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Isolation, Structure Determination and Biological Activity of 15-Deoxo-7,32-*O*-didesmethylrapamycin from the Soil Actinomycete LL-D45042

EDMUND I. GRAZIANI*, MIA Y. SUMMERS, JEFFREY E. JANSO, KER YU, VALERIE S. BERNAN, MICHAEL GREENSTEIN and GUY T. CARTER

Departments of Chemical & Screening Sciences Oncology, Wyeth Research, 401 North Middletown Road, Pearl River, New York, 10965

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Rapamycin (**1**) is a potent immunosuppressive and anti-proliferative natural product isolated from the soil actinomycete *Streptomyces hygroscopicus* NRRL 5491.[1] The final steps in the biosynthesis of rapamycin involve oxidations at C15 and C32 by cytochromes P-450 and three methylations on oxygens at C7, C32, and C41.[2] Many of the possible biosynthetic intermediates that lack full elaboration at these positions have been isolated and characterized.[3]

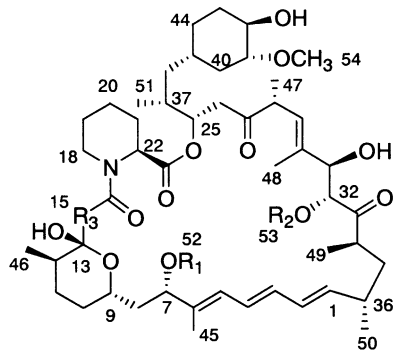
In the course of evaluating rapamycin producers in our collection of actinomycetes, we encountered a new strain, designated LL-D45042, that makes the previously unreported rapamycin intermediate, 15-deoxo-7,32-*O*-didesmethylrapamycin (**2**). We herein report the preliminary characterization of the strain, and the isolation, structure determination and biological activity of the new compound.

Culture LL-D45042 sporulated moderately to abundantly on most media studied with aerial mycelium in the Grey-color series. The 16S rDNA sequence was determined for strain LL-D45042 following isolation and direct sequencing of the amplified gene and supported classification of the strain in the genus and species of *S. hygroscopicus*.

Physiological studies[4] of culture LL-D45042 resulted in no melanin production, slow starch hydrolysis, decomposition of and fair growth on cellulose, no hydrogen sulfide production, and good digestion of casein. Carbohydrate utilization tests indicated good growth on D-glucose, L-arabinose, sucrose, *i*-inositol, D-mannitol, β-D-fructose, α-L-rhammnose and moderate growth on D-xylose and cellulose. Culture LL-D45042 exhibited abundant growth at 22°C, 28°C, and 37°C, but no growth at 45°C and 50°C. Other defining characteristics include abundant growth and dark grey-yellow surface mycelium on glycerol-asparagine agar (ISP5) and absence of soluble pigment on any of the ISP agars compared. A comparison of the above properties with previously reported[3a,5] rapamycin-producing strains revealed several different characteristics that support the creation of a new strain of *S. hygroscopicus* designated LL-D45042.[6]

Cells from a 1liter culture of LL-D45042 were pelleted by centrifugation and exhaustively extracted with methanol. A chloroform extract of this material was chromatographed on reversed phase silica employing a gradient of from 30% methanol in water to 95% methanol. The fraction containing (**2**) eluted with approximately 75% and was further purified *via* reversed phase chromatography employing a gradient from 5% acetonitrile in water to 95% to yield 0.5 mg of a white solid, 15-deoxo-7,32-*O*-didesmethylrapamycin (**2**); FT-ICRMS *m/z* 894.534230 for $C_{49}H_{77}NO_{12}Na$ $(M+Na)^+$ 894.533799 found: (−0.00043);

Fig. 1. Structures of rapamycin (**1**) and 15-deoxo-7,32-*O*-didesmethylrapamycin (**2**).



* Corresponding author: graziaei@wyeth.com

UV (acetonitrile - water): 270, 280, 292 nm; ^{1}H NMR (500 MHz, CD_3CN) see Table 1; ^{13}C NMR (125 MHz, d_6-DMSO) see Table 1.

FT-ICRMS analysis yielded the molecular formula $C_{49}H_{77}NO_{12}$ for (**2**), which was consistent with the formal loss of two methyl groups and an oxygen with reference to rapamycin. Interpretation of the NMR spectra of rapamycins is complicated by the two conformational populations observed due to *cis*-/*trans*-isomerization of the amide bond;[7] nonetheless, the assignments obtained for the major conformer are in agreement with previously reported literature values (Table 1).[8] Examination of the NMR data for **2** revealed a set of methylene protons at δ 2.74 and 2.52 in the multiplicity edited (me)-HSQC spectrum of **2** that showed correlations into a carbon at δ 39.8. These two methylene protons also showed HMBC correlations into a resonance at δ 97.7 corresponding to the hemiketal carbon C13. These methylene proton resonances showed further HMBC correlations into a carbonyl signal at δ 171.6, indicating that these signals must be assigned to a methylene group at C15. These assignments are in good agreement with the values reported previously for rapamycin analogs in which C15 is reduced to a fully saturated methylene group.[3] The presence of free hydroxyl groups at C7 and C32 was confirmed by NMR correlations from the OH protons (δ 4.63 and 4.98, respectively) into H7 (δ 4.07) and H32 (δ 4.26) in the COSY spectrum of **2**, as well as the complementary correlations into C7 (δ 72.9) and C32 (δ 75.8) in the HMBC spectrum of **2**.

Rapamycin effects its biological activities by first binding to an immunophilin such as FKBP12, and then further binding of this complex to the target-of-rapamycin protein, mTOR.[9] 15-deoxo-7,32-*O*-didesmethylrapamycin (**2**) had an IC_{50}=400 nM in an FKBP12 binding assay, a value

Table 1. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data in d_6-DMSO for 15-deoxo-7,32-*O*-didesmethylrapamycin (**2**).

C#	δ ^{13}C	δ ^{1}H	C#	δ ^{13}C	δ ^{1}H
1	137.56	5.56	29	123.5	5.20
2	131.6	6.15	30	137.9	
3	130.2	6.20	31	78.1	3.85
4	128.0	6.40	31 (OH)		
5	125.4	5.95	32	75.8	4.26
6	140.1		32(OH)		4.98
7	72.9	4.07	33	213.2	
7(OH)		4.63	34	40.3	2.57
8	41.0	1.53 1.30	35	40.4	1.35 1.07
9	65.7	3.59	36	33.5	2.30
10	31.4	1.16	37	33.5	1.77
11	27.3	1.49 1.37	38	38.3	1.13 1.00
12	37.3	1.52	39	32.6	1.27
13	97.7		40	35.3	1.95 0.60
13 (OH)		5.80	41	83.9	2.80
15	39.8	2.74 2.52	42	73.2	3.16
16	171.6		42 (OH)		4.62
18	43.4	4.03 3.10	43	32.9	1.73 1.16
19	24.7	1.65 1.38	44	31.2	1.54 0.85
20	20.4	1.67 1.31	45	10.5	1.62
21	26.6	2.13 1.64	46	16.9	0.83
22	51.3	5.23	47	15.5	0.92
23	170.4		48	13.5	1.74
25	75.1	4.96	49	13.1	0.84
26	40.9	2.58 2.44	50	20.9	0.98
27	208.8		51	15.2	0.81
28	46.2	3.33	54	56.9	3.31

approximately two orders of magnitude less potent than rapamycin. This is consistent with the observation of a crucial hydrogen bond between the C15 carbonyl of rapamycin and FKBP12 as well as with the reported binding of other rapamycin analogs that are lacking the C15 carbonyl.[10)]

In conclusion, we have isolated a previously unreported intermediate along the biosynthetic pathway to rapamycin from a new strain of *Streptomyces hygroscopicus* designated LL-D45042. Spectroscopic data for the compound are consistent with the structure of 15-deoxo-7,32-*O*-didesmethylrapamycin as assigned, and the biological activity of the compound is reduced relative to rapamycin due to the absence of an important oxygen implicated in binding to FKBP12.

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