Supplementary Material (ESI) for Organic and Biomolecular Chemistry

Rapamycin biosynthesis: elucidation of gene product function

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Isolation of strains and plasmid construction

Isolation of individual genes and gene cassettes

To create the expression vector pSGset1, first a primer dimer containing sites for the restriction enzymes *Spe*I, *Nde*I, and *Xba*I was ligated into *Pvu*II digested pSET152.[1] The P_{actI} promoter was then inserted from the vector pSG142[2] using *Nde*I and *Spe*I. This was itself digested with *XbaI* and the *Xba*I to *NheI* fragment containing the *actII*-ORF4 regulator region from pEX*oleG2*cas[3] was inserted. The vector pSGset1 was isolated, containing the *actII*-ORF4 regulator, the P_{actI} promoter and the 6xHis-tag coding sequence as well as the lambda t_0 transcriptional termination region (originating from plasmid pQE-16) which can integrate site-specifically at the ΦC31 attachment site.

Each of the genes *rapK, rapI, rapJ, rapM, rapQ, rapL* and the gene operon *rapNO* were isolated by PCR, using primers which introduced an *NdeI* site at the 5' end and an *XbaI* site at the 3' end of the construct (see Table below). After isolation and ligation into pUC18, all PCR products were confirmed by sequence analysis.

Primers

RapK	BIOSG8 5'-GGGCATATGAGGCAATTGACTCCGCCGGTCACGGCACCGTACTGCC -3' BIOSG9 5'-GGGGTCTAGAGGTCACGCCACCACACCCTCGATCTCGACC -3'
RapI	BIOSG12 5'-GGGCATATGAGCGCGTCCGTGCAGACCATCAAGCTGCC -3' BIOSG13 5'-GGGGTCTAGAGGTCAGGCGTCCCCGCGGCGGGCGACGACCT -3'
RapJ	BIOSG10 5'-GGGCATATGAGCACCGAAGCTCAGCAAGAGAGCACGCCCACCGCACGCT-3' BIOSG11 5'-GGGGTCTAGAGGTCACTCCGCTCCCCAGGTGACCCGGAGCTCGGC -3'
RapL	BIOSG6 5'-GGGCATATGCAGACCAAGGTTCTGTGCCAGCGTGACATCAAG -3' BIOSG7 5'-GGGGTCTAGAGGTCACTACAGCGAGTACGGATCGAGGACGTCCTCGGGCG -3'
RapM	BIOSG4 5'-GGGCATATGATCCAACCCGACGTCGTGACCGCCTTCACAGCGG -3' BIOSG5 5'-GGGGTCTAGAGGTCACACGCGGACGGCGATCTGGTGCCGATAGG -3'
RapN/O	BIOSG2 5'-GGGCATATGTCGACGACCGATCAGGGTGAGACCGGAAAGGCCTG -3' BIOSG3 5'-GGGGTCTAGAGGTCAGTCCTGGGGTTCGAGAAGCTCGCCGGTCTCCTT-3'
RapQ	AHL21 5'- CATATGTTGGAATTGGGTACCCGCCTG -3' AHL22 5'- TCTAGACGCTCACGCCTCCAGGGTG -3'

The first gene *rapK* (or gene *rapJ* in the case of the RapJNOQL expression plasmid) was inserted into the expression vector pSGSet1 using *NdeI* and *XbaI* sites. Additional genes were then inserted sequentially into this base plasmid by first passaging them through the plasmid pSGlit1, which added a methylation sensitive *XbaI* site to the 5' end of the gene, then removing the gene using *XbaI* and inserting this into the methylation resistant *XbaI* site at the end of the relevant gene cassette in pSGSet1.[2, 4]

Conjugation of S. hygroscopicus

The plasmid to be conjugated into *S. hygroscopicus* was transformed by electroporation into the *dam*$^-$ *dcm*$^-$ ET12567 *E. coli* strain containing either pUB307[5] or pUZ8002.[6] An overnight preculture was used to inoculate fresh 2xTY (with 50 μg/mL apramycin and 25 μg/mL kanamycin) at a dilution of 1/25 and grown with shaking at 37°C to an optical density at 595 nm of 0.25-0.6. The cells from this broth were washed twice with 2xTY and then resuspended with 0.5 ml of 2xTY per 25 mL original culture. To isolate spore suspensions of *S. hygroscopicus*, pre-dried plates of medium 1 agar were spread with *S. hygroscopicus* spores or mycelia using standard microbiological techniques followed by incubation at 26°-28ºC for 14- 21 days. Spores were harvested by addition of 1-2 mL of sterile 20 % w/v glycerol or water by standard techniques. An aliquot of 200 μL of the *S. hygroscopicus* spore suspension was washed in 500 μL of 2xTY, resuspended in 500 μL of 2xTY, subjected to heat shock at 50°C for 10 minutes then cooled on ice. An aliquot of 0.5 ml of the *E. coli* suspension was mixed with the heat-shocked spores and this mixture plated on medium 1 agar plates. These plates were incubated at 26°-28ºC for 16 hours before overlaying with 1 mg of nalidixic acid and 1 mg of apramycin per plate. Ex-conjugant colonies usually appeared after 3-7 days.

Strain	Complementation	Product
BIOT-3206	*rap*KIJNOMQL	**1**
BIOT-1839	*rap*KL	**2**
BIOT-2034	*rap*KIL	**3**
BIOT-2209	*rap*KML	**4**
BIOT-2210	*rap*KIML	**5**
BIOT-2212	*rap*KINOL	**6**
BIOT-1815	*rap*KMNOL	**7**
BIOT-3373	*rap*JNOQL	**8**
BIOT-1811	*rap*KJL	**9**

Table 1. Complementation of *S. hygroscopicus* MG2-10 deletion strain

Fermentation and isolation of compounds

General culture methods

Streptomyces lividans TK24 and its derivatives were maintained on MS plates[7] and cultivated in TSBGM (Tryptic Soy Broth with 1.0 % glucose and 100 mM MES, pH 6.0), supplemented with 100 μg/mL apramycin when required, at 26°C in side-baffled Erlenmeyer flasks with shaking at 300 rpm. *Streptomyces hygroscopicus* ATCC29253 and its derivatives were maintained on Medium 1 agar plates at 28°C and cultivated in TSB GM as above. Vegetative cultures of *S. hygroscopicus* ATCC29253 and its derivatives were also prepared in 50 mL tubes containing 10 ml Medium 2 at 28°C, 300 rpm, 48 h and inoculated at 10% v/v into 10 mL Medium 3 for production at 26°C, 300 rpm. Where BIOT-3373, the *rapJNOQL* complemented strain, was grown, cyclohexane carboxylic acid was fed to 1 mM at 24 and 48 hours after inoculation.

Medium 1

component	**Per L**
Corn steep powder	2.5 g
Yeast extract	3 g
Calcium carbonate	3 g
Iron sulphate	0.3 g
BACTO agar	20 g
Wheat starch	10 g
Water to	1 L

Medium 2 (vegetative medium)

Component	**Per L**
Soy bean flour (Nutrisoy)	5 g
Dextrin W/40	35 g
Corn Steep Solids	4 g
Glucose	10 g
$(NH_4)_2SO_4$	2 g

Lactic acid (80%)	1.6 mL
$CaCO_3$	7 g
Adjust pH to 7.5 with 1M NaOH.	

Medium 3: (production medium)

Component	**Per L**
Soy flour (Nutrisoy)	31.25 g
Dextrin W/40	18.75 g
KH_2PO_4	5 g
$(NH_4)_2SO_4$	1.25 g
$MnCl_2.4H_2O$	10 mg
$MgSO_4.7H_2O$	2.5 mg
$FeSO_4.7H_2O$	120 mg
$ZnSO_4.7H_2O$	50 mg
SAG 417	0.1 mL
pH to 6.4 with NaOH	
L-lysine	0.625 g
Glucose (40 % w/v)	50 mL

Chemical analysis of cultures

Fermentation broth was mixed with an equal volume of methanol for 20 min and then clarified by centrifugation. The resulting supernatant was taken and examined by LCMS/MS using the method described below (chemical methods and spectroscopic analysis).

Fermentation

Streptomyces hygroscopicus strains were cultured from frozen spore stocks in cryopreservative (20% glycerol, 10% lactose w/v in distilled water) on Medium 1 and spores were harvested after 10-20 days growth at 29°C. Alternatively, spores from frozen working stocks were inoculated directly into pre-culture Medium 2. A primary pre-

culture was inoculated with the harvested spores and cultured in 250 mL Erlenmeyer flasks containing 50 mL Medium 2, shaken at 250 rpm with a two-inch throw, at 28ºC, for two days. The primary pre-culture was used to inoculate secondary pre-cultures of Medium 2, at 10% v/v, which was shaken at 300 rpm with a one-inch throw, at 28ºC, for a further 24 h. Secondary pre-cultures were used to inoculate, at 10% v/v, production Medium 3 containing 0.01% v/v SAG 417 antifoam and allowed to ferment in a stirred bioreactor for five to seven days at 26ºC. Airflow was set to 0.75 volume of air per volume in vessel per minute (vvm), over pressure at 0.5 bar and the impeller tip speed was controlled between 0.98 ms^{-1} and 2.67 ms^{-1}. Additional SAG 417 was added on demand. pH was controlled at 6 – 7 with ammonia solution (10% v/v) or sulphuric acid (1 M).

Heterologous expression of rap genes and biotransformation

Cultures of *Streptomyces lividans* TK24 (control) and *S. lividans* TK24 transformed with the appropriate cassettes were grown in TSBGM with shaking at 30ºC and fed with 20 μg/mL of the appropriate rapamycin analogue. Equivalent unfed controls were run in parallel. After 5 days of further incubation the cultures were extracted with ethyl acetate and brought to dryness. Reconstitution in methanol and analysis by LCMS/MS allowed identification of all rapamycin related compounds.

Extraction and purification protocol

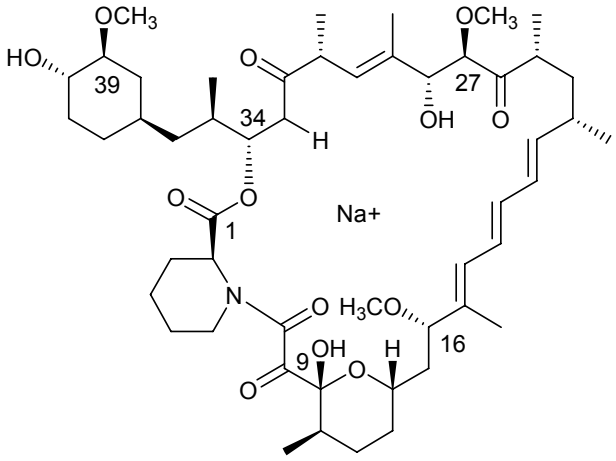
The fermentation broth was clarified by centrifugation to provide supernatant and cells. The supernatant was applied to a column (16 × 15 cm) of Diaion® HP20 resin (Supelco), washed with water followed by 75% MeOH/H_2O and then eluted with MeOH. The cells were mixed to homogeneity with an equal volume of acetone. After at least 30 minutes the acetone slurry was clarified by centrifugation and the supernatant decanted. The pelleted cells were similarly extracted twice more with acetone. The acetone extract was combined with the eluted material from the HP20 column and the solvent was removed *in vacuo* to give an aqueous concentrate. The aqueous (typically 1–2 L) was extracted with ethyl acetate (3 × equal volume) and the solvent removed *in vacuo* to give an oily crude extract (typically 20 g). The oily residue was dissolved in a minimal volume

of ethyl acetate and dried onto silica. The coated silica was applied to a silica column (400 g, ~36 × 6 cm) that was eluted sequentially with acetone/hexane mixtures ranging from 25% acetone to 100% acetone. The fractions containing rapamycin analogues were identified by HPLC using conditions described below; rapamycin and its analogues display a characteristic triene chromophore with a λ_{max} = 277 nm.

The rapamycin analogue-containing fractions were combined and the solvent was removed *in vacuo*. The residue was further chromatographed over Sephadex LH20, eluting with 10:10:1 chloroform/heptane/ethanol. The semi-purified analogues were finally purified by HPLC using a Gilson 306 HPLC system to elute a Phenomenex Luna reversed-phase column (C18 BDS, 21.2 × 250 mm, 5 μm) at 21 mL/min; isocratic mobile phases with mixtures of CH_3CN/H_2O between 50% to 70% were used depending upon the polarity of the rapamycin analogue.

Chemical methods, spectroscopic analysis and structural determination

Generic structure of rapamycin



General methods

LCMS analysis was performed on an Agilent HP1100 HPLC system in combination with a Bruker Daltonics Esquire 3000+ ion trap mass spectrometer fitted with an electrospray source. The MS was operated in both positive and negative ion modes (continuous switching) and over a mass range of 100-1000 amu; UV analysis was performed at multiple wavelengths on an Agilent DAD detector recording between 190-450 nm. Chromatography was achieved over reversed-phase silica (Hypersil C18-BDS,

150 x 4.6 mm column, 3 μ particle size) eluted at 1 mL/min using the following gradient: T=0 min, 55% B; T=15, 100% B. Mobile phase A: 10% acetonitrile:90% water, containing 10 mM ammonium acetate and 0.1% v/v formic acid; Mobile phase B: 90% acetonitrile:10% water, containing 10 mM ammonium acetate and 0.1% v/v formic acid. The content of rapamycins was calculated by comparison to a standard calibration curve.

NMR spectra were recorded on a Bruker Advance 500 spectrometer at 298 K operating at 500 MHz and 125 MHz for ^{1}H and ^{13}C respectively. Standard Bruker pulse programs were used to acquire the ^{1}H-^{1}H COSY, APT, HMQC and HMBC spectra; coupling constants are given in hertz. NMR experiments were run in $CDCl_3$ and were referenced to the residual proton resonating at δ_H 7.26 and carbon at δ_C 77.0.

Assessment of compound purity

Purity was assessed by HPLC examining both the MS total ion current trace, and multiple UV wavelengths. This assessment is complicated by the presence of multiple forms of compounds (typically 2-4 forms in varying relative amounts). These forms have the same MS/MS characteristics, and when purified from one another slowly revert to a mixture; purity was therefore calculated as a summation of the peak areas for the different forms versus overall components. All compounds were >95% purity by this method and by inspection of the ^{1}H and ^{13}C NMR spectrum.

9-Deoxo-16-O-desmethyl-27-desmethoxy rapamycin, ***3***

S. hygroscopicus MG2-10[KIL] was fermented, extracted and isolated as outlined above. The isocratic solvent system used for preparative HPLC was 60% CH_3CN/H_2O.

Isolated yield: 22 mg

UV (DAD): λ_{max} = 267, 277, 297 nm (typical rapamycin triene chromophore)

MS (ESI): *m/z*, 878.5 $[M+Na]^+$, 854.4 $[M-H]^-$

^{1}H & ^{13}C NMR data in $CDCl_3$; this compound exists as a 1:1 mixture of conformers in $CDCl_3$. The data above is for both conformers. Where a dotted line has been drawn across the table it was not possible to determine connectivity between spin systems, hence the assignment of data to a particular conformer is not possible.

Proton	**δ_H**		**multiplicity**	**coupling**		**δ_C**	
1						169.0	171.5
2	4.37	5.40				55.6	52.5
3a	1.51	1.75[a]				26.5	26.3
3b	2.40	2.19					
4a							20.9
4b							
5a	1.30	1.48					25.1
5b	1.68	1.72					
6a	4.45	3.26				39.0	44.4
6b	2.16	3.83					
8						171.7	172.4
9a	2.41	2.54				38.7	40.2
9b	2.67	2.89					
10						98.4	99.7
10-OH	6.62	5.34	br. s				
11	1.37	1.51				38.7	38.7
12a	1.67	1.62				27.3	27.6
12b	1.48	1.48					
13a	1.29	1.32					
13b							
14	4.21	3.87				71.3	69.6
15a	1.47[b]	1.50					
15b	1.66	1.65					
16	4.21	4.06		dd		76.0	75.6
17						141.6	138.4
18	6.08	6.22	d	d	11.2	122.5	125.0
19	6.38	6.31	dd	dd	14.0, 11.2	128.6	127.7
20	6.01	6.17		dd		131.1	132.2

Proton		δ_H		multiplicity		coupling		δ_C	
							10.5		
21		6.04	6.04					130.3	130.3
22		5.18	5.30	dd	dd	14.1, 9.1	14.9, 9.3	139.4	139.1
23		2.11	2.15					39.5	37.3
24a		1.34	1.35					40.3	40.3
24b		1.68	1.67						
25		2.43	2.44					45.5	46.3
26								215.2	216.1
27a		2.53	2.60					46.7	47.9
27b		2.65	2.43						
28		4.33	4.39	dd		7.9, 3.2		71.7	71.9
29								139.6	139.6
30		5.36	5.45		d		9.9	123.7	125.4
31		3.24	3.37					46.4	45.6
32								209.0	209.1
33a		2.63	2.63					39.4	39.4
33b		2.95	2.95						
34		5.13	5.38					76.0	74.2
35		1.93	1.98[b]					32.7	32.7
36a		1.04	1.03					37.8	39.8
36b		1.17	1.16						
37		1.34	1.38					33.2	33.2
38a	ax.	0.61	0.73	ddd	ddd	11.9, 11.9, 11.9	11.9, 11.9, 11.9	33.9	34.5
38b	eq.	2.04	2.09						
39		2.90	2.91					84.5	84.4
40		3.37	3.37					73.8	73.8

Proton	**δ_H**		**multiplicity**		**coupling**		**δ_C**	
41a	1.31	1.31					31.2	31.2
41b	1.97	1.97						
42a	0.97	0.97					31.7	31.7
42b								
11-CH_3	0.93	0.93	d	d	6.5	6.5	16.8 [c]	16.9 [c]
17-CH_3	1.78	1.63	s	s			15.6	12.7
23-CH_3	0.98	1.00					21.7	21.7
25-CH_3	1.00	1.02					16.7	19.1
29-CH_3	1.58	1.48	s	s			13.1	11.7
31-CH_3	1.07	1.00	d		6.9		16.2	14.6
35-CH_3	0.89	0.89	d	d	6.8	6.8	14.6[d]	15.2[d]
39-*O*-CH_3	3.37	3.37	s	s			56.5	56.5

a: may be assigned instead to H4a

b: tentative assignment

c: the assignment may be interchanged

d: the assignment may be interchanged

9-Deoxo-27-desmethoxy-39-O-desmethyl rapamycin, ***4***

S. hygroscopicus MG2-10[KML] was fermented, extracted and isolated as outlined above. The isocratic solvent system used for preparative HPLC was 60% CH_3CN/H_2O.

Isolated yield: 35 mg

UV (DAD): λ_{max} = 267, 277, 297 nm (typical rapamycin triene chromophore)

MS (ESI): *m/z*, 878.5 $[M+Na]^+$, 854.7 $[M-H]^-$

^1H & ^{13}C NMR data in $CDCl_3$:

Position	**δ_H**	**multiplicity**	**coupling**	**δ_C**
1	-			170.9
2	5.38	m		51.9

Position	**δ_H**	**multiplicity**	**coupling**	**δ_C**
3a	1.73	m		26.8
3b	2.21	m		
4a	1.39	m		20.5
4b	1.74	m		
5a	1.54	m		25.1
5b	1.78	m		
6a	3.35	m		43.5
6b	3.86	br.d	12.7	
8	-			173.3
9a	2.44	d	14.5	38.8
9b	2.44	d	14.5	
10	-			98.0
10-OH	6.07	br.s		
11	1.45	m		39.1
12a	1.47	m		27.6
12b	1.59	m		
13a	1.31	m		32.1
13b	1.47	m		
14	3.64	dd	11.1, 11.1	84.5
15a	1.57	m		38.7
15b	1.65	m		
16	3.72	dd	10.5, 4.7	84.5
17	-			134.8
18	5.99	d	9.6	130.3
19	6.38	dd	14.9, 10.2	126.9
20	6.32	dd	14.9, 9.9	133.2
21	6.12	dd	15.1, 9.5	130.5
22	5.48	dd	15.1, 8.5	139.3
23	2.23	m		35.7

Position		**δ_H**	**multiplicity**	**coupling**	**δ_C**
24a		1.30	m		40.2
24b		1.50	m		
25		2.58	m		44.8
26		-			215.1
27a		2.65	dd	16.6, 8.0	46.2
27b		2.70	dd	16.7, 4.2	
28		4.37	dd	7.8, 4.0	73.1
29		-			139.6
30		5.35	d	9.9	124.5
31		3.40	dq	9.7, 6.8	46.2
32		-			208.8
33a		2.57	dd	16.2, 5.1	41.5
33b		2.62	dd	16.2, 6.4	
34		5.06	m		75.6
35		1.95	m		33.5
36a		1.10	m		38.5
36b		1.25	m		
37		1.48	m		33.2
38a	ax.	0.90	m		38.4
38b	eq.	1.98	m		
39		3.33	m		75.2
40		3.37	m		75.8
41a		1.31	m		32.0
41b		1.96	m		
42a		1.03	m		31.9
42b		1.70	m		
11-CH_3		0.90	d	6.4	16.9
17-CH_3		1.61	s		9.9
23-CH_3		1.03	d	6.8	21.7

Position	**δ_H**	**multiplicity**	**coupling**	**δ_C**
25-CH_3	1.05	d	6.9	15.7
29-CH_3	1.69	s		12.2
31-CH_3	1.13	d	6.9	16.4
35-CH_3	0.92	d	6.7	15.6
16-*O*-CH_3	3.12	s		55.6

9-Deoxo-27-desmethoxy rapamycin, ***5***

S. hygroscopicus MG2-10[KIML] was fermented, extracted and isolated as outlined above. The isocratic solvent system used for preparative HPLC was 75% CH_3CN/H_2O.

Isolated yield: 24 mg

UV (DAD): λ_{max} = 267, 277, 297 nm (typical rapamycin triene chromophore)

MS (ESI): *m/z*, 892.5 $[M+Na]^+$, 868.6 $[M-H]^-$

^1H and ^{13}C NMR data in $CDCl_3$:

Position	**δ_H**	**multiplicity**	**coupling**	**δ_C**
1				171.0
2	5.37	m		52.0
3a	1.73	m		26.8
3b	2.22	m		
4a	1.39	m		20.5
4b	1.73	m		
5a	1.56	m		25.1
5b	1.77	m		
6a	3.34	m		43.5
6b	3.85	br. d	12.9	
8				173.4
9a	2.43	d	14.4	38.8
9b	2.74	d	14.4	
10				98.0

Position	**δ_H**	**multiplicity**	**coupling**	**δ_C**
10-OH	6.02	s		
11	1.43	m		39.1
12a	1.44	m		27.5
12b	1.58	m		
13a	1.28	m		32.2
13b	1.45	m		
14	3.61	m		65.8
15a	1.55	m		38.6
15b	1.64	m		
16	3.70	dd	10.8, 4.7	84.5
17				134.8
18	5.98	d	9.2	130.8
19	6.34	m		126.9
20	6.32	m		133.1
21	6.11	dd	15.3, 9.0	130.6
22	5.46	dd	15.2, 8.6	139.3
23	2.22	m		35.7
24a	1.28	m		40.2
24b	1.49	m		
25	2.58	m		44.8
26				215.0
27a	2.65	m		46.2
27b	2.65	m		
28	4.37	m		73.1
29				139.8
30	5.32	d	9.9	124.5
31	3.38	m		46.3
32				208.9
33a	2.59	m		41.4

Position		δ_H	multiplicity	coupling	δ_C
33b		2.59	m		
34		5.04	ddd	5.2, 5.2, 5.2	75.7
35		1.97	m		33.4
36a		1.11	m		38.6
36b		1.26	m		
37		1.41	m		33.1
38a	ax.	0.69	ddd	12.3, 12.3, 12.3	34.1
38b	eq.	2.11	m		
39		2.93	m		84.4
40		3.37	m		73.9
41a		1.32	m		31.2
41b		1.97	m		
42a		1.00	m		31.6
42b		1.68	m		
11-CH_3		0.88	d	6.4	16.9
17-CH_3		1.59	s		9.9
23-CH_3		1.02	d	7.2	20.5
25-CH_3		1.03	d	7.1	15.7
29-CH_3		1.67	s		12.2
31-CH_3		1.12	d	6.8	16.3
35-CH_3		0.92	d	6.8	15.8
16-*O*-CH_3		3.10	s		55.6
39-*O*-CH_3		3.39	s		56.5

9-Deoxo-16,27-bis-O-desmethyl rapamycin, ***6***

S. hygroscopicus MG2-10[KINOL] was fermented, extracted and isolated as outlined above. The isocratic solvent system used for preparative HPLC was 60% CH_3CN/H_2O.

Isolated yield: 77 mg

UV (DAD): λ_{max} = 267, 277, 297 nm (typical rapamycin triene chromophore)

MS (ESI): *m/z*, 894.5 $[M+Na]^+$, 870.5 $[M-H]^-$

^1H and ^{13}C NMR data in $CDCl_3$:

Position	**δ_H**	**multiplicity**	**Coupling**	**δ_C**
1				172.1
2	5.55	m		52.8
3a	1.74	m		26.0
3b	2.21	m		
4a	1.18	m		21.1
4b	1.73	m		
5a	1.44	m		25.2
5b	1.73	m		
6a	3.28	m		45.7
6b	3.87	m		
8				171.6
9a	2.41	d	12.5	42.3
9b	3.34	d	12.5	
10				99.2
10-OH	4.15	m		
11	1.61	m		38.3
12a	1.50	m		27.9
12b	1.61	m		
13a	1.36	m		31.5
13b	1.52	m		
14	3.99	m		72.5
15a	1.45	m		40.9
15b	1.70	m		
16	3.86	m		75.3
17				140.0
18	6.44	d	11.4	121.9
19	6.33	dd	14.4, 11.4	128.6

Position	δ_H	multiplicity	Coupling	δ_C
20	6.20	dd	14.8, 10.6	131.2
21	6.02	dd	14.9, 10.6	131.2
22	5.25	m		137.4
23	2.26	m		35.3
24a	1.21	m		41.1
24b	1.21	m		
25	2.37	m		40.9
26				212.8
27	4.55	d	2.3	74.9
28	4.20			77.3
29				135.8
30	5.25	m		124.9
31	3.29	m		44.9
32				208.0
33a	2.53	dd	18.2, 4.0	42.2
33b	2.81	dd	18.2, 10.6	
34	5.28	ddd	5.5, 4.0, 4.0	75.8
35	1.71	m		31.2
36a	0.92	m		36.9
36b	1.04	m		
37	1.23	m		32.6
38a ax.	0.28	ddd	11.9, 11.9, 11.9	34.2
38b eq.	1.88	m		
39	2.85			84.8
40	3.29	m		74.1
41a	1.26	m		31.3
41b	1.92	m		
42a	0.88	m		32.3

Position	**δ_H**	**multiplicity**	**Coupling**	**δ_C**
42b	1.57	m		
11-CH_3	0.98	d	6.2	16.6
17-CH_3	1.59	s		14.6
23-CH_3	1.01	d	6.4	21.4
25-CH_3	0.89	d	6.4	12.0
29-CH_3	1.90	s		15.7
31-CH_3	0.92	d	6.4	15.6
35-CH_3	0.84	d	6.8	17.6
39-*O*-CH_3	3.37	s		57.5

9-Deoxo-27,39-bis-O-desmethyl rapamycin, 7

S. hygroscopicus MG2-10[KMNOL] was fermented, extracted and isolated as outlined above. The isocratic solvent system used for preparative HPLC was 60% CH_3CN/H_2O.

Isolated yield: 6 mg

UV (DAD): λ_{max} = 267, 277, 297 nm (typical rapamycin triene chromophore)

MS (ESI): *m/z*, 894.5 $[M+Na]^+$, 870.6 $[M-H]^-$

^1H and ^{13}C NMR data in $CDCl_3$:

Position	**δ_H**	**multiplicity**	**Coupling**	**δ_C**
1				170.2
2	5.49	m		51.6
3a	1.78	m		26.9
3b	2.29	m		
4a	1.34	m		20.6
4b	1.76	m		
5a	1.55	m		25.3
5b	1.79	m		
6a	3.38	m		43.8
6b	3.92	br.d	12.4	

Position	**δ_H**	**multiplicity**	**Coupling**	**δ_C**
8				173.1
9a	2.46	d	14.2	39.3
9b	2.80	d	14.0	
10				98.0
10-OH	5.55	s		
11	1.46	m		39.2
12a	1.46	m		27.6
12b	1.58	m		
13a	1.30	m		32.0
13b	1.47	m		
14	3.61	dd	11.2, 11.2	66.0
15a	1.54	m		38.6
15b	1.67	m		
16	3.75	dd	10.5, 4.7	84.4
17				134.8
18	5.93	d	9.2	130.0
19	6.37	dd	14.8, 9.2	126.6
20	6.35	dd	14.8, 9.0	133.5
21	6.12	dd	15.2, 9.0	129.7
22	5.70	dd	15.2, 7.4	139.0
23	2.31	m		33.4
24a	1.27	m		41.1
24b	1.38	m		
25	2.92	m		40.5
26				215.9
27	4.27	d	4.7	76.4
28	4.17	br.d	4.2	77.8
29				136.5
30	5.49	m		125.0

Position		δ_H	multiplicity	Coupling	δ_C
31		3.49	dq	10.0, 6.8	46.5
32					209.2
33a		2.49	dd	15.2, 3.3	41.5
33b		2.72	dd	15.0, 8.6	
34		5.09	m		76.0
35		1.93	m		33.7
36a		1.08	m		38.9
36b		1.24	m		
37		1.46	m		33.2
38a	ax.	0.87	m		38.5
38b	eq.	1.98	m		
39		3.35	m		75.0
40		3.34	m		75.7
41a		1.32	m		32.0
41b		1.95	m		
42a		1.00	m		31.9
42b		1.69	m		
11-CH_3		0.89	d	6.3	16.9
17-CH_3		1.61	s		9.9
23-CH_3		1.03	d	6.5	20.7
25-CH_3		1.02	d	6.5	14.2
29-CH_3		1.78	s		13.9
31-CH_3		1.15	d	6.8	16.2
35-CH_3		0.90	d	6.7	15.4
16-*O*-CH_3		3.14	s		55.7

16,39-bis-O-desmethyl-27-desmethoxy rapamycin, ***9***

S. hygroscopicus MG2-10[KJL] was fermented, extracted and isolated as outlined above. The isocratic solvent system used for preparative HPLC was 55% CH_3CN/H_2O.

Isolated yield: 176 mg (mixture of inter-converting isomers)

UV (DAD): λ_{max} = 267, 277, 297 nm (typical rapamycin triene chromophore)

MS (ESI): *m/z*, 878.6 $[M+Na]^+$, 854.6 $[M-H]^-$

^1H and ^{13}C NMR data in $CDCl_3$:

Due to the presence of inter-converting forms of this molecule we were unable to definitively assign the entire NMR spectrum of this compound. Fortunately the MS/MS fragmentation of this compound clearly identified the location of oxidation to the C9 region, and in addition, we were able to identify the oxidation at C9 through a limited number of observations and 2D NMR connectivities. Briefly, the pro-chiral methylene signals around 2.4-2.8 ppm for the C-9 protons of pre-rapamycin (**2**) were clearly missing from the proton NMR as expected; similarly, the signal at around 40 ppm in the ^{13}C NMR corresponding to a C-9-methylene was missing. A new carbon signal resonating at 193.7 ppm was observed in the ^{13}C NMR. This showed a clear two bond HMBC correlation to a proton resonating at 2.09 ppm which we identified as the H-11 proton through its chemical shift and connection to the C-11 methyl group.

16-O-desmethyl-39-desmethoxy rapamycin, ***8***

S. hygroscopicus MG2-10[JNOQL] was fermented with the addition of cyclohexanecarboxylic acid, extracted and isolated as outlined above. The isocratic solvent system used for preparative HPLC was 60% CH_3CN/H_2O.

Isolated yield: 55 mg

UV (DAD): λ_{max} = 267, 277, 297 nm (typical rapamycin triene chromophore)

MS (ESI): *m/z*, 892.5 $[M+Na]^+$, 868.7 $[M-H]^-$

^1H and ^{13}C NMR data in $CDCl_3$:

Position	**δ_H**	**multiplicity**	**coupling**	**δ_C**
1				170.4
2	5.22	br.d	5.0	51.9
3a/b	2.25	m		27.3
4a	1.79	m		20.8
4b	1.44	m		
5a	1.70	m		24.8

Position	**δ_H**	**multiplicity**	**coupling**	**δ_C**
5b	1.38	m		
6a	3.51	ddd	16.0, 10.5, 5.0	45.6
6b	3.25	ddd	16.0, 9.5, 6.0	
8				167.2
9				194.3
10				98.7
10-OH	5.10	br.s		
11	2.06	m		33.6
12	1.60	m		26.3
13a	1.64	m		31.1
13b	1.35	m		
14	3.99	m		70.0
15a	1.80	m		37.6
15b	1.44	m		
16	4.04	dd	5.5, 5.5	76.1
16-OH	3.48	br.s		
17				135.2
18	6.15	d	11.0	130.8
19	6.31	dd	14.5, 11.0	126.0
20	6.18	dd	14.5, 10.5	132.4
21	6.06	dd	15.0, 10.5	132.8
22	5.37	dd	15.0, 8.0	138.4
23	2.25	m		35.3
24a	1.88	m		35.1
24b	1.17	m		
25	2.46	m		41.8
26				212.8
27	3.96	d	4.0	84.4
28	4.21	d	4.0	76.0

Position		**δ_H**	**multiplicity**	**coupling**	**δ_C**
28-OH		3.48	br.s		
29					139.2
30		5.28	d	11.0	124.4
31		3.29	dq	11.0, 6.5	45.8
32					207.8
33a		2.74	dd	17.5, 5.5	40.8
33b		2.49	dd	17.5, 4.0	
34		5.17	ddd	7.0, 5.5, 4.0	70.5
35		1.88	m		32.3
36a		1.44	m		40.2
36b		1.20	m		
37		1.35	m		38.2
38a	ax.	1.46	m		32.5
38b	eq.	0.69	m		
39a		1.46	m		40.4
39b		0.69	m		
40		3.99	m		75.0
40-OH		3.51	br.s		
41a		1.46	m		40.4
41b		0.69	m		
42a		1.46	m		32.5
42b		0.69	m		
11-CH_3		0.92	d	6.5	15.5
17-CH_3		1.66	s		12.9
23-CH_3		0.96	d	6.5	21.4
25-CH_3		0.90	d	6.5	14.2
27-*O*-CH_3		3.28	s		58.3
29-CH_3		1.76	s		12.9
31-CH_3		1.00	d	6.5	16.1

Position	δ_H	multiplicity	coupling	δ_C
35-CH_3	0.81	d	6.5	16.1

Activity assays

Solubility assay and Caco-2 permeation assay

These assays were performed at Inpharmatica Ltd., Cambridge, U.K., for protocols refer to http://www.inpharmatica.com/pdfs/Admensa_Laboratory_Sciences.pdf

NCI 60 cell line panel in vitro *bioassay for anticancer activity*

Assays were performed as described at http://dtp.nci.nih.gov/branches/btb/ivclsp.html.

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