J=6 Hz)
6'				2.13 (3H, s)
4-OCH_3	3.41 (3H, s)	3.32 (3H, s)	3.32 (3H, s)	3.32 (3H, s)
5-CH_3	1.02 (3H, d, J=7.5 Hz)	1.10 (3H, d, J=7 Hz)	1.10 (3H, d, J=7.5 Hz)	1.10 (3H, d, J=7.5 Hz)
7-CH_3	1.83 (3H, d, J=1 Hz)	1.78 (3H, d, J=1 Hz)	1.77 (3H, d, J=1 Hz)	1.78 (3H, d, J=1 Hz)
$NH_{(2)}$		3.30 (2H, br s)	5.88 (1H, br s)	6.19 (1H, br s)

	Ro 09-1547 (**5**)	Ro 09-1549 (**6**)	Ro 09-1544 (**7**)
Position			
2	3.43 (1H, d, J=9 Hz)	3.51 (1H, d, J=9 Hz)	3.49 (1H, d, J=9 Hz)
3	3.53 (1H, dt, J=1, 9 Hz)	3.43 (1H, t, J=9 Hz)	3.63 (1H, dt, J=1, 9 Hz)
4	3.64 (1H, dd, J=5, 9 Hz)	3.46 (1H, m)	3.27 (1H, dd, J=5, 9 Hz)
5a	2.11 (1H, m)	1.57 (1H, m)	2.22 (1H, m)
5b		2.06 (1H, m)	
6a	3.62 (1H, dd, J=2, 11 Hz)	3.25 (1H, ddd, J=4.5, 8, 11 Hz)	3.62 (1H, dd, J=2, 11 Hz)
6b	3.75 (1H, dd, J=2, 11 Hz)	4.01 (1H, ddd, J=2, 4.5, 11 Hz)	3.82 (1H, dd, J=2, 11 Hz)
8	6.12 (1H, dq, J=11, 1 Hz)	6.14 (1H, dq, J=11, 1 Hz)	6.18 (1H, br d, J=11 Hz)
9	6.34 (1H, dd, J=11, 15 Hz)	6.35 (1H, dd, J=11, 15 Hz)	6.43 (1H, dd, J=11, 15 Hz)
10	6.23 (1H, dd, J=11, 15 Hz)	6.22 (1H, dd, J=11, 15 Hz)	6.23 (1H, dd, J=11, 15 Hz)
11	6.11 (1H, ddt, J=11, 15, 1 Hz)	6.11 (1H, ddt, J=11, 15, 1 Hz)	6.20 (1H)
12	5.74 (1H, dt, J=15, 7.5 Hz)	5.72 (1H, dt, J=15, 7.5 Hz)	6.20 (1H)
13	2.09 (2H, m)	2.06 (1H, m)	6.10 (1H, ddq, J=11, 15, 1.5 Hz)
14	1.42 (2H, sextet, J=7.5 Hz)	1.42 (1H, sextet, J=7.5 Hz)	5.73 (1H, dq, J=15, 7.5 Hz)
15	0.91 (3H, t, J=7.5 Hz)	0.91 (3H, t, J=7.5 Hz)	1.84 (3H, dd, J=1.5, 7.5 Hz)
4-OCH_3		3.46 (3H, s)	3.40 (3H, s)
5-CH_3	1.02 (3H, d, J=7.5 Hz)		1.05 (3H, d, J=7.5 Hz)
7-CH_3	1.83 (3H, d, J=1 Hz)	1.82 (3H, d, J=1 Hz)	1.84 (3H, d, J=1 Hz)
OH	1.78 (1H, d, J=1 Hz)		2.08 (1H, d, J=1 Hz)
	2.45 (1H, br s)		

culture filtrate increased in parallel with the growth, reaching about 28 mg/liter after 96 hours.

Isolation

The culture filtrate (120 liters) of strain NR6564 was extracted with ethyl acetate at pH 7. The organic layer was concentrated under reduced pressure and then partitioned between hexane and water at pH 2. The hexane layer (crude extract 1) contained the neutral components, Ro 09-1469 (**1**), Ro 09-1543 (**3**), Ro 09-1547 (**5**), Ro 09-1549 (**6**) and Ro 09-1544 (**7**), whereas the water layer contained the basic components, Ro 09-1470 (**2**) and Ro 09-1545 (**4**). The components **1**, **2**, **4** and **7** were purified by silica gel column chromatography, followed by medium pressure silica gel chromatography using LiChroprep Si60 (Merck). The purification of components, **3**, **5** and **6** was achieved by 1) silica gel column chromatography, 2) Sephadex LH-20 column chromatography and 3) preparative HPLC.

Physico-chemical Properties

The physico-chemical properties of compounds **1**~**7** are summarized in Table 1. The molecular formulae were determined by high resolution electron impact mass spectrometry (HREI-MS). Their ^{1}H NMR spectral data are summarized in Table 2. The ^{13}C NMR spectral data for the representative compounds, **1**, **2** and **4** are summarized in Table 3. The UV spectra of compounds **1**~**6** showed characteristic absorption maxima at around 264, 274 and 285 nm assignable to a conjugated triene, whereas the UV spectrum of **7** showed maxima at 260 (sh), 291, 304 and 319 nm, suggesting the presence of a conjugated tetraene (Table 1). The IR spectra of **2**, **3** and **4** showed ester carbonyl absorption at around 1748 cm^{-1}, whereas no carbonyl absorption was observed in the IR spectra of the other components.

Table 3. ^{13}C NMR spectral data for **1**, **2** and **4** in $CDCl_3$.

Position	**1**	**2**	**4**
	δ_C (m)[a]	δ_C (m)[a]	δ_C (m)[a]
2	86.7 (d)	85.3 (d)	85.4 (d)
3	67.6 (d)	69.9 (d)	69.2 (d)
4	84.1 (d)	81.6 (d)	81.5 (d)
5	31.8 (d)	32.4 (d)	32.5 (d)
6	71.0 (t)	70.8 (t)	70.8 (t)
7	133.4 (s)	134.3 (s)	133.0 (s)
8	129.8 (d)	129.8 (d)	129.7 (d)
9	125.8 (d)	125.8 (d)	126.0 (d)
10	134.2 (d)	134.3 (d)	134.0 (d)
11	130.7 (d)	130.7 (d)	130.6 (d)
12	135.6 (d)	135.9 (d)	135.7 (d)
13	34.9 (t)	34.9 (t)	34.9 (t)
14	22.4 (t)	22.4 (t)	22.4 (t)
15	13.7 (q)	13.7 (q)	13.7 (q)
1′		171.7 (s)	171.5 (s)
2′		42.9 (t)	51.0 (t)
3′			43.9 (t)[b]
4′			44.0 (t)[b]
5′			208.0 (s)
6′			30.1 (q)
4-OCH_3	56.0 (q)	56.4 (q)	56.3 (q)
5-CH_3	10.9 (q)	10.8 (q)	10.8 (q)
7-CH_3	12.2 (q)	11.8 (q)	11.7 (q)

[a] Multiplicity.
[b] Assignment may be reversed.

Structures of Ro 09-1469 (1) and Ro 09-1470 (2)

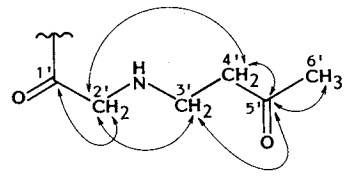
SCHWARTZ *et al.* have recently reported the isolation of a novel antifungal agent, restricticin containing conjugated triene, tetrahydropyran and glycyl ester functionalities, and its desglycyl product, restrictinol[1]. The physico-chemical and spectrocopic properties of **2** and **1** are almost identical with those of restricticin and restrictionol, respectively. Our independent structural and stereochemical analyses of **2** and **1** led to the same structures proposed by HENSENS *et al.*[5]. Thus, **1** and **2** were identified as restrictinol and restricticin, respectively.

Structure of Ro 09-1543 (3)

The IR spectrum of **3** showed absorption bands at 1746, 1670 and 1548 cm^{-1}, suggesting the presence of ester and amide functionalities. When the ^{1}H NMR spectral data of **3** was compared with those of **2** (Table 2), a singlet methyl signal at δ 1.98 and an exchangeable proton signal at δ 5.88 were observed in

the ^{1}H NMR spectrum of **3**. These facts together with the molecular formula ($C_{21}H_{33}NO_5$) elucidated by HREI-MS (Table 1) suggested that **3** was an acetyl derivative of **2**. Since AB quartet signals (at δ 3.81 and 4.12) assignable to methylene protons of the glycine residue were observed at lower field than those of **2** (at δ 3.50 and 3.77), the acetyl group was determined to be attached to the glycine moiety.

Fig. 5. CH long range couplings (⟷) observed in the HMBC spectrum of **4**.



On the basis of these findings, the structure of Ro 09-1543 (**3**) was determined to be the *N*-acetyl-derivative of **2**.

Structure of Ro 09-1545 (**4**)

The IR spectrum of **4** characteristically showed absorption bands at 1746 and 1716 cm^{-1} assignable to ester and ketone, respectively. The presence of ester and ketone functionalities was also supported by the ^{13}C NMR spectral data (Table 3) for **4** (at δ 171.5 and 208.0). In the ^{1}H NMR spectrum of **4**, a singlet methyl (at δ 2.13) and $-CH_2-CH_2-$ signals (at δ 2.73, 2.77 and 2.55) were observed when comparing the ^{1}H NMR spectral data of **4** with those of **2** (Table 2). The connectivities of these functionalities, CO, CH_3 and $-CH_2-CH_2-$ were determined by the heteronuclear multiple bond correlation (HMBC) experiment of **4**. The observed long range CH couplings are shown in Fig. 5. Based on these data, the structure of Ro 09-1545 (**4**) was determined to be the *N*-(3-oxobutyl)-derivative of **2**.

Structure of Ro 09-1547 (**5**)

In the ^{1}H NMR spectrum of **5**, no methoxy signal was observed, however the ^{1}H NMR spectral data for **5**, except for the absence of the methoxy signal were almost identical with those of **1** (Table 2). With this information and the molecular formula ($C_{16}H_{26}O_3$) established by HREI-MS (Table 1), the structure of Ro 09-1547 (**5**) was determined to be *O*-desmethyl-derivative of **1**.

Structure of Ro 09-1549 (**6**)

Ro 09-1549 (**6**) has the same molecular formula ($C_{16}H_{26}O_3$) as **5** (Table 1), suggesting that **6** is also a desmethyl derivative of **1**. In the ^{1}H NMR spectrum of **6**, 4-OCH_3 signal was observed at δ 3.46, but no 5-CH_3 signal was observed at around δ 1.0. Since proton signals at δ 1.57 (1H, m) and 2.06 (1H, m) observed in the ^{1}H NMR spectrum of **6** were attributed to methylene protons at C-5 (Table 2), the structure of Ro 09-1549 (**6**) was determined to be the 5-desmethyl-derivative of **1**.

Structure of Ro 09-1544 (**7**)

The UV spectrum of **7** showed absorption maxima at 260 (sh), 291, 304 and 319 nm, suggesting the presence of a conjugated tetraene (Table 1). In the ^{1}H NMR spectrum of **7**, two allyl methyl signals at δ 1.84 (6H) and seven olefinic proton signals at δ 5.73, 6.10, 6.18, 6.20 (2H), 6.23 and 6.43 were observed (Table 2). The other ^{1}H NMR spectral data for tetrahydropyran moiety of **7** were almost identical with those of **1**. With these data and the molecular formula ($C_{17}H_{26}O_3$) of **7** (Table 1), **7** was suggested to be the 13,14-dehydro-derivative of **1**.

The geometries of the Δ^9 and Δ^{13} double bonds were determined to be *E*, based on the coupling constants of the olefinic protons ($J_{9,10}$ = 15 Hz and $J_{13,14}$ = 15 Hz). The geometry of the Δ^{11} double bond was also elucidated to be *E*, based on the chemical shifts of the olefinic protons, 10-H (δ 6.23), 11-H

(δ 6.20), 12-H (δ 6.20) and 13-H (δ 6.10)[6]. The *E* geometry was also assigned to the Δ^7 double bond because nuclear Overhauser effect (NOE) between 7-CH_3 and 9-H was observed in the ^{1}H NMR of **7**. Thus, the structure of Ro 09-1544 was determined to be **7**.

The Detection of Ro 09-1469 (1) in the HPLC Analysis

During the antifungal screening, several strains of *Aspergillus sclerotiorum* also showed strong inhibitory activity against *Candida albicans* 652. Since it was suspected that the activity was derived from similar compounds, we analyzed the active principles in the broth filtrate of these strains. Ro 09-1469 (**1**) was accordingly detected from *Aspergillus sclerotiorum* IFO 5863, 7542, 8863 and 9198.

Antifungal Activity

The IC_{50} of Ro 09-1470, Ro 09-1543 and Ro 09-1545 were 1.5, 46 and 1.2 μg/ml respectively against *Saccharomyces cerevisiae* ATCC 9763. The rest 4 compounds showed no activity at 100 μg/ml. Detailed biological activity will be described in the paper which follows this one[2].

Discussion

SCHWARTZ *et al.* have recently reported the isolation of novel antifungal agents, restricticin, its *N,N*-dimethyl derivative and its desglycyl hydrolysis product restrictinol as an artifact from the fermentation broth of *Penicillium restrictum* Gilman MF5261[1]. We have also isolated restricticin (Ro 09-1470, **2**), restrictinol (Ro 09-1469, **1**), and novel congeners, Ro 09-1543 (**3**), Ro 09-1545 (**4**), Ro 09-1547 (**5**), Ro 09-1549 (**6**) and Ro 09-1544 (**7**) from the fermentation broth of *Penicillium* sp. NR6564. Ro 09-1469 (**1**), a hydrolysis product of Ro 09-1470, Ro 09-1543 or Ro 09-1545, was also detected in the fermentation broth of several strains of *Aspergillus sclerotiorum*. Moreover, strain NR6564 was found to be clearly different from *Penicillium restrictum* which belongs to the subgenus *Aspergilloides* Pitt having monoverticillate penicilli. This suggests that the series is found in the broth of *Aspergillus* as well as in that of *Penicillium*. The detailed taxonomic studies on NR6564 will be reported elsewhere.

Ro 09-1470 (**2**), the most active compound of the series, was proved to inhibit the fungal P450 lanosterol C-14 demethylase. Since the natural antifungals showing this mode of action have hitherto been unknown, Ro 09-1470 (**2**) is the first natural antifungal that inhibits the fungal P450 lanosterol C-14 demethylase. The detailed biological activity and the mode of action will be reported in the paper which follows this one[2].

References

1) SCHWARTZ, R. E.; C. DUFRESNE, J. E. FLOR, A. J. KEMPF, K. E. WILSON, T. LAM, J. ONISHI, J. MILLIGAN, R. A. FROMTLING, G. K. ABRUZZO, R. JENKINS, K. GLAZOMITSKY, G. BILLS, L. ZITANO, S. M. DEL VAL & M. N. OMSTEAD: Restricticin, a novel glycine-containing antifungal agent. J. Antibiotics 44: 463~471, 1991
2) AOKI, Y.; T. YAMAZAKI, M. KONDOH, Y. SUDOH, N. NAKAYAMA, Y. SEKINE, H. SHIMADA & M. ARISAWA: A new series of natural antifungals that inhibit P450 lanosterol C-14 demethylase. II. Mode of action. J. Antibiotics 45: 160~170, 1992
3) PITT, J. I.: The Genus Penicillium and its Teleomorphic States, *Eupenicillium* and *Talaromyces*. pp. 1~634, Academic Press, 1979
4) YAMAGUCHI, H.; T. HIRATANI, M. BABA & M. OSUMI: Effect of aculeacin A, a wall-active antibiotic, on synthesis of the yeast cell wall. Microbiol. Immunol. 29: 609~623, 1985
5) HENSENS, O. D.; C. F. WICHMANN, J. M. LIESCH, F. L. VANMIDDLESWORTH, K. E. WILSON & R. E. SCHWARTZ: Structure elucidation of restricticin, a novel antifungal agent from *Penicillium restrictum*. Tetrahedron 47: 3915~3924, 1991
6) ALBRIKTSEN, P. & R. K. HARRIS: NMR studies of conjugated linear trienes II. Hexatriene and 1,6-dimethylhexatriene. Acta Chem. Scand. 27: 1875~1882, 1973