27-O-Demethylrapamycin, an Immunosuppressant Compound Produced by a New Strain of *Streptomyces hygroscopicus*

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As part of a programme to produce new immunosuppressant agents we have grown a new rapamycin producing strain of *Streptomyces hygroscopicus* and have found that it also produces 27-O-demethylrapamycin (**I**) as well as 27-demethoxyrapamycin. The latter compound was originally reported as 29-demethoxyrapamycin[1] but is here renumbered following the recommendations of MCALPINE *et al.*[2]

The new strain designated NCIMB 40319 was isolated from termite hill soil in Gambia. The main biochemical differences between the new strain and the original rapamycin producing strain, NRRL 5491[3] are given in Table 1.

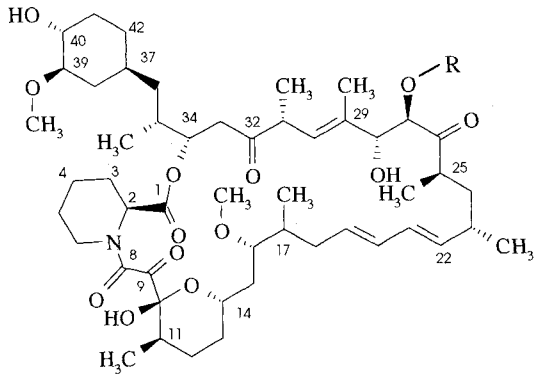
To produce **I** frozen vegetative culture (1 ml) was used to inoculate 500 ml flasks containing 100 ml seed medium consisting of soy peptone 1%, glucose monohydrate 2%, baker's yeast 0.5%, NaCl 0.2%, $ZnSO_4 \cdot 7H_2O$ 0.005%, $MgSO_4 \cdot 7H_2O$ 0.0125%, $MnSO_4 \cdot 4H_2O$ 0.001%, $FeSO_4 \cdot 7H_2O$ 0.002%, pH7. Flasks were incubated at 25°C and shaken at 240 rpm (50 mm throw).

After 72 hours, 2 litre flasks containing 400 ml of the above seed medium were each inoculated with 16 ml of the primary seed culture and incubated as above for 48 hours. Production fermentations were carried out in a 450-litre fermenter using a final stage medium which consisted of soya bean flour (Arkasoy 50) 2%, glucose 2%, baker's yeast 0.6%, L-lysine monohydrochloride 0.6%, K_2HPO_4 0.25%, KH_2PO_4 0.25%, $MgSO_4 \cdot 7H_2O$ 0.0125%, $ZnSO_4 \cdot 7H_2O$ 0.005%, $MnSO_4 \cdot 4H_2O$ 0.001%, $FeSO_4 \cdot 7H_2O$ 0.002%, NaCl 0.5%, glycerol 3%, soyabean oil 2% and NOPCO antifoam 0.05%. pH was adjusted to 6.4 after sterilisation using 50% ammonia solution. During fermentation pH was maintained above 6.0 by the further addition of ammonia solution. Fermenters were run for 120 hours at 25°C, 150 litres/minute airflow and agitator speeds of 350~400 rpm.

I was isolated from the mycelial phase by extraction with dichloromethane. The extract after concentration to an oil was extracted with methanol and **I** was purified by chromatography on silica gel eluting with a step gradient of acetone in hexane followed by high resolution chromatography on a Dynamax 8 μm C_{18} column (methanol-water, 72:28) and finally on a Microsorb 5 μm C_{18} column (methanol-water, 74:26). 200 mg **I** was obtained from 320 litres fermentation broth and had the physicochemical properties summarised in Table 2.

Identification of **I** was carried out primarily by

Fig. 1. Structures of rapamycin and 27-O-demethylrapamycin.



27-O-demethylrapamycin (**I**), R=H
Rapamycin, R=CH_3

Table 1. Comparison of NCIMB 40319 and NRRL 5491.

	NCIMB 40319	NRRL 5491
Mycelial pigments	None	Yellowish
Hydrolysis of starch (ISP4)	−	Slow
H_2S production (ISP6)	+	−
Tyrosinase production (ISP7)	+	−
L-arabinose utilisation (ISP9)	+	+/−
Xylose utilisation (ISP9)	+	+/−
Sucrose utilisation (ISP9)	+	−
Raffinose utilisation (ISP9)	+	+/−

Table 2. Physicochemical properties of 27-O-demethylrapamycin.

Appearance	White crystals
Molecular formula	$C_{50}H_{77}NO_{13}$
FAB-MS	922 ($M+Na^+$)
UV λ_{max} (MeOH) nm (ε)	269 (35,240), 278 (44,950), 291(34,791)
TLC (Rf value)*	0.22 (CH_2Cl_2-Me_2CO, 3:1)
$[\alpha]_D^{25}$ (*c* 0.5, MeOH)	−107°

* Merck TLC plate type 5635.

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Table 3. NMR data on 27-O-demethylrapamycin.

Carbon	δ_C (major)	δ_C (minor)	δ_H (major)	Carbon	δ_C (major)	δ_C (minor)	δ_H (major)
1	169.5	169.7		27	75.7	75.6	4.34
2	51.4	56.7	5.23	28	78.0	78.4	4.19
3	27.0	**	2.32, 1.72	29	135.8	136.6	
4	20.7	**	1.77, 1.47	30	125.7	126.3	5.43
5	25.2	24.5	1.69, 1.45	31	46.2	46.0	3.34
6	44.3	41.1	3.51, 3.38	32	208.2	207.9	
8	166.6	166.0		33	40.8	**	2.62, 2.59
9	194.4	195.8		34	75.6	75.7	5.12
10	98.7	98.9		35	33.7	32.7	1.86
11	34.3	**	2.08	36	38.9	38.4	1.29, 1.10
12	27.1	27.9	1.62	37	33.2	33.0	1.37
13	31.6	31.8	1.72, 1.30	38	34.4	34.2	2.05, 0.65
14	67.3	68.0	3.99	39	84.5	84.5	2.92
15	39.7	39.4	1.92, 1.73	40	74.0	74.1	3.37
16	83.8	83.8	3.63	41	31.3	31.3	1.97, 1.32
17	137.2	137.2		42	30.8	30.5	1.67, 0.96
18	128.4	128.6	6.01	43	16.1	**	0.95
19	126.9	126.5	6.37′	44	10.4	10.6	1.67
20	133.0	133.1	6.25	45	21.2	21.4	1.03
21	130.3	130.1	6.11	46	13.7	14.1	0.99
22	139.3	139.9	5.49	47	13.9	13.4	1.80
23	34.8	35.2	2.31	48	16.0	15.9	1.08
24	38.4	**	1.50, 1.25	49	15.6	**	0.89
25	40.7	40.6	2.70	50	55.9	55.8	3.12
26	215.1	215.17		52	56.8	57.0	3.40

The spectra were obtained in $CDCl_3$ solution referenced to TMS (ppm).
** Could not be unambiguously assigned.

Table 4. Immunosuppressant activity of 27-O-demethylrapamycin.

	27-DMR IC_{50}	Rapamycin IC_{50}
Inhibition of FKBP 12 PPIase activity	0.3 nM	0.5 nM
Inhibition of ConA-stimulated proliferation of murine splenic T cells	3 nM	1 nM
Inhibition of LPS-stimulated proliferation of murine splenic B cells	0.5 nM	0.1 nM

Table 5. Comparative antifungal data on 27-O-demethylrapamycin and rapamycin.

	MIC μg/ml	
	27-DMR	Rapamycin
Aspergillus fumigatus AF1	64	32
A. niger AF1	8	<0.125
Candida albicans 73/079	0.25	<0.125
C. albicans ATCC 10231	1.0	<0.125
Cryptococcus humicolus CBS 1896	<0.125	>128
Fusarium culmorum MF 39	>128	<0.125
F. oxysporum WO 920	>128	<0.125
F. semitectum CMI 160602	16	<0.125

high-field NMR spectroscopy. All NMR experiments were carried out on a 50 mg/0.5 ml solution in $CDCl_3$/TMS. All measurements were carried out on a Bruker AM 400 NMR spectrometer equipped with a $^{13}C/^{1}H$ 5 mm dual probe using standard software, at a probe temperature of 300 K.

A complete assignment of the ^{1}H and ^{13}C NMR spectra is given in Table 3. The data was obtained by the use of standard 1-D and 2-D assignment techniques. The complexity of the spectra is due to hindered rotation about the amide bond giving rise to peaks from the two rotamers. In summary **I** was identified from the following observations:

a) molecular weight is 14 less than rapamycin
b) loss of methoxyl resonance in both carbon and proton spectra
c) upfield shift of methyne carbon from ~84 ppm to ~75 ppm
d) addition of one exchangeable proton
e) positive identification of the two remaining methoxyl bearing carbons as C-16 and C-39
f) positive identification of C-28/H-28 by correlation spectroscopy by long range coupling (COLOC) to unambiguously assigned H-30
g) identification of C-27 *via* COLOC to H28
h) confirmation of H-27 coupling to an exchangeable resonance
i) confirmation of C-27 bearing a hydroxyl by use of SIMPLE[4] data.

Affinity of **I** and rapamycin for their intracellular receptor, FKBP 12, was determined by inhibition of

recombinant human FKBP 12 peptidyl prolyl isomerase as described by BOSSARD *et al.*[5].

The antiproliferative effects of **I** and rapamycin were evaluated on the response of B6D2F1 spleen cells to mitogens, using concanavalin A (ConA) for T cells and lipopolysaccharide (LPS) for B cells. Spleen cells from B6D2F1 mice were established in RPMI with 10% fetal bovine serum at 5×10^6/ml and 100 μl aliquots of this suspension (5×10^5 cells) were dispensed into 96 well round bottomed microtitre plates (Linbro Flow Laboratories). For the response to T cell mitogen, ConA at 5 μg/ml was added and for the response to LPS, 50 μg of *E. coli* LPS was added. The final volume in the wells was adjusted to 200 μl. Cell cultures were incubated for 72 hours and pulsed with 0.5 μCi ^{3}H-thymidine during the last 18 hours. The cells were harvested on an automated multiple sample harvester and cell-associated radioactivity counted in a Beckman liquid scintillation counter. The results obtained are summarised in Table 4.

I also had weak anti-fungal activity but was generally less active than rapamycin as indicated in Table 5. Minimum inhibitory concentrations were determined by incorporating the compound in Sabouraud Dextrose Agar (Oxoid). Inoculation was with a heavy suspension of 5~7 day fungal culture applied by a Multipoint Inoculator (Denley Tec Limited, U.K.). The plates were incubated at 32.5°C for seven days and observed daily for growth. The MIC was the lowest concentration recorded where the organism did not grow after growth on similarly inoculated controls had been observed.

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