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Antimicrobial Characterization and Interrelationships of Dirithromycin and Epidirithromycin

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Dirithromycin is the 9-*N*,11-*O*-oxazine adduct formed from 9(*S*)-erythromycylamine and 2-(2-methoxyethoxy)acetaldehyde in which the methoxyethoxymethyl substituent on the oxazine ring possesses the *R* configuration. Epidirithromycin is its isomer in which the methoxyethoxymethyl substituent has the opposite (*S*) configuration. Both compounds readily epimerize in solution, reaching an equilibrium ratio of 85:15 in favor of dirithromycin, given sufficient time. The rate of interconversion is dependent upon pH, temperature, and solvent. An enriched sample of epidirithromycin (95% purity) was synthesized by condensing erythromycylamine and 2-(2-methoxyethoxy)acetaldehyde in diethyl ether as the reaction solvent, and the product was fully characterized by nuclear magnetic resonance spectroscopy and high-pressure liquid chromatographic (HPLC) analysis. Both oxazine derivatives readily hydrolyze to erythromycylamine, so all three compounds exhibit the same antibiotic activity in vitro. In order to determine whether dirithromycin itself possesses significant antimicrobial activity without initial hydrolysis to erythromycylamine, inhibition of cell-free ribosomal protein synthesis was measured under conditions which were adapted to minimize hydrolysis, as measured by analytical HPLC in parallel experiments. Under these particular conditions, inhibition of ribosomal protein synthesis by dirithromycin was <10% of the value measured for erythromycylamine.

Dirithromycin is a new macrolide antibiotic undergoing clinical development that displays relatively high and prolonged tissue concentrations of antimicrobial activity (4, 7). It is a semisynthetic derivative of erythromycin that is prepared by first converting erythromycin to 9(*S*)-erythromycylamine and then condensing this intermediate with 2-(2-methoxyethoxy)-acetaldehyde, thereby creating a 9-*N*,11-*O*-oxazine ring system (Fig. 1) (3, 8, 11). The stereochemistry of the methoxyethoxymethyl substituent attached to the newly formed oxazine ring has been firmly established as the *R* configuration by X-ray crystallography (8). More recently, the various polymorphic, isomorphic, and solvated forms of dirithromycin in the solid state have been described (14).

Epidirithromycin is the isomer of dirithromycin in which the methoxyethoxymethyl substituent on the oxazine ring possesses the opposite (*S*) stereochemistry (Fig. 1). It was initially detected as a minor constituent by chromatographic and nuclear magnetic resonance (NMR) spectroscopic studies of the isomerization of dirithromycin in solution (5). The structure of epidirithromycin was deduced by comparisons with the NMR spectra of another pair of epimeric oxazine derivatives of erythromycylamine (5, 9). This article reports the synthesis and isolation of epidirithromycin in a highly enriched form, thereby permitting a more complete characterization of its physicochemical and spectral features and its antibiotic properties.

Dirithromycin and epidirithromycin readily interconvert to an equilibrium mixture, and both hydrolyze in vitro and in vivo to their common precursor, 9(*S*)-erythromycylamine, itself a potent and well-known antibiotic (3, 10). All three compounds generally exhibit the same antibacterial activity under standard conditions for testing in vitro susceptibility. This circumstance made it impossible to conclusively determine whether dirithromycin possessed antimicrobial activity without being first hydrolyzed to erythromycylamine. To better answer this question regarding the mechanism of action, the well-established inhibition of ribosomal protein synthesis by macrolides was employed (15). However, the procedures had to be modified in order to find suitable conditions for rapidly measuring inhibition of protein synthesis while minimizing the hydrolysis of dirithromycin. By using these conditions for rapid measurement, inhibition of protein synthesis by dirithromycin was then determined while its degree of epimerization and hydrolysis was simultaneously monitored by analytical high-pressure liquid chromatography (HPLC).

(These results were presented at the 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy, New Orleans, La., 17 to 20 October 1993).

MATERIALS AND METHODS

Antibiotics. Erythromycylamine, dirithromycin, and intermediate synthetic reagents were prepared as previously described (3). Epidirithromycin was synthesized as follows. To a slurry of erythromycylamine (20.0 g, 27.2 mmol) in ether (100 ml) was added 2-(2-methoxyethoxy)acetaldehyde (6.0 g, 30.5 mmol) in one portion at ambient temperature. Although the slurry thinned, a complete solution was not obtained, and the reaction mixture again thickened within 15 to 20 min. The mixture was stirred for a total of 30 min. The solid material was filtered, washed with fresh ether, and vacuum dried at 40°C to yield 10.9 g (48%) of solid product; mp, 119 to 124°C (dec.); field-desorption mass spectrum (*m/z*), 836 ($M + H^+$); infrared spectroscopy (KBr), 3546, 2972, 2938, 2895, 1373, 1179, 1163, 1127, 1112, 1078, 1056, 1033, and 993 cm^{-1}. ^1H and ^{13}C NMR assignments are given in Table 1. Elemental analysis calculated for $C_{42}H_{78}N_2O_{14}$: C, 60.41; H, 9.42; N, 3.35. Found: C, 60.46; H, 9.35; N, 3.49.

Analytical HPLC. HPLC analyses were conducted on a Hypersil octadecyl silane column (25 cm by 4.6-mm inside diameter; ≤5-μm packing) in a Hitachi 655A-11 liquid chromatograph equipped with an L-5000 controller and Waters Associates TCM column heater set at 40°C. Column output was detected with a

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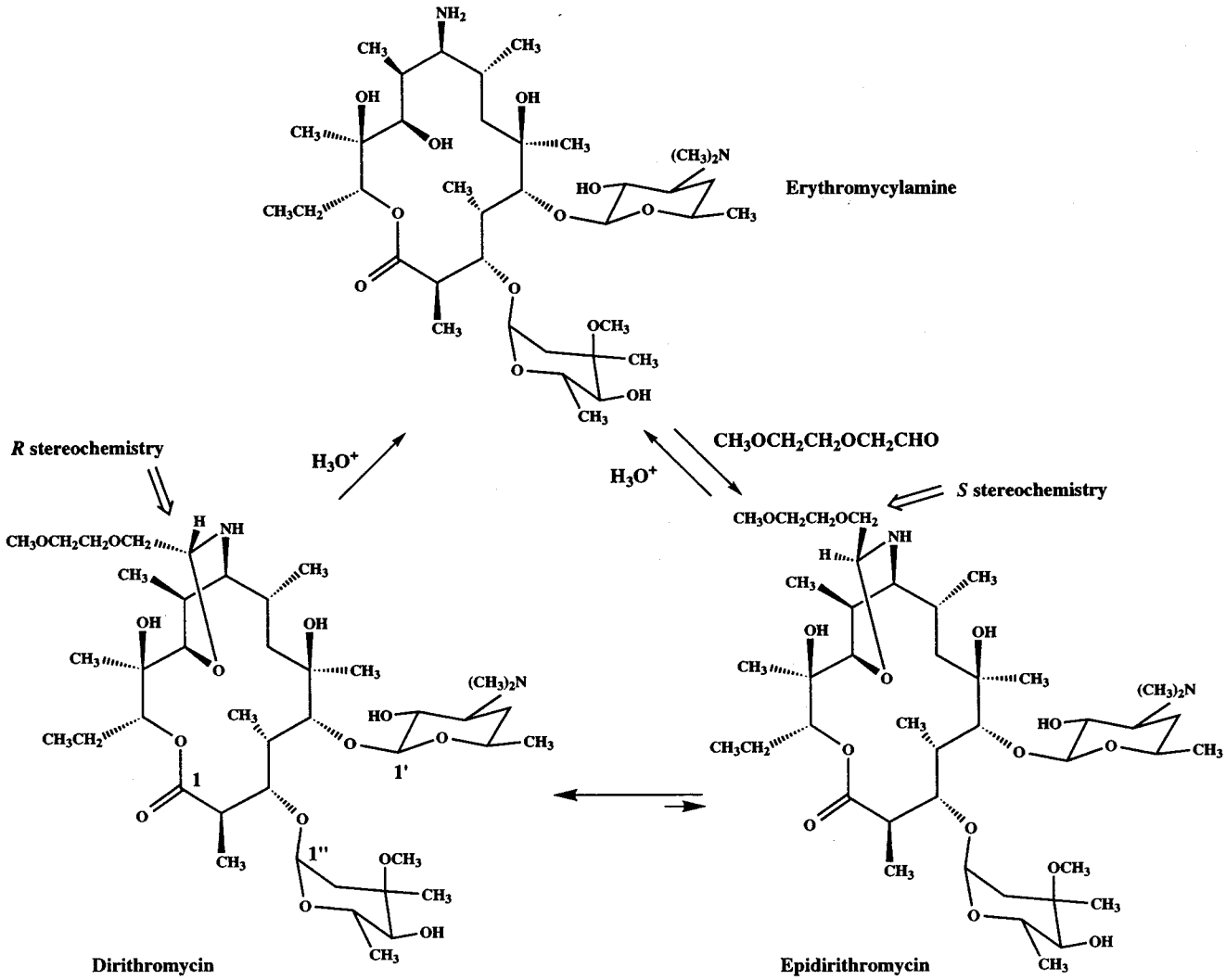

FIG. 1. Structures of dirithromycin, epidirithromycin, and erythromycylamine.

Waters Associates 410 differential refractometer set at 40°C and connected to a Hitachi D-2000 chromatointegrator. The column was eluted with a mobile phase of 50 mM potassium phosphate buffer (pH 7.5)–acetonitrile–methanol (37:44:19) at a flow rate of 2.0 ml/min and a run time of approximately 25 min. The phosphate buffer was prepared rather than purchased since commercial buffers often contain formalin, which reacts with erythromycylamine even at low concentrations to form the 9-*N*,11-*O*-oxazine derivative from formaldehyde.

Instrumentation. Radioactivity was measured with a Beckman LS 3801 scintillation counter. NMR spectra were determined on a Bruker AMX-500 NMR spectrometer. Elemental analyses were conducted with a Control Equipment Corp. 440 elemental analyzer. Mass spectra were obtained on a Finnigan MAT 731 mass spectrometer interfaced to a Finnigan MAT SS-200 data system.

Epimerization studies. Dirithromycin was prepared in acetonitrile as previously reported (3). Samples of the reaction mixture, including any solid that formed, were collected at various times and analyzed by HPLC. The samples were diluted in the HPLC mobile phase to a concentration of 1 to 2 mg/ml and assayed immediately. The peak areas corresponding to dirithromycin, epidirithromycin, and erythromycylamine were used to calculate the relative percentage of each component (Fig. 2). The dirithromycin was filtered, washed with cold acetonitrile, and vacuum dried at 40°C; it was 95 to 97% pure by HPLC assay and contained less than 0.3% epidirithromycin. The purity of dirithromycin was increased by recrystallization from acetone. HPLC analysis of the reaction filtrate showed an 85:15 ratio of dirithromycin to epidirithromycin for the material remaining in the filtrate.

Solutions of epidirithromycin were prepared at a concentration of 0.5 g/10 ml in methanol, isopropanol, dichloromethane, or the HPLC mobile phase. The solutions were maintained at ambient temperature, and samples were withdrawn periodically. One drop of sample was diluted with 1 ml of mobile phase and assayed by HPLC. The peak areas of epidirithromycin and dirithromycin were used to calculate the relative percentage of each component (Fig. 3). In an analogous manner, the rates of epimerization of epidirithromycin were measured as a function of temperature and of added mineral acid (0.5 g/10 ml in methanol; 15 mol% hydrochloric acid).

Bacterial strains. All bacterial strains used in this study were clinical isolates from the Lilly culture collection that had been obtained from numerous sources with a broad geographical distribution. Isolates were maintained in liquid nitrogen or at −70°C in electric freezers.

Susceptibility testing. Antibiotic susceptibility was determined by an agar dilution method (19) on Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, Md.). The medium was supplemented with 1% supplement C (Difco Laboratories, Detroit, Mich.) to enhance the growth of *Haemophilus influenzae* and nonenterococcal streptococci. The antibiotics were incorporated directly into the melted agar prior to the pouring of plates. An inoculum of approximately 10^4 CFU was prepared by the appropriate dilution of cultures grown overnight in fresh brain heart infusion broth (Difco Laboratories) and was applied to plates via a 36-prong Cathra inoculating device. Plates were examined for growth after 18 to 20 h of incubation at 37°C.

Stability studies. Tris buffer was preheated to 37°C. Dirithromycin was dissolved at the required concentration, and the solution was maintained at 37°C. At the appropriate times, samples were taken and quickly extracted with ethyl acetate, and the organic extract was immediately injected onto the HPLC column for analysis.

Inhibition of ribosomal protein synthesis. Inhibition of cell-free protein synthesis on ribosomes from *Escherichia coli* was measured with Amersham kit N.380, in which the progression of protein synthesis from a DNA template was followed by measuring the amount of tritiated leucine incorporated into protein.

TABLE 1. Assignments of ^{1}H and ^{13}C NMR spectra of dirithromycin and epidirithromycin[a]

Position	Dirithromycin		Epidirithromycin	
	^{1}H	^{13}C	^{1}H	^{13}C
1		176.97		175.08
2	2.68	44.37	2.91	44.66
3	4.00	76.86	4.24	80.12
4	1.71	44.50	1.98	39.09
5	3.94	79.17	3.58	83.91
6		74.49 (A)		73.86 (B)
7	1.27/1.38	39.22	1.30	34.29
8	2.12	29.33	2.05	33.00
9	2.13	65.94	2.55	63.70
10	1.78	27.51	1.95	29.28
11	3.23	72.34	3.67	69.89
12		74.30 (A)		73.50 (B)
13	4.91	76.36	5.07	75.72
13-CH_2	1.39/1.89	21.30	1.41/1.87	20.85
13-CH_3	0.80	11.14	0.81	10.66
2-CH_3	1.18	12.82	1.20	16.28
4-CH_3	1.09	8.93	1.10	9.27
6-CH_3	1.12	24.64	1.35	27.27
8-CH_3	1.33	20.70	0.98	21.31
10-CH_3	1.14	14.07	1.08	19.27
12-CH_3	1.08	14.81	1.09	15.31
Oxazine-1	4.57	82.77	4.73	82.42
Oxazine-2	3.54/3.60	72.86 (C)	3.43/3.56	71.46 (D)
Oxazine-4	3.52	71.95 (C)	3.35/3.64	72.95 (D)
Oxazine-5	3.59/3.73	71.05 (C)	3.63	70.44 (D)
Oxazine-7	3.55	58.90	3.40	59.09
1′	4.78	100.93	4.41	103.05
2′	3.28	70.93	3.27	70.64
3′	2.51	64.89	2.45	65.15
4′	1.26/1.64	28.95	1.22/1.68	28.20
5′	3.60	69.34	3.54	68.69
6′	1.24	21.00	1.25	21.43
$N(CH_3)_2$	2.27	40.31	2.29	40.18
1″	5.19	94.31	4.95	96.32
2″	1.56/2.40	34.41	1.60/2.39	34.76
3″		72.67		72.35
4″	2.99	78.42	3.06	77.73
5″	3.84	65.70	4.05	65.19
6″	1.24	18.31	1.36	18.58
3″-CH_3	1.27	21.79	1.25	21.43
3″-OCH_3	3.35	49.14	3.32	49.43

[a] NMR spectra were measured in $CDCl_3$ solution at −10°C in order to minimize epimerization. Chemical shifts were recorded as δ values (parts per million) measured from the $CHCl_3$ solvent signal (proton: 7.26 δ; carbon: 77.0 δ) that had been calibrated with respect to tetramethylsilane. Assignments followed by the same letter were not unambiguous and may be reversed.

The effects of the antibiotic on protein synthesis were then compared with analogous results from drug-free controls. The requisite components were assembled at 0°C and mixed in the following order: (i) 1 μl of DNA (pBluescript II SK−), (ii) 1.5 μl of supplement solution, (iii) 0.6 μl of leucine, (iv) 0.4 μl of [^{3}H]leucine, and (v) 1.0 μl of 30S ribosome extract. The mixture was incubated at 37°C for 15 min to initiate protein synthesis. The antibiotics were dissolved in dilution buffer (1 mM Tris acetate, pH 7.5) at a concentration of 3.7 mg in 925 μl, from which 1:10 serial dilutions were prepared. Because of the lability of dirithromycin, the solutions of dirithromycin were prepared last, a process that required approximately 45 s. Aliquots of 1.5 μl of the antibiotic solutions were then added to the protein synthesis mixture, with the solutions of dirithromycin being the first ones used. Incubation was continued for 5 min, after which protein synthesis was quenched by submersion of the reaction tubes in an ice bath. After the addition of 500 μl of 1 N sodium hydroxide to each reaction tube, the reaction mixtures were incubated at 37°C for 15 min and then treated with 1 ml of 25% trichloroacetic acid containing 1 mg of casein hydrolysate per ml. After incubation for 30 min at 0°C, the precipitated protein was collected on Whatman glass-fiber filters and washed with 5% trichloroacetic acid and ethanol, and radioactivity was counted in a liquid scintillation counter. All experiments were performed in triplicate.

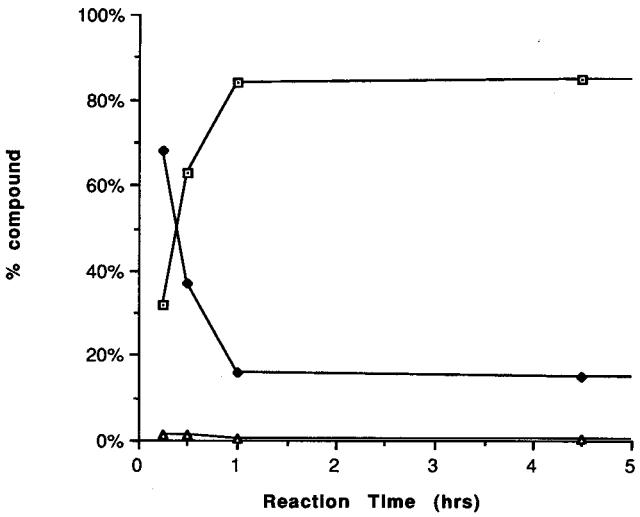

FIG. 2. Reaction profile for synthesis of dirithromycin and epidirithromycin. Reaction of erythromycylamine with 2-(2-methoxyethoxy)acetaldehyde in acetonitrile. ⊡, dirithromycin; ◆, epidirithromycin; △, erythromycylamine.

RESULTS AND DISCUSSION

Synthesis and characterization. Dirithromycin is synthesized by treating erythromycylamine with 2-(2-methoxyethoxy)-acetaldehyde for several hours in organic solvents such as acetonitrile (3, 8, 11). HPLC analysis of the reaction mixture revealed that epidirithromycin is very rapidly formed as the initial reaction product and that it subsequently epimerizes until an 85:15 equilibrium ratio in favor of dirithromycin is reached within approximately 1 h in solution (Fig. 2). The formation of epidirithromycin as the initial, kinetically controlled reaction product has been rationalized on the basis of the solution conformations adopted by the reactants and products (12). (Regarding reference 12, please note that because of typographical errors, the assignments for epidirithromycin at C-26 [49.4 ppm] and C-8″ [59.1 ppm] should be interchanged in

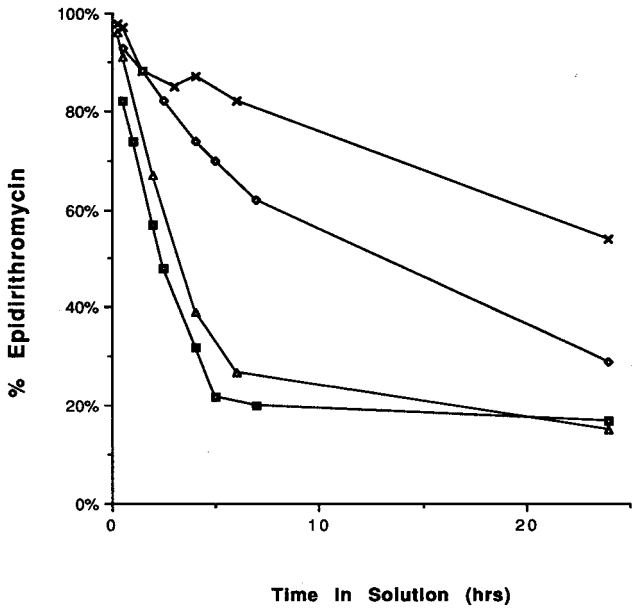

FIG. 3. Epimerization of epidirithromycin in various solvents. ◆, isopropanol; ■, dichloromethane; ×, methanol; ▲, HPLC mobile phase.

TABLE 2. In vitro susceptibility evaluation of dirithromycin and epidirithromycin

Strain	MIC (μg/ml) of:			
	Dirithromycin	Epidirithromycin	Erythromycylamine	Erythromycin
Streptococcus pyogenes C203	0.06	0.06	0.06	0.06
Streptococcus pneumoniae PARK	0.015	0.03	0.03	0.03
Staphylococcus aureus X1.1	1.0	0.5	0.5	0.25
Staphylococcus aureus V41	>128	>128	>128	>128
Staphylococcus epidermidis 222	1.0	0.5	0.5	0.25
Staphylococcus epidermidis 270	>128	>128	>128	>128
Enterococcus faecium 2041	8.0	8.0	8.0	1.0
Haemophilus influenzae C.L.	8.0	4.0	4.0	2.0

Table 1 on p. 3859; in Table 2 on p. 3860, the assignments for dirithromycin at H-7 should be 1.35/1.25 ppm and for epidirithromycin at H-11 should be 3.68 ppm [11a]). Precipitation of dirithromycin from solution then drives the synthetic reaction toward formation of dirithromycin as the thermodynamically controlled product (3, 12). In contrast, with diethyl ether as solvent and a shorter reaction period, epidirithromycin is obtained at approximately 95% purity, with the other 5% consisting of dirithromycin (3%) and unreacted erythromycylamine (2%). If the reaction mixture in ether is stirred for a longer period, the initial precipitate of epidirithromycin dissolves and a new precipitate of dirithromycin is subsequently formed.

After its isolation by filtration, epidirithromycin is stable when stored cold in the solid state. Physicochemical and spectral characterization of epidirithromycin established its coidentity with the compound whose structure had previously been elucidated as a minor component in solutions of dirithromycin (Table 1) (5, 9). NMR assignments for the purified macrolides were consistent with those from other studies, but as also found by others, assignments for some protons and carbon atoms have proven difficult to establish unambiguously (5, 12). However, the NMR spectra of dirithromycin and epidirithromycin did prove to be characteristic for their respective stereochemistries of the oxazine substituent, a consistency that permits the assignment of relative stereochemistry to other pairs of 9-*N*,11-*O*-oxazine derivatives of erythromycylamine (6).

Dirithromycin and epidirithromycin are easily separated from each other by reversed-phase HPLC analysis (13). This HPLC separation was employed to monitor the facile interconversion between epidirithromycin and dirithromycin under a variety of conditions. Epimerization is solvent dependent (Fig. 3) and occurs more rapidly at higher temperatures (60°C versus 25°C) and in the presence of acid (data not shown). Epimerization of the oxazine substituent coincides with a conformational change of the 14-membered lactone ring system (12).

In vitro activity. Susceptibility testing of dirithromycin, epidirithromycin, and erythromycylamine demonstrated that they possessed equivalent in vitro activities within the experimental error of the test (±1 twofold dilution) (Table 2). They also had the same spectrum as and a potency similar to that exhibited by erythromycin. Both inducibly and constitutively macrolide-lincosamide-streptogramin B-resistant strains of staphylococci showed complete cross-resistance to all of the compounds. However, the facile epimerization and hydrolysis that were described above for dirithromycin and epidirithromycin suggested that both derivatives would probably have completely hydrolyzed to erythromycylamine well before the overnight endpoints of the agar dilution tests had been measured, so erythromycylamine was likely the test substrate in all three instances.

Hydrolysis studies. The rate of hydrolysis of dirithromycin in Tris buffer at 37°C was measured by analytical HPLC and was found to exceed 50% hydrolysis within 1 h (Fig. 4). Since Tris buffer was the reaction medium that would be used to measure inhibition of ribosomal protein synthesis, this initial result clearly indicated that the experimental conditions required modification in order to permit more rapid determination of protein synthesis inhibition. Additional experimentation showed that hydrolysis of dirithromycin could be kept to within approximately 10% over a 10-min period (Fig. 5). The rate of hydrolysis was relatively constant over the concentration range of 0.102 to 6 mM. Isomerization to epidirithromycin was also kept to approximately 10% under these conditions. In separate studies, epidirithromycin was found to hydrolyze to erythromycylamine at a higher rate than dirithromycin, so no epidirithromycin was built up during the course of these experiments.

Inhibition of protein synthesis. The antimicrobial activity of all macrolide antibiotics is presumably exerted through their inhibition of the microbe's ribosomal protein synthesis (1). Direct examination of the effects of a compound on its mechanism of action provides a means for examining that com-

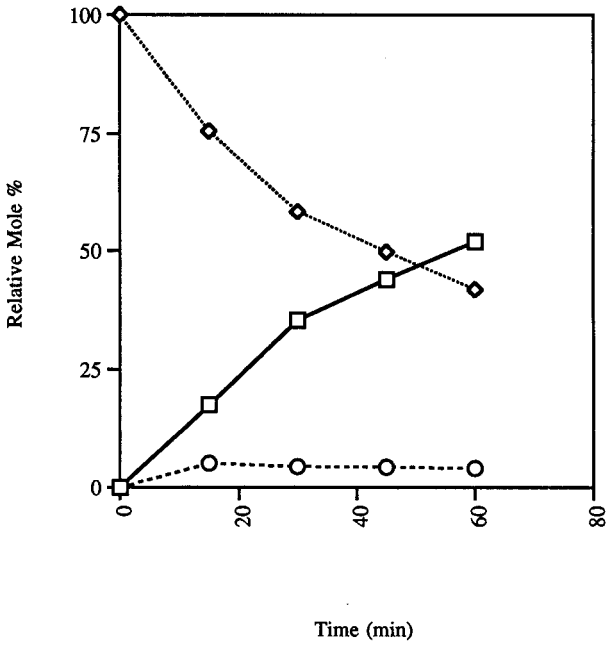

FIG. 4. Hydrolysis of dirithromycin over a 60-min period at 6 mM in Tris buffer at 37°C. □, erythromycylamine; ◇, dirithromycin; ○, epidirithromycin.

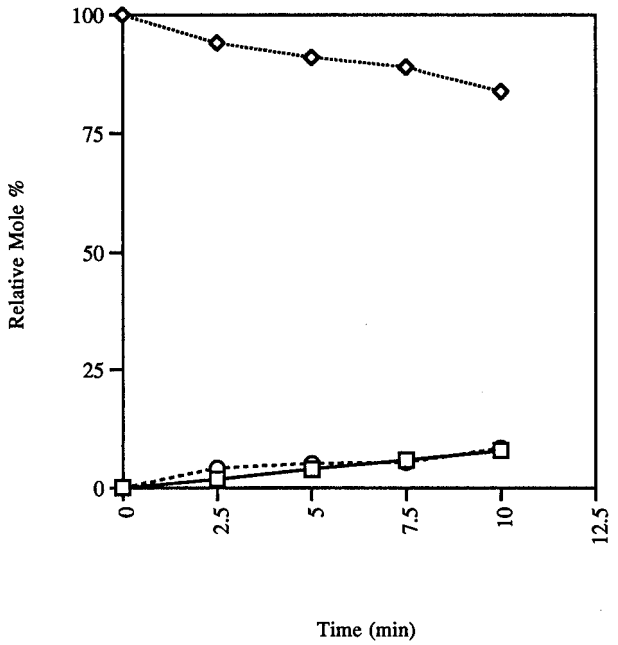

FIG. 5. Hydrolysis of dirithromycin over a 10-min period at 0.102 mM in Tris buffer at 37°C. □, erythromycylamine; ◇, dirithromycin, ○, epidirithromycin.

pound's inherent antibiotic activity under conditions which minimize or eliminate questions concerning chemical stability and intracellular penetration, etc. The well-known classical example is that provided by the 2′ esters of erythromycin (15). The limitation of this approach is that relatively unstable compounds may behave quite differently in water or aqueous buffer solutions from the way they do in biological fluids, even at the same pH, a phenomenon that has also been observed with 2′ esters of erythromycin (16, 17).

Following this well-established example in the present case, cell-free protein synthesis was conducted in the usual manner with sensitive ribosomes derived from *E. coli*, as provided in an Amersham kit. On the basis of the results of HPLC analysis revealing relatively rapid hydrolysis of dirithromycin, a protocol which minimized the time in which dirithromycin was held in solution was developed. Thus, the necessary components for protein synthesis were first assembled and maintained at 0°C. The DNA template, leucine, tritiated leucine, and ribosome extract were then mixed and incubated at 37°C for 15 min in order to initiate protein synthesis. At the appropriate time and working as quickly as possible with dirithromycin, the antibiotics being tested were dissolved in dilution buffer, 1:10 serial dilutions were prepared, and the solutions were rapidly added to the protein synthesis mixture. Incubation was continued for 5 min, at which time the reaction was immediately terminated by immersion of the reaction tubes into an ice bath followed by the addition of 1 N sodium hydroxide solution. The reaction mixtures were then processed in the usual manner in order to collect trichloroacetic acid-precipitated protein for counting radioactivity in a liquid scintillation counter. The experimental results are given in Table 3.

The dose-response profiles for inhibition of protein synthesis exhibited by erythromycylamine and dirithromycin were very similar except that a concentration of dirithromycin 1 order of magnitude greater than that of erythromycylamine was required to produce the same degree of inhibition of protein synthesis. Since the parallel HPLC analyses which measured

TABLE 3. Inhibition of protein synthesis by dirithromycin and erythromycylamine

Antibiotic	Concn (μg/ml)	Dpm	% Inhibition
None (control)	0	2,833	0
Erythromycylamine	100	1,057	63
	10	852	70
	1.0	1,061	63
	0.1	2,833	0
	0.01	1,953	32
	0.001	2,141	25
Dirithromycin	1,000	1,121	61
	100	1,102	72
	10	1,094	62
	1.0	4,676	0
	0.1	3,648	0
	0.01	2,785	0

the rate of hydrolysis of dirithromycin had indicated that approximately 10% hydrolysis to erythromycylamine was occurring under these conditions, the majority of the protein synthesis inhibition observed for dirithromycin could be ascribed to the erythromycylamine that was being generated via hydrolysis. Consequently, under these particular experimental conditions, we conclude that inhibition of ribosomal protein synthesis by dirithromycin itself is less than 10% of the value measured for erythromycylamine. Since these measurements are obviously only an approximation, they can provide only an order-of-magnitude qualitative assessment of the activity of dirithromycin itself as an inhibitor of bacterial protein synthesis. However, it would be of interest to determine if the lack of inhibition observed in this functional assay correlates with results from ribosomal binding studies (2).

In conclusion, the advantages of dirithromycin result from its unusual and complex pharmacokinetics, clinical efficacy, and low incidence of drug interactions (4, 7). Its facile hydrolysis to erythromycylamine described above may explain its complex pharmacokinetic profile and may fit a recently proposed model (18). Dirithromycin may more rapidly penetrate cells and tissues and then subsequently undergo internal hydrolysis to erythromycylamine, a more basic and more highly protonated molecule whose efflux from cells and tissues may be substantially slowed relative to dirithromycin. This study indicates that the facile hydrolysis of dirithromycin is also responsible for much of its antimicrobial efficacy.

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