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INCORPORATION OF ACETATE, PROPIONATE, AND METHIONINE INTO RAPAMYCIN BY *STREPTOMYCES HYGROSCOPICUS*

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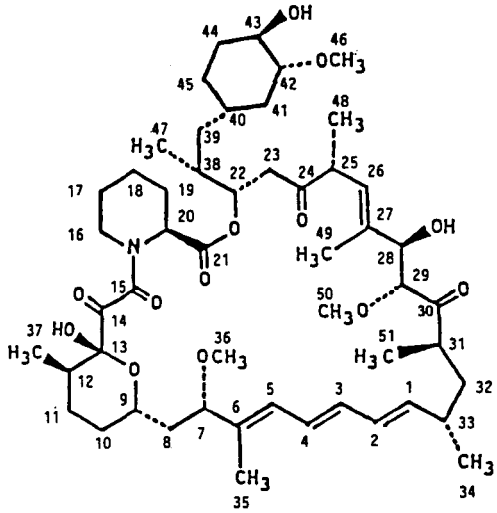
ABSTRACT.—Labelling experiments with ^{13}C-labelled precursors demonstrated that the majority of the macrolide ring of rapamycin [**1**] is formed from six acetate and seven propionate units. A two-carbon unit in the ring was not labelled by acetate, propionate, glycine, or methionine. Methionine and glycine did, however, label the three methoxy groups of rapamycin to a high degree. Results from the incorporation experiments demonstrated that there were errors in the published ^{13}C-nmr spectral assignments for rapamycin; these have now been corrected.

Rapamycin [**1**] was first reported as an antifungal agent in 1975 by Vezina *et al.* (1). It is produced by a strain of *Streptomyces hygroscopicus* isolated from an Easter Island soil sample. It is very active against *Candida* species (common human pathogenic yeasts) and moderately active against dermatophytes, while having no activity against bacteria (2). Its specificity, relatively low toxicity, and good oral absorption in mice and dogs (3) make rapamycin a potentially valuable antifungal agent. Rapamycin was also shown to have good activity against mammary, colon, and brain tumor model systems (4) and against transplanted tumors (5). It inhibits the immune response in rats, apparently by inhibiting the lymphatic system (6). The antifungal mode of action of rapamycin is still unclear, although it appears to differ from those of many known antifungal agents (7,8).



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The structure of rapamycin [**1**] was determined by a combination of X-ray crystallography and ^{13}C- and ^{1}H-nmr experiments (9,10). A major portion of the molecule is a very large (31-membered) "macrolide ring." Three conjugated double bonds (C-1 to C-6) allow rapamycin to be classified as a "triene," an uncommon type of polyene. A rare α-ketoamide functionality (C-14 and C-15) is located just outside a hemiketal ring, resulting in a series of three highly oxidized carbons. C-15 forms an amide bond with the sole nitrogen atom present in the antibiotic. The heterocyclic ring including this nitrogen and C-16 to C-20 plus the C-21 carboxyl can be excised by acid- or base-catalyzed hydrolysis as L-pipecolic acid (10). Outside the macrolide ring is a trisubstituted cyclohexane ring (C-40 to C-45). Many antibiotics contain C_6 rings, but they are usually aromatic. A few other antibiotics contain cyclohexane rings, such as asukamycin and manumycin, but little has been published about their biosynthesis (11,12). At the time of its discovery, many structural features of rapamycin were unique. Recently, FK506, a promising immunosuppressant from *Streptomyces tsukubaensis*, was found to have many structural features identical to rapamycin (13). Information learned about the biosynthesis of rapamycin may also apply to the same moieties of FK506.

Little was known about the biosynthesis of rapamycin when this work was begun. Based on what was known about the biosynthesis of macrolides, ansamycins, and other similar natural products, we proposed a pathway for rapamycin biosynthesis (Figure 1). We hypothesized that a cyclic C_7 unit derived from the shikimic acid pathway would serve as the starter unit. The long polyketide chain (macrolide ring) would then be built up from the condensation of seven acetate and seven propionate units. Chain growth

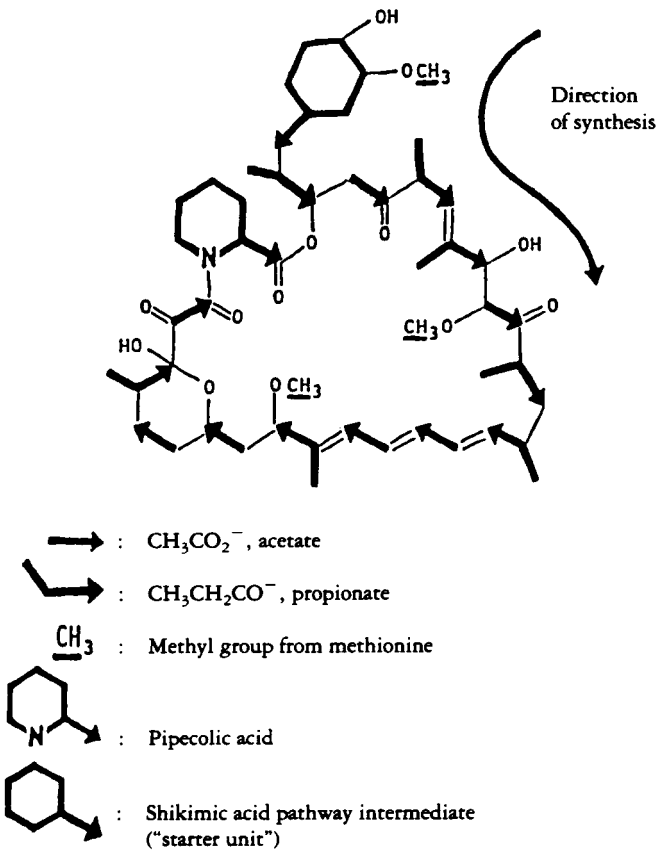


FIGURE 1. Proposed scheme for the biosynthesis of rapamycin [**1**].

would be terminated by the attachment of the pipecolate moiety or by lysine, which would later be cyclized to form the pipecolate moiety. The pipecolate carboxy group and the C-22 hydroxyl would then interact to close the ring by lactone formation. Some additional oxidations and reductions would occur, and three hydroxyl groups would be methylated, presumably with *S*-adenosylmethionine (SAM) acting as the methyl donor.

In this paper we report the results of experiments designed to test parts of the above proposed pathway and to determine the biosynthetic origins of a large number of the carbons of rapamycin.

EXPERIMENTAL

STRAINS.—*S. hygroscopicus* strain AY-B1206 was obtained from the Ayerst Research Laboratories, Montreal, Canada. This strain is a soil isolate found during a re-examination of the soil sample which contained the original rapamycin-producing strain (AYB994, deposited as ATCC 29253 and NRRL 5491). It was reported (personal communication from S.N. Sehgal) to produce higher levels of rapamycin and little or no demethoxyrapamycin when compared to the original isolate.

MEDIA.—*Sporulation agar.*—One liter of agar contains 20 g glucose, 1 g KNO_3, 0.5 g K_2HPO_4, 0.1 g KH_2PO_4, 0.5 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, and 22 g agar (pH 6.8). The agar was melted and distributed into square bottles; after autoclaving, the bottles were placed on their sides to provide a thin layer of agar.

Fermentation medium for labelling studies.—The following were added to 900 ml distilled H_2O: 2.0 g K_2HPO_4, 2.0 g KH_2PO_4, 5.0 g NaCl, 1.5 g $CaCO_3$, 20 ml glycerol, 1.0 g L-leucine, 2.0 g L-glutamic acid, 4.0 g L-lysine·HCl, 5.0 g yeast extract (Difco), 0.06 g $ZnSO_4$, 0.256 g $MgSO_4 \cdot 7H_2O$, 0.012 g $MnSO_4 \cdot H_2O$, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.018 g $(NH_4)_6Mo_7O_{24} \cdot H_2O$, 0.01 g $Na_2B_4O_7 \cdot 10H_2O$, 0.01 g $CoCl_2 \cdot 6H_2O$, 0.0013 $CuCl_2 \cdot 2H_2O$, 0.51 g $MgCl_2 \cdot 6H_2O$, and 0.36 g $NaSO_4$. The pH of the solution was adjusted to 6.0. This solution (45 ml) was placed into 500-ml unbaffled Erlenmeyer flasks. The flasks were autoclaved for 20 min at 121°. After cooling, 5.0 ml of sterile 20% glucose was added to each flask.

CULTURE CONDITIONS.—Fermentation flasks were inoculated directly with a spore suspension. To prepare the inoculum for fermentations, several bottles of sporulation agar were inoculated with spore stock. Bottles were incubated for 14–21 days at 25°. During this time, the culture sporulated, changing from white to dark grey. A spore suspension was prepared by adding sterile H_2O to the bottles and suspending the spores by scraping the agar surface with an inoculating loop or spatula. Spores from 200 to 250 cm^2 of agar were suspended in approximately 15 ml H_2O. For labelling work, one ml of the suspension was used to inoculate 50 ml of fermentation medium in 500-ml unbaffled flasks. Fermention flasks were shaken at 25° (2″ diameter, 250 rpm) until the pH reached 7.0, usually 6 to 7 days. During the course of the fermentation, 1-ml samples of broth were removed from at least duplicate flasks. The pH of each sample was measured. Samples were assayed for rapamycin as described below. The final concentration of rapamycin was 50 to 60 mg/liter.

To determine the dry cell weight of the cultures, 2 ml of 3 N HCl was added to each 50-ml flask to dissolve any remaining $CaCO_3$. The complete contents of a flask were then collected by filtration, dried at 90° for 24 h, and weighed.

HPLC ASSAY FOR RAPAMYCIN.—MeOH (9 ml) was added to each 1-ml sample of fermentation broth, and the tubes were tightly sealed and shaken several times during 20 min to extract the rapamycin. The tubes were centrifuged and the supernatant fluids filtered through glass fiber filters or solvent-resistant 0.45-μm filters. These samples were either analyzed immediately or stored at −20° until analysis. The rapamycin content was determined by reversed-phase hplc (Waters μBondapak C-18 column, 4 mm × 30 cm; mobile phase 70% MeOH/30% H_2O, 2 ml/min; detected at 254 nm and 280 nm, Waters Model 440 UV and Waters Lambda-Max Model 480 absorbance detectors at 0.01 aufs); 50 μl of extract was injected. Rapamycin has a retention time of approximately 30 min.

Purified rapamycin (a gift of Ayerst Laboratories) was dissolved in MeOH and used to prepare a series of standard solutions. The concentration of rapamycin was found to be directly proportional to peak height. A 10 μg/ml standard solution gave a peak of approximately 70% of the full scale.

SMALL-SCALE RECOVERY AND PURIFICATION OF RAPAMYCIN.—To prepare rapamycin samples for nmr and tlc analysis, the contents of several fermention flasks were combined. The mycelium was collected by centrifugation for 10 min (4000 *g*, GSA rotor) at room temperature. The pellet was resuspended in distilled H_2O and centrifuged again. The pellet was then resuspended in approximately 4 times its volume of MeOH. This mixture was shaken to extract the rapamycin and then centrifuged for 10 min. The superna-

tant (MeOH) was removed, and the extraction repeated two more times with similar volumes of fresh MeOH. The extracts were combined and the MeOH removed by rotary evaporation. The resulting oil was dissolved in CH_2Cl_2 (150 ml per liter of broth being processed) to which an equal volume of H_2O was added. A dark emulsion layer often appeared at the interface. The CH_2Cl_2 layer was removed, and the oil/emulsion was extracted 2 more times with similar volumes of CH_2Cl_2. The CH_2Cl_2 extracts were pooled and the solution evaporated to dryness. The resulting residue was extracted with several portions of hexane. The hexane was removed by rotary evaporation, leaving a dark yellowish oil. At this stage, the sample was redissolved in a known volume of hexane and a portion applied to tlc plates (as in the experiments for determining ^{14}C incorporation). Alternatively, the sample was dissolved in approximately 2 ml MeOH and further purified by semipreparative-scale hplc (Waters μBondapak C18, 8 mm × 30 cm; 254 and 280 nm UV detectors; typically 65% MeOH/35% H_2O, 5 to 7 ml/min). The center portion of the rapamycin peak was collected.

The collected fractions were pooled and concentrated by one of two procedures. For most of the samples, the fractions were chilled in an Me_2CO/dry ice bath, and the MeOH/H_2O evaporated under vacuum in a lyophilizer. For two samples (2-^{13}C-propionate and $^{13}C_2$-glycine experiments), C-18 reversed-phase cartridges (Maxi-Clean Cartridges, Alltech, Deerfield, Illinois) were used. Sufficient H_2O was added to the pooled fractions to bring their concentration to approximately 40% MeOH, and the solution was passed through the cartridge to bind the rapamycin to the C-18 packing. Air was passed through the cartridge briefly to remove as much solvent as possible, and the rapamycin was eluted with pure MeOH. The MeOH was removed by rotary evaporation without heating, and any remaining traces of H_2O were removed by lyophilization. After concentration, the rapamycin was collected by dissolving in CH_2Cl_2, the solution filtered and transferred to a small flask, and the CH_2Cl_2 removed by rotary evaporation. The sample was stored at −20° until analyzed by ^{13}C nmr.

^{13}C-NMR SPECTROSCOPY.—Two different spectrometers were used for acquiring ^{13}C-nmr spectra of rapamycin in $CDCl_3$ (unless otherwise noted) in 10 mm tubes: a Bruker 270 Spectrometer (67.9 MHz) and a home-built wide-bore 360 (90.4 MHz). At 67.9 MHz acquisition parameters included a 16 kHz sweep width, 16K data points, 40° pulse width (12–16 μsec), and a relaxation delay of 1.5 sec. ^1H noise-decoupled spectra were recorded at ambient probe temperature (30–33°). The number of transients accumulated ranged from 14,000 to 16,000 for the natural abundance sample (100 mg) and 8000 (for highly enriched) to 17,000 for the enriched samples (8 to 12 mg). To enhance sensitivity, each free induction decay was treated with a 4-Hz line broadening. For acquisition of ^{13}C spectra at 90.4 MHz the following parameters were used: 20 kHz sweep width, 16K data points, 90° pulse width (18.5 μsec), and 5 sec relaxation delay. Protons were decoupled by a WALTZ sequence. Probe temperature was 22° in this spectrometer. The number of transients ranged from 4000 to 5000 for natural abundance (100 mg) and 5000–6000 for ^{13}C-enriched (approximately 10 mg) samples. To enhance sensitivity, a 2-Hz line broadening was applied to free induction decays.

RESULTS

GROWTH, PRODUCTION, AND pH.—A typical fermentation profile is shown in Figure 2. Because rapamycin accumulation was first detectable between 2 and 2.5 days, this was the time chosen for the addition of labelled precursors. Rapamycin is unstable at alkaline pH; cultures were harvested before pH 7 was reached.

INCORPORATION OF LABELLED ACETATE.—Initial efforts were focused on the origins of the polyketide chain, beginning with the simplest available precursor, acetate. Preliminary experiments showed that the maximum amount of sodium acetate that could be added to the culture at 2.5 days was 0.5 g/liter; higher concentrations caused decreased rapamycin production and cell death. Experiments with 1-[^{14}C]- plus 2-[^{14}C]-acetate indicated that this concentration of acetate should provide suifficient, although low, enrichment of rapamycin in the corresponding ^{13}C experiment. In separate experiments, 1-[^{13}C]- and 2-[^{13}C]-acetate were added to cultures, and the labelled rapamycin was recovered and analyzed. We obtained 7% total incorporation of 1-[^{14}C]- and 2-[^{14}C]-acetate or an estimated 1% at each of seven sites. The relative ^{13}C enrichments are shown in Table 1. The maximum uncertainty in these values is 25%. Due to the small sample size, the signal-to-noise ratios were relatively low. However, careful comparison of the spectra revealed that several carbons were significantly labelled. C-5, C-9, C-15, C-24, and C-30 were enriched by 1-^{13}C-acetate as predicted by the pro-

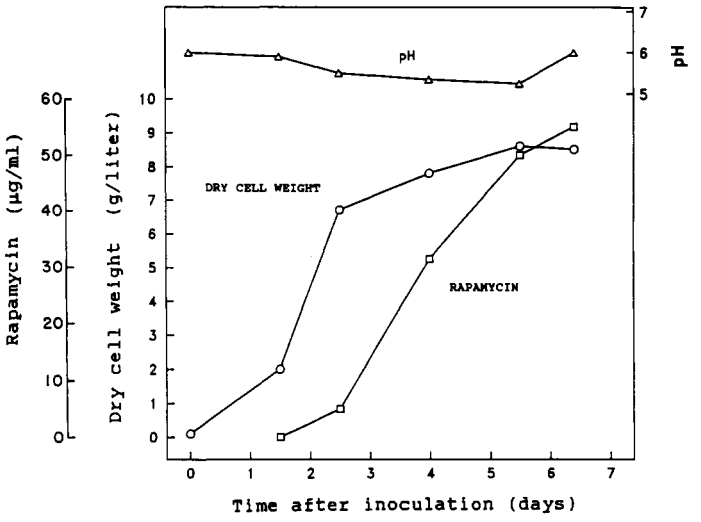


FIGURE 2. Rapamycin production, growth, and pH during fermentation.

posed scheme. Surprisingly, C-3 and C-11 (predicted to be derived from C-1 of acetate) were not significantly enriched, while C-4 (predicted to be derived from C-2 of acetate) was highly enriched. For the sample prepared with 2-^{13}C-acetate, similar results were obtained. C-2, C-8, C-14, C-23, and C-29 were enriched by 2-^{13}C-acetate as predicted, while C-10 and C-4 (predicted to be labelled) were not, and C-3 (predicted to be derived from C-1 of acetate) was definitely enriched. In addition, in both samples, several carbons thought to be derived from propionate were also apparently enriched by labelled acetate. The latter enrichment of propionate carbons by acetate could be attributed to metabolic scrambling of the labelled acetate carbons into propionate/methylmalonyl-CoA via the citric acid cycle. The lack of significant incorporation at C-10 and C-11 suggested that these two carbons are not derived from acetate. The enrichment of the resonance assigned to C-3 by 2-[^{13}C]-acetate and that assigned to C-4 by 1-[^{13}C]-acetate was most surprising; it implied that a strange rearrangment involving C-C bond breakage and formation might have occurred. An alternative explanation was that an error had been made in the ^{13}C-nmr assignments of C-3 and C-4 (10). Eight of the carbons that had been labelled as predicted (C-23, C-24, C-29, C-30, C-8, C-9, C-14, and C-15) correspond to four of the predicted seven acetate units, in the predicted orientation (Figure 3).

In order to determine if any error had been made in the published ^{13}C-nmr spectral assignments (10) and to confirm the above conclusions, doubly labelled acetate (1,2-[$^{13}C_2$]-acetate) was incorporated into rapamycin using the same procedures as for the singly labelled samples. If the acetate is incorporated intact, the nuclei of the two adjacent ^{13}C atoms will still exhibit ^{13}C-^{13}C coupling, resulting in the appearance of a satellite doublet ("coupling pattern") flanking the singlets corresponding to the two rapamycin carbons enriched. If the acetate C-C bond is broken and the two ^{13}C atoms are separated by scrambling reactions, subsequent incorporation will increase the intensity of the singlet resonance without the appearance of the ^{13}C-^{13}C doublet. As indicated in Table 1, a total of 12 "coupled" carbons (J_{CC} = 40 to 60 Hz) were identified after incorporation of 1,2-[$^{13}C_2$]-acetate into rapamycin, indicating the incorporation of six intact acetate units. Four of these are the four acetate units labelled as predicted by singly labelled acetate. The other two correspond to C-2 through C-5, implying the incorporation of two intact acetate units into the olefinic region. This contradicts the re-

TABLE 1. Relative ^{13}C-Enrichment Factors.[a]

Chemical shift (ppm)	Published[b] carbon assignment	1-[^{13}C]-acetate	2-[^{13}C]-acetate	1,2-[$^{13}C_2$]-acetate		1-[^{13}C]-propionate	2-[^{13}C]-propionate	Reassigned[d] carbon no.
				uncoupled	coupled[c]			
215.5	30	3.0[e]	1.0	1.5	yes			
208.1	24	3.9[e]	1.2	1.5	yes			
192.5	14	0.9	2.3[e]	1.5	yes			
169.2	21	1.0	0.9	1.5				
166.8	15	2.4[e]	0.8	1.4	yes			
140.1	26	2.0[f]	1.2	2.1		3.0[e]		1
136.1	27	1.3	1.9[f]	2.2			3.0[e]	
135.5	6	1.4	1.7[f]	2.4			2.5[e]	
133.6	4	3.5[g]	0.8[g]	1.5	yes			3
130.2	2	1.3	2.4[e]	1.7	yes			A[h]
129.6	5	3.0[e]	0.9	1.7	yes			A
126.7	1	1.9[f]	2.1[f]	2.6		3.0[e]		26
126.4	3	1.1[g]	3.3[g]	1.6	yes			4
98.5	13	2.4[f]	1.3	2.3		2.0[e]		
84.9	29	1.3	2.2[e]	1.4	yes			
84.4	7	1.6[f]	1.4	1.9		2.5[e]		
84.4	42							
77.2	28	(obscured by solvent peaks)				2.5[e]		
75.7	22	1.9[f]	0.7	—		2.7[e]		
73.9	43	1.2	1.2	1.0				
67.2	9	3.1[e]	1.1	1.7	yes			
59.4	36	1.0	0.9	1.0				
56.5	50	1.0	1.1	0.9				
55.8	46	0.9	1.1	1.1				
51.4	20	1.1	0.8	1.2				
46.5	25	1.3	2.1[f]	2.3			3.0[e]	
44.2	16	1.3	0.9	1.2				A
41.4	31	1.4	2.0[f]	2.7			2.2[e]	
40.7	23	1.1	2.4[e]	1.7	yes			A
40.2	39	1.4	1.3	2.0		2.1[g]		32
38.9	8	0.8	1.7[e]	1.6	yes			A
38.4	10	1.1	0.7[g]	1.1	no[g]			39
35.2	33	1.1	2.8[f]	2.6			2.1[e]	
34.2	41	0.9	2.6[e]	1.2				A
33.7	38	1.1	1.9[f]	2.2			2.0[e]	
33.2	40	0.9	1.5	1.6				
33.2	12						2.0[e]	
31.7	32	0.7	0.8	1.0		[g]		45
31.3	45	0.8	1.4	1.3				10
31.3	44							A
27.3	19	2.4[f]	1.6[f]	1.8				A
27.0	11	1.4[g]	0.9	1.7	no[g]			A
25.3	17	1.0	0.7	1.2				A
21.5	48	0.7	1.7[f]	1.6				34
20.6	18	0.8	0.8	0.9				A
16.2	47	0.6	1.3	1.8				37
15.9	51	0.8	1.3	1.9				47
15.9	34							48
13.8	37	0.6	1.4	1.7				51
13.0	35	0.9	1.8[f]	2.1				49
10.1	49	0.8	1.2	1.6				35

[a]Natural abundance = 1.0. Reference: average of three methoxy carbon signals.

[b]These assignments are from Findlay and Radics (10).

[c]The average coupling constants (J_{cc}) after peak reassignments for the six pairs of carbons were $J_{2,3} = 53$, $J_{4,5} = 58$, $J_{8,9} = 41$, $J_{14,15} = 61$, $J_{23,24} = 39$, $J_{29,30} = 41$ Hz.

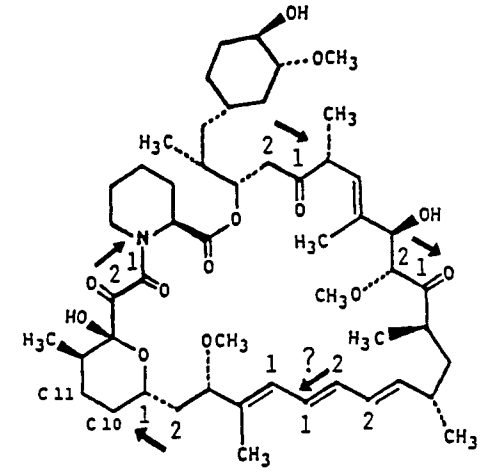
[d]Dr. R.P. Borris and Dr. B.H. Arison, personal communication.

[e]Significantly enriched, as predicted by proposed scheme.

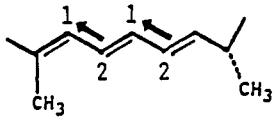
[f]Enrichment attributed to incorporation after metabolic scrambling.

[g]Not as predicted; possible nmr misassignment.

[h]Indicates assignments agree with those of Findlay and Radics (10).



Expected labelling pattern of olefinic region



1 : Enriched by 1-[^{13}C]-acetate

2 : Enriched by 2-[^{13}C]-acetate

$\overrightarrow{2\ 1}$: $CH_3CO_2^-$; orientation of acetate unit

FIGURE 3. Combined results of 1-^{13}C-acetate and 2-^{13}C-acetate incorporation experiments.

sults of the singly labelled acetate incorporation, and demonstrates that there is indeed an error in the published ^{13}C-nmr assignments (10). There are at least three ways to interchange the assignments for these very similar carbons to make them consistent with the proposed scheme and the labelling results; thus an absolute reassignment of the carbons was not possible from these data alone. No coupling patterns were observed for any other carbons, confirming that C-10 and C-11 are not derived from acetate, and that the incorporation into other carbons was through scrambling reactions.

INCORPORATION OF LABELLED PROPIONATE.—According to the biosynthetic scheme proposed, seven propionate units should be incorporated into rapamycin. It was also possible that 1-[^{13}C]- and 2-[^{13}C]-propionate might label C-11 and C-10; a C_2 unit in rifamycin is derived from two carbons of a propionate unit, the methyl group of which is oxidatively removed after it is incorporated (14). Rapamycin samples enriched by 1-[^{13}C]- and 2-[^{13}C]-propionate were prepared using the same procedures as for the acetate samples, except that 0.5 g/liter was added to the cultures at 2.5 days and an additional 0.25 g/liter was added at 3.5 days of incubation.

In the case of 1-[^{13}C]-propionate, six carbons (C-1, C-7, C-13, C-22, C-26, C-39) showed significant enrichment, as indicated in Table 1. (The chemical shifts of C-7 and C-42 overlap; therefore, it was assumed that the increase in peak intensity is due to enrichment of C-7, not C-42.) Five of these carbons were predicted to be derived from the carboxyl groups of propionate. C-39 corresponds to the carboxyl groups of the proposed C_7 unit and was not predicted to be enriched. Also, C-32 corresponds to the carboxyl of one of the predicted propionates, but no enrichment was observed. These results could

be explained by an error in the nmr assignments of these two carbons (10); they are very similar carbons and the assignments might be interchanged.

One of the carbons predicted to be derived from 1-[^{13}C]-propionate, i.e., C-28, was obscured by the solvent ($CDCl_3$) peaks. In order to determine whether there was any incorporation into this carbon, the ^{13}C-nmr spectra of this sample and an unenriched sample were also obtained in CD_2Cl_2, a very similar solvent. The pattern of the rapamycin peaks was not altered much, and the same six peaks were enriched. In addition, a seventh peak at approximately 77.2 ppm was enriched. Based on the published assignments it was concluded that this peak corresponded to C-28, the seventh predicted propionate carbon.

In the case of 2-[^{13}C]-propionate, seven carbons were enriched. These correspond to the seven carbons predicted by the proposed scheme (C-38, C-25, C-27, C-31, C-33, C-6, C-12). These results confirm the incorporation of the seven propionates and support the possibility that C-32 and C-39 were misassigned (10). It is highly unlikely that C-31 and C-33 could be labelled by 2-[^{13}C]-propionate without C-32 being labelled by 1-[^{13}C]-propionate, nor is there a likely route for C-32 to be exchanged with C-39.

Unlike labelled acetate, labelled propionate did not seem to be scrambled into other non-propionate-derived carbons. Perhaps propionate, once activated, is less likely to enter the citric acid cycle.

CORRECTION OF THE ^{13}C-NMR SPECTRAL ASSIGNMENTS.—The results of the above incorporation studies strongly suggested that there were errors in the published ^{13}C-nmr assignments (10). However, absolute reassignments were not possible from the available data alone. A sample of rapamycin was used to generate HETCOR, COSY, and NOESY maps (Dr. R.P. Borris and Dr. B.H. Arison, personal communication; subject of a future publication). Analysis of the data revealed several errors in the assignments (10), some of which are indicated in Table 1. The assignments of C-3 and C-4 were interchanged, and the peak assigned to C-39 was not assigned to C-32, as suggested by the above incorporation data.

INCORPORATION OF LABELLED METHIONINE.—According to the proposed scheme, the three methoxy carbons in rapamycin are derived from the methyl group of methionine. Unlabelled L-methionine was added to the fermentation medium after 2.5 days of incubation to see if it affects rapamycin production. The addition of 0.5 g/liter methionine caused no marked effect on rapamycin production, while 1.0 and 2.0 g/liter clearly decreased production (approximately 35 and 45% decrease, respectively). These findings were at first surprising because addition of a true precursor is expected to increase production if that precursor is rate-limiting or to have no effect if it is not limiting. However, despite the fact that methionine is the precursor of the side chain of thienamycin and the methoxy group of cephamycin C, it inhibits the production of these antibiotics by *Streptomyces catteleya* (15).

Experiments with L-^{14}C-[methyl]-methionine indicated that addition of 0.5 g/liter of labelled methionine resulted in very high enrichment of rapamycin. The specific activities of the added ^{14}C-[methyl]-methionine and the resulting rapamycin were equal, indicating 100% total incorporation or 33% incorporation at each of the three sites. A rapamycin sample enriched by L-^{13}C-[methyl]-methionine was prepared from cultures which received 0.3 g/liter L-^{13}C-[methyl]-methionine at 2.5 days of incubation. The peaks assigned to C-36, C-50, and C-46, the three methoxy carbons, showed a 15- to 20-fold increase in height, confirming that methionine acts as the methyl donor for these carbons. No other carbons were enriched.

INCORPORATION OF LABELLED GLYCINE.—C-10 and C-11 were not significantly enriched by any of the above precursors. It was thought that glycine might enrich this

two-carbon unit, either after conversion to glycollate (transamination and reduction) or after incorporation into glycerate (via serine). When added to cultures of *S. hygroscopicus* var. *geldanus* var. *nova*, both 1-^{13}C-glycerate and 1-^{13}C-glycollate labelled carbons in geldanamycin which acetate had previously failed to label (16). 1-^{13}C-glycine was found to label a two-carbon unit in saframycin A, a heterocyclic quinone antibiotic synthesized by *Streptomyces lavendulae* (17) but did not label the analogous carbons in naphthyridomycin, a structurally similar antibiotic produced by *Streptomyces lusitanus* (18). In the case of leucomycin biosynthesis by *Streptomyces kitasatoensis*, 1-^{13}C-glycine, 1-^{13}C-glycollate, and 1-^{13}C-glycerate did not enrich the carbons of a previously unlabelled C_2 unit, but this was attributed to the failure of these compounds to enter the cells, because 2-^{13}C-glycerol was shown in later experiments to label these carbons (19).

When added to cultures at 2.5 days of incubation, glycine (up to 2 g/liter) had no effect on rapamycin production. A sample of rapamycin enriched by 1,2-$^{13}C_2$-glycine was prepared from cultures receiving 0.75 g/liter at 2.5 days and 0.25 g/liter at 3.5 days. Only the resonances assigned to the three methoxy carbons (C-36, C-50, C-46) showed an increase in intensity (9- to 10-fold). No other resonances showed any increased intensity over natural abundance or any evidence of ^{13}C-^{13}C coupling. Clearly, glycine is entering the cells but is only incorporated into rapamycin after conversion to the methyl group of methionine (via glycine synthase and tetrahydrofolate reactions).

DISCUSSION

The origins of most of the carbons of rapamycin have been identified, as summarized in Figure 4. The results match those of the predicted scheme quite well, except for the lack of acetate incorporation into C-10 and C-11.

Most of the carbons of the lactone ring of rapamycin are derived from six acetate and seven propionate units, condensed in a "head to tail" fashion. This pattern of incorpora-

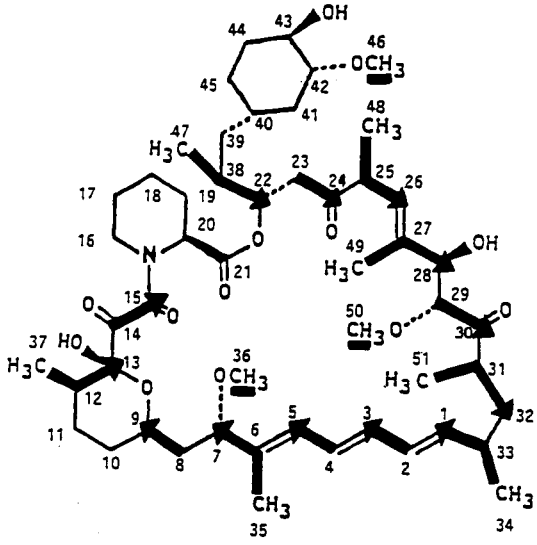


FIGURE 4. Summary of the incorporation results.

tion implies a polyketide pathway mechanism of biosynthesis, as has been found for many acrolides and polyenes. Of note is the fact that the rare α-ketoamide functionality (C-14 and C-15) is indeed derived from acetate, while the high oxidation state of these two carbons and the adjacent carbon might have suggested a carbohydrate origin. The incorporation experiments revealed errors in the published ^{13}C-nmr assignments (10), and these errors were confirmed by further nmr analysis. Those carbons originally misassigned are chemically very similar, and spectroscopic assignments based on similar compounds (or fragments) could easily be wrong.

The seventh predicted acetate unit (C-10 and C-11) was not labelled significantly by any form of acetate, nor by any other precursor tested, and the original of these two carbons remains unknown. It is likely that they are derived from glycollate or glycerate, as has been shown for two-carbon units in geldanamycin and leucomycin (16,19).

The three methoxy carbons of rapamycin were shown to be derived from the methyl group of methionine. Methionine was found to suppress rapamycin production if high concentrations were added to young cultures, but this is consistent with observations for other systems (15). Glycine, while highly enriching the same methoxy carbons after metabolic scrambling, did not decrease rapamycin production.

The substituted cyclohexane moiety (C-39 to C-45) and the heterocyclic ring (C-16 to C-22) were not labelled by any of the precursors tested, as is consistent with their predicted shikimate pathway and pipecolate origins.

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