THE PREPARATION OF ALANINE PEPTIDES OF ERYTHROMYCIN A

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Alanine peptides were attached to the desosamine hydroxyl group of erythromycin A and to the 9-amino group of 9-(S)-erythromycylamine in an attempt to facilitate transport of the antibiotic into Gram-negative bacterial cells. Antibacterial activity and ribosome binding data are reported for these compounds.

Erythromycin A (1) inhibits the growth of bacteria by blocking protein synthesis. The antibiotic acts by specifically binding to 50 S ribosomes in both Gram-positive (Bacillus and Staphylococcus) and Gram-negative (Escherichia) bacteria. In cell free preparations, the binding occurs at very low erythromycin concentrations and about one molecule of erythromycin is bound per 50 S ribosome. However, against whole cells antibacterial activity of erythromycin is limited to Gram-positive bacteria because of its inability to penetrate the outer membrane of many Gram-negatives. If a means of facilitating transport into Gram-negative cells could be discovered, erythromycin would presumably be fully active.

We wish to report an attempt to confer Gram-negative activity on erythromycin A and 9-(S)-erythromycylamine by attachment of alanine peptides. An easily accessible site for attachment of peptides is the desosamine hydroxyl by an ester linkage. Erythromycin 2'-esters are well absorbed orally but must hydrolyze in vivo to be therapeutically effective. Treatment of erythromycin A with carbobenzyloxy-L-alanine in the presence of dicyclohexylcarbodiimide (DCC) followed by hydrogenolysis of the protecting group gave the 2'-alanine ester 2. Because of difficulty in removing the carbobenzyloxy group in the conversion of 2 to 3 and 4, the fluorenylmethyloxycarbonyl (FMOC) protecting group was used. Coupling of FMOC-L-alanine with 2 in the presence of DCC followed by treatment with morpholine to remove the FMOC group gave the 2'-alanylalanine ester 3. Treatment of 2 with FMOC-L-alanyl-L-alanine and DCC followed by removal of the protecting group with diethylamine gave the 2'-tripeptide ester 4.

Another site for attachment of peptides is the 9-amino group of 9-(S)-erythromycylamine. The mixed anhydride of FMOC-L-alanine reacted smoothly with 5 to provide the 9-alanylamide 6 after removal of the protecting group. The dipeptide 7 and the tripeptide 8 amides were prepared in a similar manner. Any 2'-ester formed under the reaction conditions was hydrolyzed with aqueous methanol.

The compounds were tested for antimicrobial activity in an in vitro agar diffusion well assay against Bacillus subtilis 558 in antibiotic medium 31 and the activities are shown in Table 1. No activity was observed for any of the compounds against Escherichia coli 94 or Pseudomonas aeruginosa 56 in vitro at 1 mg/ml, the highest level tested. The in vitro activities against Streptococcus pyogenes were determined in mice as described and are shown in Table 1. No activity was observed against E. coli in vivo at 100 mg/kg, the highest level tested. In addition the compounds were tested for competition with [14C]erythromycin in an Escherichia coli ribosome binding assay. The results are given in Table 1.
A previous study\textsuperscript{10} has demonstrated that erythromycin analogs which show 50\% inhibition at less than 80 \(\mu\text{M}\) have at least 1\% the activity of erythromycin A against Bacillus subtilis. Therefore the binding data in Table 1 predict that if the compounds are capable of penetrating the bacterial membrane, they should show some antibacterial activity.

Erythromycin 2'-esters have been shown\textsuperscript{11} to have substantially less antibiotic activity than the parent erythromycin. In fact the activity which is observed against intact cells may be due in great part to unesterified antibiotic freed by hydrolysis. Although the extent of hydrolysis was not measured in our study, it is possible that cleavage of the peptides occurs before compounds 2, 3, or 4 show antimicrobial activity. Because of potential hydrolysis, the compounds were dissolved in DMSO and only diluted with water immediately before analysis in the binding assay. We believe that hydrolysis of 2, 3, and 4 was minimal during this assay. Compound 2 binds better to ribosomes than erythromycin A and this data casts some doubt on the necessity for a free 2'-hydroxyl for binding. It is interesting that 2 binds substantially better than 2'-acetyl erythromycin (\(I_{50}=7.9 \mu\text{M}\)).\textsuperscript{19} Some hydrolysis of 4 undoubtedly occurs during the determination of antimicrobial activity \emph{in vitro} since its activity is higher than one would expect from its ribosome binding. Since the peptide esters 2, 3, and 4, show progressively less activity than erythromycin A, they are apparently cleaved to erythromycin at different rates with cleavage being slowest with the tripeptide derivative 4. Alternatively, 4 might be transported less readily than 3 and 2 to explain its lower activity. \emph{In vivo} compounds 2, 3, and 4 seem to be readily cleaved since they all exhibit the same activity as erythromycin A.

The complete loss of antibiotic activity of the peptide amides 6, 7, and 8 indicates that these compounds apparently are not readily cleaved to 9-(S)-erythromycylamine (5) \emph{in vitro} or \emph{in vivo} since 5 exhibits good activity relative to 1.\textsuperscript{17} The lack of activity \emph{in vitro} for 6, 7 and 8 was not due to peptides competing with the amides for bacterial transport because these amides were also inactive in defined minimal medium.

Present knowledge indicates\textsuperscript{12,13} that the outer membrane of Gram-negative bacteria constitutes a permeability barrier. Embedded in the membrane are nonspecific pores with an exclusion limit of about 550~650 daltons for enteric bacteria but extending up to several thousand daltons for \emph{Pseudomonas}. In addition to nonspecific pores, there are specific transporter proteins in the outer membranes of Gram-negative bacteria and vitamin B\textsubscript{12}, enterochelin-Fe\textsuperscript{3+} and maltodextrins have been shown to be transported by these proteins. Therefore, while peptide derivatives of erythromycin might penetrate the outer membrane of \emph{Pseudomonas}, transport across the outer membrane of the enteric bacteria would probably

\begin{table}
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\caption{Table 1.}
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Compound} & \textbf{Ribosome binding} & \textbf{\% of} & \textbf{Activity} & \\
 & \textbf{\(I_{50} \ (\mu\text{M})\)} & \textbf{Erythromycin A} & \textbf{\(\text{CD}_{50} \ (\text{mg/kg})\)} & \\
 & & \textbf{\(\text{\% of Erythromycin A}\)} & \textbf{\emph{In vitro vs. B. subtilis}} & \textbf{s.c.} \textbf{p.o.} \\
\hline
1 (Erythromycin A) & 0.79 & 100 & 1.1~3.0 & 15~35 \\
2 & 0.49 & 97 & 2.3 & 30 \\
3 & 2.29 & 88 & 1.6 & 23 \\
4 & 10.96 & 41 & 2.1 & 25 \\
6 & 10.96 & <1 & 43 & >50 \\
7 & 18.20 & <1 & 43 & >50 \\
8 & 12.30 & <1 & >50 & >50 \\
\hline
\end{tabular}
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require the assistance of a transporter protein.

Peptide transport systems have been demonstrated to be present on the inner membrane of many microorganisms. These systems have been successfully employed to transport biosynthetic intermediates and antimicrobial compounds.

In this study it was hoped that if the erythromycin peptides could gain access to the inner membrane, even if only at low concentration, their transport into the cell might be facilitated by the peptide transport system and once inside the cell, peptidase cleavage would free the active antibiotic. Unfortunately, the desired transport and cleavage does not appear to have occurred since none of the derivatives showed Gram-negative activity.

Experimental

Spectral data (IR and NMR) were recorded for the new compounds and were in accord with the assigned structures. In the IR spectra, the 2'-esters 2~4 showed the expected bands at 1730~1735 cm\(^{-1}\) (lactone+ester) and at 1695~1705 cm\(^{-1}\) (ketone). In addition 3 and 4 gave an amide band at 1675~1680 cm\(^{-1}\). Compounds 6~8 showed a lactone band at 1725~1730 cm\(^{-1}\) and an amide band at 1665~1670 cm\(^{-1}\). The most distinguishing feature of the complicated NMR spectra was the shift of the dimethylamino resonance from \(\delta 2.30\) in compounds 6~8 to \(\delta 2.20\) to \(\delta 2.22\) in compounds 2~4 which have a substituent on the 2'-hydroxyl.

Erythromycin 2'-L-Alanine Ester (2)

To a solution of 4.00 g (5.45 mmole) of erythromycin A and 2.09 g (9.37 mmole) of carbobenzyloxy-L-alanine in 80 ml of methylene chloride was added 1.94 g (9.41 mmole) of N,N'-dicyclohexylcarbodiimide and the mixture was stirred at room temperature for 16 hours. After cooling in an ice bath and filtration, the filtrate was concentrated in vacuo. Crystallization from methylene chloride-ether-hexane gave 5.25 g, mp 100~108°C, of erythromycin 2'-N-carbobenzyloxy-L-alanine ester. The carbobenzyloxy protecting group was removed by shaking in a Parr hydrogenation apparatus at a hydrogen pressure of 3.5 kg/cm\(^2\) with 5 g of 10% Pd/C in 125 ml of dioxane for 3.5 hours. The reaction mixture was filtered through a Celite pad and the filtrate was concentrated in vacuo. The residue was dissolved in ether and cooled at \(-18^\circ\)C overnight. Filtration gave 2.08 g, mp 140~145°C, of 2.
Anal. Found: C 59.49, H 9.01, N 3.29.
Caled. for CaH9N2O14: C 59.68, H 9.02, N 3.48.

**Erythromycin 2'-l-Alanyl-l-alanine Ester (3)**

To a solution of 151 g (1.88 mmole) of 2 and 0.69 g (2.23 mmole) of 9-fluorenylmethoxycarbonyl-L-alanine in 30 ml of methylene chloride was added 0.47 g (2.26 mmole) of N,N'-dicyclohexylcarbodiimide and the mixture was stirred at room temperature for 40 minutes. After filtration, the filtrate was concentrated in vacuo and the residue was treated with 20 ml of morpholine and left at room temperature for 60 minutes. The morpholine was removed on the oil pump and 20 ml of ether was added to the residue. After cooling at -18°C for 17 hours, the resultant solid was filtered and the filtrate was concentrated in vacuo to an oil which was chromatographed on 75 g of silica gel which had been prewashed with acetone - hexane (1:1). Elution with acetone gave several pure fractions which were combined and concentrated in vacuo to yield 0.82 g of a foam. A portion was crystallized from ether - hexane to give analytically pure 3, mp 114~120°C.

Anal. Found: C 59.06, H 9.02, N 4.72.
Caled. for C43H77N3O15: C 58.95, H 8.86, N 4.80.

**Erythromycin 2'-l-Alanyl-l-alanyl-l-alanine Ester (4)**

To a solution of 1.00 g (1.24 mmole) of 2 and 0.57 g (1.49 mmole) of 9-fluorenylmethoxycarbonyl-L-alanyl-L-alanine in 20 ml of dimethoxyethane and 1 ml of dimethylformamide was added 0.32 g (1.49 mmole) of N,N'-dicyclohexylcarbodiimide and the mixture was stirred at room temperature for 3 days. After filtration, the filtrate was concentrated in vacuo. Crystallization from ether gave 1.38 g, mp 130~137°C. This was dissolved in 15 ml of diethylamine and left at room temperature for 1 hour. The diethylamine was removed in vacuo and the residue was dissolved in chloroform and washed with saturated sodium bicarbonate solution. The chloroform solution was dried (MgSO4) and concentrated in vacuo to a foam which was triturated with ether to give 0.77 g, mp 129~135°C, of 4.

Caled. for C46H82N4O16·1.25H2O: C 57.04, H 8.69, N 5.78, H2O 2.32.

**9-(S)-Erythromycylamine N-L-Alaninamide (6)**

To a solution of 4.22 g (0.014 mole) of 9-fluorenylmethoxycarbonyl-L-alanine and 1.90 ml (0.014 mole) of triethylamine in 125 ml of anhydrous acetone cooled in an ice bath was added 1.63 ml (0.013 mole) of isobutyl chloroformate with stirring. After stirring 1 hour in the ice bath, the triethylamine hydrochloride was removed by filtration. The filtrate was cooled in an ice bath and stirred while 7.50 g (0.010 mole) of 9-(S)-erythromycylamine (5) was added. After stirring in an ice bath for 30 minutes and at room temperature for 17 hours, the solvent was removed in vacuo. The crude foam was dissolved in 80 ml of 90% methanol and left at room temperature for 24 hours. After concentration in vacuo, the resultant oil was dissolved in 80 ml of morpholine and stirred at room temperature for 4 hours. The solid was removed by filtration and the filtrate was concentrated in vacuo to a semi solid. This was chromatographed on 200 g of silica gel. Elution with methylene chloride - methanol - conc. NH3OH (90:10:1) gave several fractions containing pure 6. The pure fractions were combined and crystallized from methylene chloride - ether to give 5.90 g, mp 149~155°C, of 6.

Anal. Found: C 58.82, H 9.03, N 5.01, H2O 1.12.
Caled. for C48H90N2O15·0.5H2O: C 58.94, H 9.40, N 5.16, H2O 1.11.

**9-(S)-Erythromycylamine N-L-Alanyl-l-alaninamide (7)**

To a solution of 0.622 g (2.0 mmole) of 9-fluorenylmethoxycarbonyl-L-alanine and 0.28 ml (2.0 mmole) of triethylamine in 20 ml of anhydrous acetone cooled in an ice bath was added 0.24 ml of isobutyl chloroformate with stirring. After stirring 1 hour in the ice bath, the triethylamine hydrochloride was removed by filtration. The filtrate was cooled in an ice bath and stirred while 1.210 g (1.5 mmole) of 6 was added. The reaction mixture was stirred in the ice bath for 30 minutes and at room temperature for 3.5 hours. After concentration in vacuo, the crude product was dissolved in 10 ml of 90% methanol and left at room temperature for 24 hours. The solvent was removed in vacuo and the residue was dissolved in 30 ml of diethylamine and left at room temperature for 4 hours. After concentration in vacuo, the residue was dissolved in chloroform and washed with saturated sodium bicar-
bonate solution. The residue obtained on concentration of the chloroform was triturated with ether and filtered to yield 0.825 g, mp 148°-155°C, of 7. An analytical sample, mp 147°-151°C, was obtained by recrystallization from ethyl acetate - hexane.

**Anal. Found:**
- C 57.96, H 9.00, N 6.21, H₂O 0.90.
- Calcd. for C₄₃H₈₀N₄O₁₄·0.5H₂O: C 58.28, H 9.21, N 6.32, H₂O 1.02.

9-(S)-Erythromycylamine N-Alanyl-L-alany1-L-alaninamide (8)

To a solution of 1.200 g (1.5 mmole) of 6 and 0.688 g (1.8 mmole) of 9-fluorenylmethoxycarbonyl-L-alany1-L-alanine in 40 ml of acetone was added 0.372 g (1.8 mmole) of N,N'-dicyclohexylcarbodiimide and the mixture was stirred at room temperature for 4 days. After filtration, the filtrate was concentrated in vacuo. The residue was dissolved in 40 ml of diethylamine and left at room temperature for 3 hours. The diethylamine was removed in vacuo and the residue was dissolved in chloroform and washed with saturated sodium bicarbonate solution. After drying (MgSO₄), the chloroform was removed in vacuo, and the residue was triturated with ether and filtered to yield 1.069 g, mp 162°-172°C.

**Anal. Found:**
- C 59.59, H 9.01, N 3.29.

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