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Effect of Erythromycin Analogues on Binding of [^{14}C]Erythromycin to *Escherichia coli* Ribosomes

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The relative ability of 44 erythromycin analogues to bind to ribosomes was determined by their effect on [^{14}C]erythromycin binding to *Escherichia coli* ribosomes. The association and dissociation constants of each of these erythromycin derivatives were determined as well as their interaction coefficient for their binding to ribosomes. Substitutions were made on various portions of the erythromycin molecule with retention of substantial activity as measured by inhibition of [^{14}C]erythromycin binding to ribosomes. Since the effect of erythromycin analogues on [^{14}C]erythromycin binding to ribosomes provides a relatively sensitive assay for these compounds, erythromycin analogues with relatively little affinity for ribosomes could be detected. Compounds with association constants of 10^4 M^{-1} were detectable; the association constant for erythromycin binding to ribosomes was approximately 10^8 M^{-1}. Thus, compounds with 0.0001 the association constant of erythromycin were detectable. This assay could be used alone or in conjunction with microbiological assays for primary screening of active analogues or other compounds which interfere with [^{14}C]erythromycin binding to ribosomes. It permits an estimate of the general activity of compounds rapidly and directly. Variables such as metabolic modifications of the compounds and permeability are excluded. The present assay reflects the ability of the compounds to interact directly with their target organelle and may serve as a useful adjunct in developing new compounds.

To use erythromycin as a probe of topological relationships on the ribosome, some erythromycin analogues were synthesized. These analogues permit us to determine which areas of the erythromycin molecule could be modified with retention of ability to bind to ribosomes. Since it was not convenient to synthesize each analogue in radioactive form, the relative activity of each analogue was determined by its effect on [^{14}C]erythromycin binding to ribosomes. Such studies allow us to determine in which areas of the erythromycin molecule we could attach reactive groups for affinity labeling of ribosomal components. Reactive groups with radioactive labels could be attached to different areas of erythromycin. The ribosomal proteins or oligonucleotides to which these labels become linked should comprise the erythromycin binding site. Furthermore, it may be possible to cross-link proteins by the use of two or more reactive groups on a single erythromycin molecule. By extension of these techniques, it should be possible to develop a topological map of the erythromycin binding site and adjacent areas.

By using a cell-free system for studying the relative binding activity of erythromycin analogues rather than studying their effect on intact bacteria, variations in transport, solubility, permeability, degradation, and modification are excluded. Thus, the relative activity of the above analogues on [^{14}C]erythromycin binding to ribosomes might also provide a method of screening these compounds at the ribosomal level, their site of action. With these concepts in mind, this communication evaluates the effect of a number of analogues of erythromycin on the binding of [^{14}C]erythromycin to ribosomes.

MATERIALS AND METHODS

Materials. The erythromycin analogues shown in Fig. 1, 2, and 3 were prepared as described (R. A. LeMahieu, M. Carson, R. W. Kierstead, L. M. Fern, and E. Grunberg, J. Med. Chem., in press; R. A. LeMahieu, M. Carson, and R. W. Kierstead, manuscript in preparation). The known compounds **1** to **7** and **21** had melting points identical to those reported in the literature and had spectral properties consistent with their structures. Thin-layer chromatography indicated that these materials were not contaminated by more than 1% of erythromycin A. The new compounds gave satisfactory microanalytical data, and

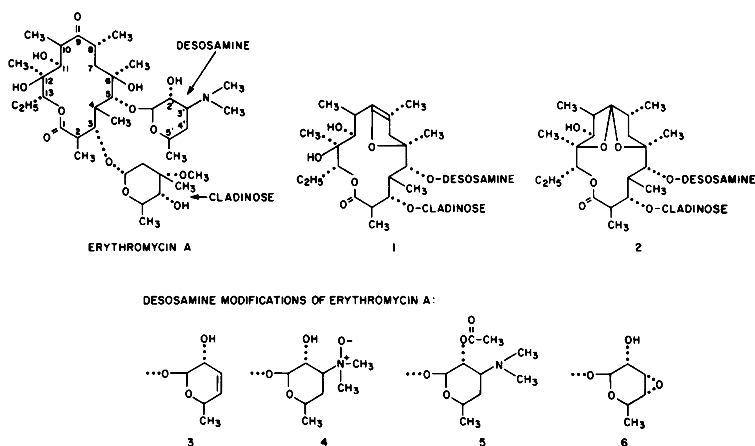

FIG. 1. *Structures of erythromycin analogues.*

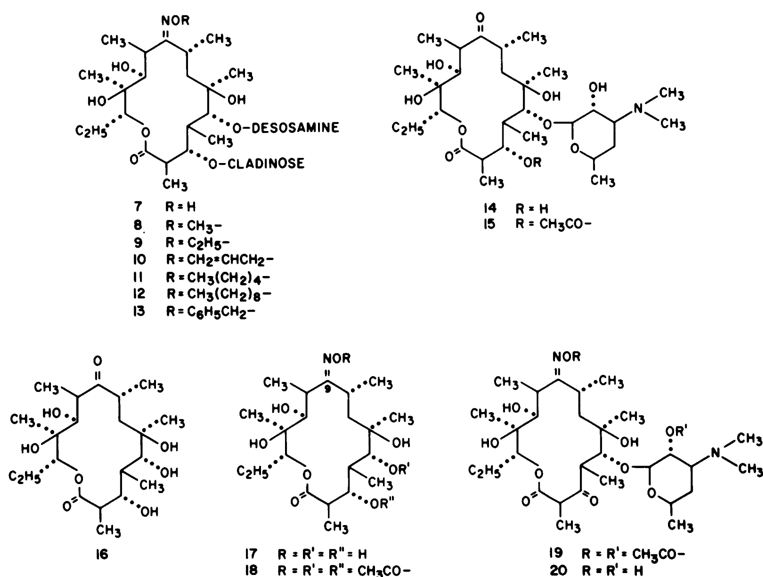

FIG. 2. *Structures of erythromycin analogues.*

their nuclear magnetic resonance and infrared and mass spectra were consistent with their structures. Compounds **8** to **13** were prepared from erythromycin A, but thin-layer chromatographic analysis showed them to be free of residual starting material. Compounds **14** to **20** and **27** to **44** were prepared by reaction schemes which involve glycoside cleavage reactions of erythromycin A oxime (**7**). Compounds **22** to **26** were prepared from **21**. The starting materials, **7** and **21** for these reactions were demonstrated by thin-layer chromatography to be free of erythromycin A. The erythromycin analogues were dissolved in dimethyl sulfoxide to make stock solutions of 0.01 M. Dilutions were made directly into water. [^{14}C] erythromycin A (45.7 mCi/mmol) was synthesized as described in the accompanying communication (10). Ribosomes were prepared as described previously (9).

Determination of [^{14}C]erythromycin binding to ribosomes. Binding of [^{14}C]erythromycin to ribosomes was determined as described in the accompanying report (10). Each reaction mixture for determination of binding of [^{14}C]erythromycin to ribosomes contained the following components in a volume of 0.50 ml unless otherwise specified: 0.004 M $MgCl_2$; 0.1 M KCl; 0.01 M NH_4Cl; 0.01 M tris(hydroxymethyl)aminomethane-chloride, pH 7.2; about 5.6 to 7.5 absorbancy units of NH_4Cl-washed *E. coli* B or *E. coli* A19 ribosomes at 260 nm; about 1.2 μM [^{14}C]erythromycin A; and erythromycin A or erythromycin A analogues as indicated. Reactions were started by adding ribosomes last to the reaction mixtures. Incubations were performed at 24 C for 30 min. At the end of the incubation, reactions were stopped by diluting the reaction mixture with 3 ml of

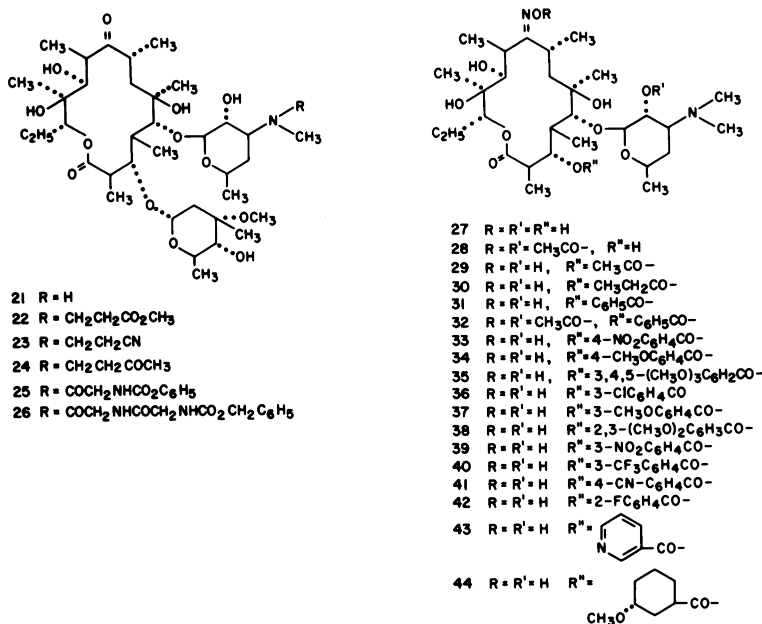

FIG. 3. *Structures of erythromycin analogues.*

cold solution A (0.005 M $MgCl_2$, 0.15 M KCl, and 0.01 M Tris-chloride, pH 7.2). The diluted reaction mixture was filtered through a 25-mm diameter membrane filter (0.45-μm pore size; HAWP, Millipore Corp.); the tube and filter were immediately washed an additional three times with 3 ml of cold solution A. The filters were then dried under an infrared lamp and radioactivity was determined in a scintillation spectrometer as previously reported (10).

RESULTS

Effect of erythromycin analogues on [^{14}C]erythromycin binding to ribosomes. The effects of antibiotics on [^{14}C]erythromycin A binding to ribosomes are shown in Fig. 4, 5, and 6. From these graphs the concentration at which 50% inhibition of [^{14}C]erythromycin binding to ribosomes occurred was determined. In Table 1, the concentration at which 50% inhibition occurred as well as the $pK_{50\%}$ is summarized for all these erythromycin analogues. The 50% inhibitory concentrations and the interaction coefficients given in Table 1 were determined from Hill plots of the data in Fig. 4 to 6. In the succeeding paragraphs, "activity" refers to the ability of an analogue to inhibit [^{14}C]erythromycin binding to ribosomes.

Two derivatives of erythromycin A containing oxygen bridges are analogues **1** and **2**. The enol ether **1** (5) was about 1/30 as active as erythromycin A in inhibiting erythromycin binding to ribosomes. The ketal **2** (13) was about 1/34 as active as erythromycin A in inhibiting [^{14}C] erythromycin binding.

Known analogues in which the desosamine moiety has been modified include compounds **3** to **6**. When the dimethylamino group was eliminated and a double bond introduced between C-3′ and C-4′ **(3)** (4), the activity of the compound in ability to inhibit [^{14}C]erythromycin binding to ribosomes was reduced to approximately 1/130 that of erythromycin A. When an epoxide is present between carbons 3′ and 4′ **(6)** (3), the activity was further reduced to less than 1/2,000 the activity of erythromycin A. The compound was essentially inactive. When the dimethylamino group was converted into an *N*-oxide **(4)** (4), the compound retained substantial activity; it was about 1/50 as active as erythromycin A. Mao and Putterman (7) reported no activity for the *N*-oxide under the condition of their assay. Acetylation of the 2′-hydroxyl group **(5)** (12) reduced the activity of the compound to one-sixth that of erythromycin A.

Oxime derivatives of erythromycin A include analogs **7** to **13**. Erythromycin A oxime **(7)** (8) was slightly less active (one-third) than erythromycin A in inhibiting erythromycin binding to ribosomes. Substitution of the oxime hydrogen with a methyl **(8)** or ethyl **(9)** group enhanced the activity so that the compounds were slightly more active than erythromycin A in inhibiting erythromycin binding to ribosomes. Substitution by an allyl group **(10)** also enhanced the activity compared to the unsubstituted oxime; this compound was approximately equivalent to

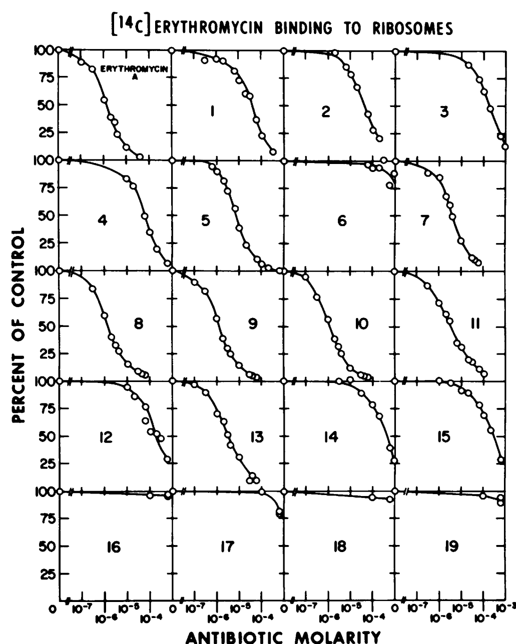

FIG. 4. *Effect of erythromycin analogues on [14C]erythromycin binding to ribosomes. Each 0.50-ml reaction mixture contained the following components: 0.1 M KCl; 0.01 M NH_4Cl; 0.004 M $MgCl_2$; 0.01 M Tris-chloride, pH 7.2; 1.1 μM [14C] erythromycin; 7.5 absorbancy units of NH_4Cl-washed ribosomes at 260 nm; and erythromycin analogues at the concentrations indicated on the abscissas. Reactions were started by the addition of ribosomes and incubated at 24 C for 30 min. In the case of analog* **28**, *the reaction was incubated at 24 C for 15 min to minimize hydrolysis of the erythromycin derivative. Assays were performed as described. The data are presented as a percentage of the [14C]erythromycin bound to ribosomes in the absence of any nonradioactive erythromycin or erythromycin derivatives.*

erythromycin A in inhibiting erythromycin binding to ribosomes. Enlarging the size of the aliphatic group to a pentyl moiety (**11**) also enhanced the activity compared to the underivatized oxime; the compound was approximately one-third to one-half as active as erythromycin A. However, enlarging the size of the aliphatic group to a nonyl residue (**12**) reduced the activity of the compound greatly; this compound was approximately 1/150 as active as erythromycin A. On the other hand, substitution of the oxime with a benzyl moiety (**13**) resulted in a derivative which was one-third to one-half as active as erythromycin A, although it was slightly more active than the underivatized oxime.

It has been possible to remove the cladinose residue from erythromycin A (R. A. LeMahieu, M. Carson, R. W. Kierstead, L. M. Fern, and E. Grunberg, J. Med. Chem., in press). Some analogues were then prepared with various substituents on the hydroxyl group at C-3. The oxime derivative of erythromycin A in which cladinose was removed (**27**) was about 1/385 as active as erythromycin A. Acetylation of the oxime and the 2′-hydroxyl groups (**28**) increased the activity of the compound so that it was 1/120 as active as erythromycin A. Acetylation of only the 3-hydroxyl group (**29**) increased the activity of the compound somewhat compared to 5-*O*-desosaminylerythronolide A oxime; the compound, however, was about 1/150 as active as erythromycin A. The 3-propionate (**30**) exhibited increased activity compared to the oxime; this compound was approximately 1/100 as active as erythromycin A in inhibiting [14C] erythromycin binding to ribosomes. Additional derivatives of 5-*O*-desosaminylerythronolide A oxime in which there are substituents on the 3-hydroxyl, as well as on the oxime and on the desosamine hydroxyls, were synthesized. With a benzoyl group on the 3-hydroxyl (**31**), the compound showed about 1/77 the activity of erythromycin A. It was slightly more active than the corresponding acetyl and propionyl derivatives (**29** and **30**). The analogous compound with acetylated oxime and 2′-hydroxyl groups (**32**), containing a benzoyl group on the 3-hydroxyl, was much less active; it was approximately 1/300 as active as erythromycin A. Various additional substituents were evaluated at the 3-hydroxyl position on 5-*O*-desosaminylerythronol-

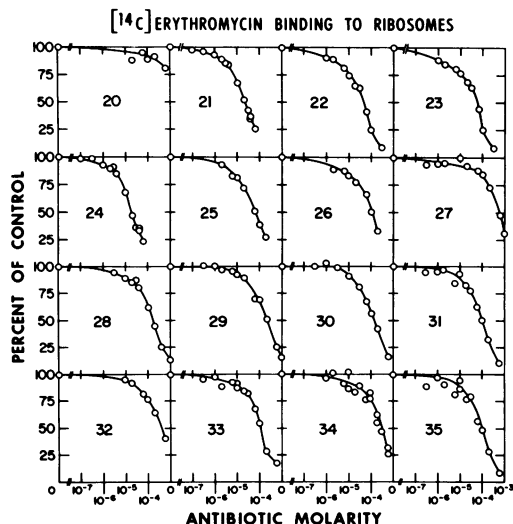

FIG. 5. *Effect of erythromycin analogues on [14C]erythromycin binding to ribosomes. Reaction mixtures and conditions are described in Fig. 4.*

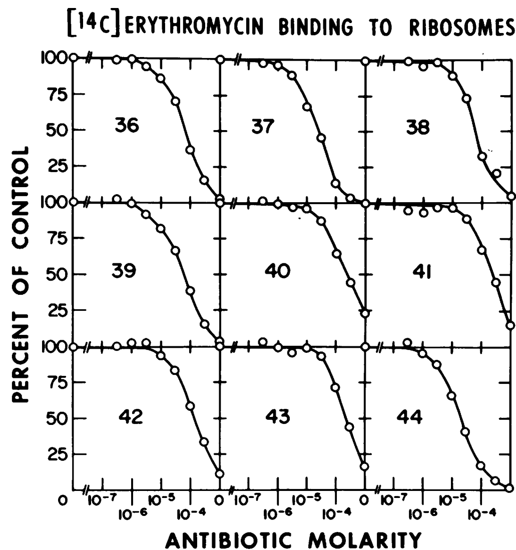

FIG. 6. *Effect of erythromycin analogues on [14C]erythromycin binding to ribosomes. Reaction mixtures and conditions are described in Fig. 4.*

ide A oxime. These included the *p*-nitrobenzoyl group (**33**), the *p*-methoxybenzoyl group (**34**), and the 3,4,5-trimethoxybenzoyl group (**35**). Although the *p*-nitrobenzoyl and *p*-methoxybenzoyl derivatives were slightly less active than the benzoyl derivative (**31**), the trimethoxybenzoyl compound was slightly more active than the benzoyl. The 3-chlorobenzoyl (**36**), the 3-methoxybenzoyl (**37**), and the 2,3-dimethoxybenzoyl (**38**) compounds were all slightly more active than the corresponding benzoyl derivative (**31**). The 2,3-dimethoxybenzoyl (**38**) and the 3,4,5-trimethoxybenzoyl (**35**) derivatives were approximately equal in activity. The 3-methoxybenzoyl derivative (**37**) was more active than the di- or trimethoxybenzoyl derivatives. The 3-nitro-benzoyl derivative (**39**) was approximately equivalent to the 3-chloro derivative (**36**) in its activity. The 3-trifluoromethylbenzoyl (**40**), the 4-cyanobenzoyl (**41**), the 2-fluorobenzoyl (**42**), and the nicotinoyl (**43**) derivatives had approximately 1/100 to 1/170 the activity of erythromycin A and were less active than the corresponding benzoyl or methoxybenzoyl derivatives. The *trans*-3-methoxycyclohexane carbonyl derivative (**44**) was about as active as the 3-methoxybenzoyl derivative (**37**) and both were approximately 1/15 as active as erythromycin A.

A comparison of 5-*O*-desosaminylerythronolide A with 5-*O*-desosaminylerythronolide A oxime was made. The 9-ketone (**14**) was 1/286 as active as erythromycin A and slightly more active than the corresponding 9-oxime (**27**). The

TABLE 1. *Concentration of erythromycin analogues which produce 50% inhibition of [14C]erythromycin binding to ribosomes*[a]

Erythromycin analogue	$pK_{50\%}$	50% Inhibition (μM)	Interaction coefficient from Hill plot (n)
Erythromycin A	5.90	1.3	0.98
1	4.42	38.0	1.03
2	4.35	44.7	0.94
3	3.77	170	0.98
4	4.22	60.2	1.18
5	5.10	7.9	1.09
6	<2.5	>3,000	
7	5.40	4.0	1.09
8	5.95	1.1	0.80
9	5.92	1.2	0.91
10	5.90	1.3	0.98
11	5.47	3.4	0.75
12	3.70	200	0.97
13	5.50	3.2	0.78
14	3.43	372	0.95
15	3.67	214	0.96
16	<2.0	>10,000	
17	<3	>1,000	
18	<2.5	>3,000	
19	<2.5	>3,000	
20	2.45	3,550	0.83
21	4.65	22.4	0.93
22	4.43	37.2	0.94
23	4.42	38.0	1.04
24	4.66	21.9	1.03
25	4.20	63.1	0.87
26	4.00	100	1.18
27	3.30	501	1.05
28	3.80	158	1.03
29	3.70	200	1.07
30	3.86	138	0.91
31	4.00	100	1.09
32	3.42	380	0.87
33	3.90	126	1.04
34	3.57	269	1.29
35	4.08	83.2	1.15
36	4.23	58.9	1.01
37	4.72	19.0	1.18
38	4.10	79.4	1.02
39	4.22	60.3	1.02
40	3.65	224	0.91
41	3.65	224	0.98
42	3.85	141	1.01
43	3.65	224	1.10
44	4.68	20.9	1.01

[a] The 50% inhibition and $pK_{50\%}$ values were determined from the data of Fig. 4,5, and 6. The $pK_{50\%}$ is the negative log of the molar 50% inhibition value.

3-acetyl derivative (**15**) was approximately as active as its corresponding oxime (**29**) in inhibiting erythromycin binding to ribosomes.

Derivatives in which both the desosamine and cladinose were removed showed little or no activity in inhibiting erythromycin binding to ribosomes. These include erythronolide A oxime (**17**) and its acetylated derivative (**18**). Similarly, erythronolide A (**16**) was also inactive. Similar results were reported by Mao and Putterman (7).

De-cladinose derivatives in which C-3 contains a ketone rather than a hydroxyl moiety were also synthesized. The unacetylated (**20**) and acetylated (**19**) compounds were practically inactive in inhibiting erythromycin binding to ribosomes.

Various substituents on the dimethylamino group of desosamine were evaluated. When one of the methyl groups was replaced by a hydrogen (**21**) (1), the compound was approximately 1/17 as active as erythromycin A. Replacement of the methyl group by $CH_3COCH_2CH_2$— (**24**) produced a derivative with activity equivalent to that of **21**. Replacement of the methyl group by $—CH_2CH_2CO_2CH_3$ (**22**) or by $—CH_2CH_2CN$ (**23**) produced analogues with approximately 1/30 the activity of erythromycin A. Replacement of the methyl group by $—COCH_2NHCO_2 \cdot CH_2C_6H_5$ (**25**) and $—COCH_2NHCOCH_2 \cdot NHCO_2CH_2C_6H_5$ (**26**) produced compounds with approximately 1/48 and 1/77 the activity of erythromycin A, respectively.

Determination of association constants for analogues. By using the method of Harris and Pestka (2), it was possible to compute the association (K_I) and dissociation (K_d) constants as well as the number of sites (n) on ribosomes for each analogue. In Fig. 7, the results of representative computations are plotted for several of the erythromycin analogues; and in Table 2, K_I, K_d, pK_d, and n are given for each analogue. In general, the $pK_{50\%}$ and pK_d values are related to each other (Fig. 8). The K_I and K_d values are constants whose determination is independent of ribosome and [^{14}C]erythromycin concentration. In contrast, $pK_{50\%}$ values depend critically on the ribosome and [^{14}C]erythromycin concentrations and are merely convenience parameters determined under an arbitrary and fixed set of conditions.

It can be seen that the values for n are usually close to 1.0. This agrees with the expectation that the analogues would compete with [^{14}C]erythromycin for binding to ribosomes. Nevertheless, several of the analogues have values substantially lower or higher than 1.0. Analogues with n values significantly greater than 1.0 ($>$1.15) may be binding to ribosomes at more than one site (analogues **17**, **26**, **31**, **35**, and **43**). Analogues with n values significantly lower than 1.0 ($<$0.85) include **11** and **22**. Since K_I and K_d were calculated with the assumption that n equals 1.0, significant variations from 1.0 would produce errors in these estimates. Alternative explanations for high and low n values are possible.

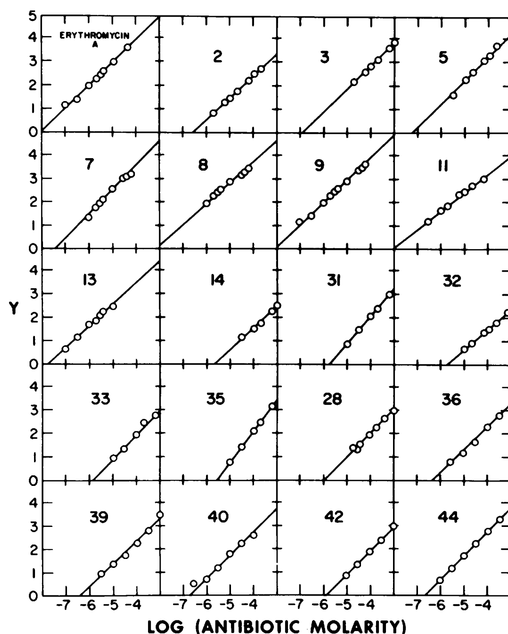

FIG. 7. *Graph of y as a function of antibiotic concentration for computation of association and dissociation constants. The equation* $\log \{(K_o [A]([R_o] - [RA])/[RA]) - 1\} = n \log [I] + \log K_I = y$ *as derived by Harris and Pestka (2) was used to calculate* K_I*, the association constant for the binding of inhibitor to ribosomes;* K_o *is the association constant for the binding of [^{14}C]erythromycin to ribosomes (9.9* $\times 10^7 M^{-1}$ *was used for these calculations, 10);* n *is the number of inhibitor molecules binding to ribosomes;* $[A]$ *is the free [^{14}C]erythromycin concentration;* $[R_o]$ *is the total concentration of ribosomes potentially active in binding [^{14}C]erythromycin; and* $[RA]$ *is the concentration of the [^{14}C]erythromycin-ribosome complex. From the data of Fig. 4, 5, and 6, y was plotted as a function of log* $[I]$*. Some representative results are presented in this figure and the results for all the analogues are summarized in Table 2. A computer program was used to determine the line of best fit by the method of least squares. When* $y = 0$, $n \log [I] = -\log K_I$*; thus assuming that* $n = 1$, $\log K_I = -\log [I]$ *at the baseline intercept. The baseline intercept was used to determine* K_I *for the reasons previously stated (2). When* $n = 1$*, the* K_I *values determined by the intercept of the abscissa or the ordinate would be equivalent.* K_I *for erythromycin was determined independently to be* 9.9×10^7 M^{-1} *and as computed above with a nonradioactive competing sample as* $10^8 M^{-1}$.

Comparison of effects of erythromycin analogues on [^{14}C]chloramphenicol and [^{14}C]erythromycin binding to ribosomes. A comparison of the effects of the erythromycin analogues on [^{14}C]chloramphenicol and [^{14}C]erythromycin binding to ribosomes is pre-

TABLE 2. *Association constants (K_I), dissociation constants (K_d), and interaction coefficients for erythromycin analogues*

Erythromycin analogue	K_I (M^{-1})	K_d (M)	pK_d	n
Erythromycin A	1.01×10^8	9.91×10^{-9}	8.00	0.99
1	5.66×10^6	1.77×10^{-7}	6.75	0.90
2	3.34×10^6	2.99×10^{-7}	6.52	0.96
3	6.74×10^5	1.48×10^{-6}	5.83	1.00
4	1.25×10^6	8.00×10^{-7}	6.10	1.12
5	1.79×10^7	5.59×10^{-8}	7.25	1.01
6	7.9×10^3	1.3×10^{-4}	3.9[a]	
7	2.11×10^7	4.74×10^{-8}	7.32	1.08
8	1.25×10^8	8.02×10^{-9}	8.10	0.94
9	1.08×10^8	9.28×10^{-9}	8.03	0.97
10	1.31×10^8	7.64×10^{-9}	8.12	0.95
11	1.04×10^8	9.63×10^{-9}	8.02	0.80
12	9.48×10^5	1.06×10^{-6}	5.98	1.01
13	5.27×10^7	1.90×10^{-8}	7.72	0.93
14	4.73×10^5	2.11×10^{-6}	5.67	0.92
15	4.86×10^5	2.06×10^{-6}	5.69	1.00
16	7.9×10^3	1.3×10^{-4}	3.9[a]	
17	5.07×10^4	1.97×10^{-5}	4.70	1.28
18	1.6×10^4	6.3×10^{-5}	4.2[a]	
19	1.6×10^4	6.3×10^{-5}	4.2[a]	
20	4.0×10^4	2.5×10^{-5}	4.6[a]	
21	5.07×10^6	1.97×10^{-7}	6.70	1.01
22	1.10×10^7	9.06×10^{-8}	7.04	0.77
23	4.67×10^6	2.14×10^{-7}	6.67	0.87
24	4.45×10^6	2.25×10^{-7}	6.65	1.04
25	3.44×10^6	2.91×10^{-7}	6.54	0.89
26	4.75×10^5	2.11×10^{-6}	5.68	1.20
27	1.21×10^5	8.24×10^{-6}	5.08	1.13
28	7.22×10^5	1.38×10^{-6}	5.86	1.05
29	3.23×10^5	3.09×10^{-6}	5.51	1.11
30	5.51×10^5	1.82×10^{-6}	5.74	1.10
31	5.28×10^5	1.90×10^{-6}	5.72	1.19
32	5.43×10^5	1.84×10^{-6}	5.73	0.88
33	7.10×10^5	1.41×10^{-6}	5.85	1.08
34	2.38×10^5	4.20×10^{-6}	5.38	1.12
35	3.61×10^5	2.77×10^{-6}	5.56	1.33
36	1.66×10^6	6.01×10^{-7}	6.22	1.02
37	3.63×10^6	2.75×10^{-7}	6.56	1.05
38	9.70×10^5	1.03×10^{-6}	5.99	1.11
39	2.15×10^6	4.66×10^{-7}	6.33	1.01
40	3.58×10^5	2.80×10^{-6}	5.55	1.11
41	2.96×10^5	3.38×10^{-6}	5.47	1.13
42	5.73×10^5	1.75×10^{-6}	5.76	1.07
43	2.40×10^5	4.16×10^{-6}	5.38	1.17
44	4.07×10^6	2.45×10^{-7}	6.61	1.05

[a] Estimated from a graph similar to that of Fig. 7; however, because of low activity and few appropriate data points, the estimate is only approximate.

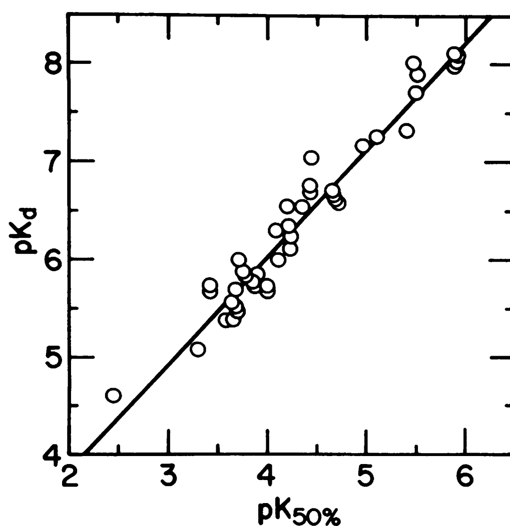

FIG. 8. *Correlation of $pK_{50\%}$ and pK_d values. The $pK_{50\%}$ and pK_d values from Tables 1 and 2 were plotted for the erythromycin analogues. The equation for the straight line is $pK_d = 1.098\ pK_{50\%} + 1.618$. This was determined by the method of least squares with the use of a computer program. The index of determination was 0.94.*

sented in Table 3. The results are presented graphically in Fig. 9. It can be seen that the group I_b, II, and III antibiotics had similar effects on chloramphenicol and erythromycin binding. Compounds in groups IV, V, and VII, which represent derivatives of 5-*O*-desosaminylerythronolide A, showed greater inhibition of [^{14}C]chloramphenicol than of [^{14}C]erythromycin binding to ribosomes. Derivatives in which the dimethylamino group of desosamine was altered (group VI) also showed greater inhibition of chloramphenicol binding than of erythromycin binding to ribosomes.

DISCUSSION

The ability of compounds to interfere with erythromycin binding to ribosomes provides an estimate of their ability to interact with the erythromycin ribosomal binding site. Thus, Wilhelm et al. (14) showed that other macrolides and lincomycin could inhibit [^{3}H]erythromycin binding to ribosomes from *Bacillus subtilis*. Also, Mao and Putterman (7) and Mao (6) reported that several macrolide antibiotics as well as some derivatives of erythromycin inhibited [^{14}C]erythromycin binding to ribosomes. Since they only tested low concentrations of the derivatives, relatively inactive derivatives such as **4** and **2** showed little or no inhibition of [^{14}C]erythromycin binding to ribosomes from *Staphylococcus aureus* (6, 7). In the studies reported in this communication, quantitative binding constants were assigned to these and other even less active derivatives.

Substitutions on various portions of the erythromycin molecule could be made with retention of substantial activity in inhibiting [^{14}C]erythromycin binding to ribosomes. Thus, after removal of cladinose, various substituents can be attached to the 3-hydroxyl group. Also, various substituents can be placed on the oxime of erythromycin A oxime with retention of much activity. Furthermore, the desosamine residue can be modified in at least two places with retention of good activity. Substitutions could be made on the 2′-hydroxyl group as well as on the dimethylamino group. Thus, in at least four separate positions, substantial substitutions were made with retention of activity. It should be possible to incorporate reactive groups for affinity labeling in these various positions singly and in various combinations to provide knowledge about the topological site of interaction of erythromycin A with ribosomes. A molecule carrying such groups in two or more distinct sites on the erythromycin A molecule might be expected to cross-link proteins or other components which comprise or are adjacent to the erythromycin A binding site. Furthermore, by extending the size and length of the groups with reactive moieties at the end, it should be possible to topologically map the ribosomal surface with respect to the erythromycin binding site.

It is apparent that removal of the dimethylamino group from desosamine reduced activity enormously. Structural alterations produced by the introduction of oxygen bridges within the macrolide ring also generally reduced ability to interact with ribosomes (compare analogues **1** and **2**). Removal of both cladinose and desosamine groups abolished activity. Substantial activity was retained, however, upon removal of only the cladinose residue. Introduction of a 3-keto group in the macrolide ring essentially abolished activity of the molecule (compare **19** and **28** as well as **20** and **27**).

Acetylation of the 3-hydroxyl group increased the activity of 5-*O*-desosaminylerythronolide A (compare **27** and **29**). The activity was further increased by substituents such as a benzoyl or substituted benzoyl group in this position (**31, 36, 37, 38, 39**).

Because of the relatively rapid hydrolysis of acetyl substituents on the oxime and 2′-hydroxyl group, it was necessary to evaluate these compounds only when freshly dissolved in aqueous solutions. If these solutions were per-

TABLE 3. *Comparison of concentrations for 50% inhibition of [14C]chloramphenicol and [14C]erythromycin binding to ribosomes*

Group	Antibiotic	[14C]Chloroamphenicol binding		[14C]Erythromycin binding	
		$pK_{50\%}$	μM	$pK_{50\%}$	μM
	Erythromycin A	5.45	3.6	5.90	1.3
Ia	1	5.10	8.0	4.42	38
Ib	2	4.27	54	4.35	45
	3	3.70	200	3.77	170
	4	4.05	89	4.22	60
II	5	4.48	33	5.10	7.9
	6	2.85	1,400	<2.5	>3,000
	7	5.20	6.3	5.40	4.0
	8	5.38	4.2	5.95	1.1
	9	5.45	3.6	5.92	1.2
III	10	5.51	3.1	5.90	1.3
	11	4.95	11	5.47	3.4
	12	4.30	50	3.70	200
	13	5.32	4.8	5.50	3.2
IV	14	4.50	32	3.43	372
	15	4.85	14	3.67	214
	16	<2.5	>3,000	<2.0	>10,000
	17	<2.5	>3,000	<3	>1,000
	18	<2.5	>3,000	<2.5	>3,000
V	19	<3.0	>1,000	<2.5	>3,000
	20	3.63	190	2.45	>3,000
	21	5.43	3.7	4.65	22
	22	4.90	13	4.43	37
VI	23	4.90	13	4.42	38
	24	5.46	3.5	4.66	22
	25	4.47	34	4.20	63
	26	4.45	35	4.00	100
	27	4.60	25	3.30	501
	28	4.67	21	2.75	1,770
	29	4.96	11	3.70	200
	30	5.04	9.1	3.86	138
VII	31	5.10	8.0	4.00	100
	32	4.00	100	3.42	380
	33	5.10	8.0	3.90	126
	34	4.86	14	3.57	269
	35	5.06	8.7	4.08	83.2

mitted to stand, the results obtained corresponded to the hydrolyzed derivatives.

When the effect of erythromycin analogues on [14C]chloramphenicol and [14C]erythromycin binding to ribosomes are compared, it can be seen that the two effects are not strictly proportional (Table 3 and Fig. 9). Although a few compounds appear to affect the binding of both similarly, most of the erythromycin derivatives inhibited chloramphenicol binding more than erythromycin binding. Thus, compounds which inhibit [14C]chloramphenicol binding relatively well may not inhibit [14C]erythromycin binding to a similar extent. Those compounds which inhibited chloramphenicol binding to a greater extent than [14C]erythromycin binding include compounds in groups IV, V, and VII. Those in group IV represent derivatives of 5-*O*-desosaminylerythronolide A. Those in group VII represent 5-*O*-desosaminylerythronolide A oxime and its derivatives. The results therefore indicate that removal of the cladinose interfered

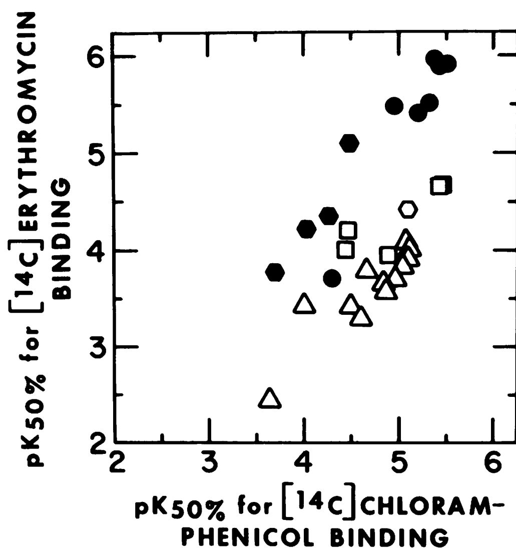

FIG. 9. *Comparison of effects of erythromycin analogues on [14C]chloramphenicol and [14C]erythromycin binding to ribosomes. The data for the $pK_{50\%}$ values of Table 3 are plotted here. The following symbols represent the groups designated in Table 3: ●, erythromycin A and group III; ⬢, groups Ib and II; △, groups IV and VII; ○, group Ia; □, group VI.*

with binding to ribosomes, but it was unexpected that some of these derivatives would inhibit [14C]chloramphenicol binding relatively better than [14C]erythromycin binding. This observation is not consistent with a single site for binding of all these derivatives to ribosomes. It is, thus, conceivable that some of these erythromycin derivatives may bind at additional sites rather than exclusively to the single erythromycin binding site. Furthermore, competition between the erythromycin derivatives on the one hand and [14C]erythromycin or [14C]chloramphenicol on the other may involve conformational changes of the ribosome rather than or in addition to direct competition for ribosomal sites. If, however, any specific portion of the erythromycin molecule is responsible for inhibition of chloramphenicol binding by direct competition for ribosomal binding sites, it is certainly not the cladinose residue.

Since the effect of erythromycin analogues on [14C]erythromycin binding to ribosomes provides a relatively sensitive assay for these compounds, erythromycin analogues with relatively poor binding to ribosomes can be detected. Compounds with association constants of 10^4 M^{-1} were detectable. The sensitivity of the assay could be increased by using a concentration of [14C]erythromycin below the saturation level. In this way, compounds with even less activity might be detected. This assay can be used alone or in conjunction with microbiological assays for primary screening of active analogues. It permits an estimate of the general activity of compounds rapidly and directly. Variables such as metabolic conversions or modifications of the compounds, permeability, and similar parameters are excluded. In general, results obtained with this assay parallel results of microbiological assays (11). The present assay reflects the ability of the compounds to interact directly with their target organelle and may serve as a useful adjunct in developing new compounds.

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