SYNTHESIS OF A TIGHT-BINDING, MULTISUBSTRATE ANALOG INHIBITOR OF GENTAMICIN ACETYLTRANSFERASE I

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(Received for publication June 14, 1979)

Gentamicin acetyltransferase I will catalyze acyl transfer from chloroacetylcoenzyme A to form 3-N-chloroacetylgentamicin. This product can be linked to coenzyme A to form a multisubstrate analog by nucleophilic displacement of the chlorine by the sulfur of coenzyme A. The analog can be purified by selective binding to cationic and anionic ion exchange resins. Kinetic analysis of a time-dependent onset and reversal of inhibition of gentamicin acetyltransferase I by the purified multisubstrate analog yields an inhibition constant of $5 \sim 20 \times 10^{-10}$ M. The inhibitor does not potentiate antibiotic activity against resistant *Escherichia coli*. Nevertheless, the effectiveness of the tight-binding between the enzyme and the multisubstrate analog demonstrates that inhibitors of resistance can be designed and prepared by specific enzymatic synthesis.

Resistance to aminoglycoside antibiotics in R factor-containing bacteria is determined by a family of recently discovered enzymes which inactivate the antibiotics[1]. Since bacteria which display resistance due to the presence of a drug inactivating enzyme are not resistant *per se*, but remain sensitive to the unchanged form of the antibiotic, resistance to aminoglycoside antibiotics might be counteracted by the concurrent administration of specific inhibitors of the inactivating enzymes. The design of such inhibitors is dependent upon specific knowledge of the target enzymes.

Gentamicin acetyltransferase I*** was found in resistant clinical isolates of *Pseudomonas aeruginosa* and catalyzes the following reaction[3].

Gentamicin + Acetyl-Coenzyme A → 3-N-Acetylgentamicin + Coenzyme A.

Kinetic studies of gentamicin acetyltransferase I revealed the following information with relevance to inhibitor design[4,5]. First, the enzymatic mechanism is sequential, meaning that both substrates (antibiotic and coenzyme) must be present on the enzyme before any products are released, and suggesting that no covalent bonds are formed between enzyme and substrate. Second, the binding of substrates is highly synergistic. The presence of antibiotic greatly increases the binding of coenzyme and *vice versa*. Third, antibiotic binding is primarily derived from amine groups of the purpurosamine and garosamine rings of gentamicin, with only a minor contribution derived from the 2-deoxystreptamine ring. Fourth, the acyl transfer specificity of the enzyme includes propionyl-CoA, but not butyryl-CoA or malonyl-CoA. Hence, the active site will accommodate non-bulky additions to the transferred acetyl

This investigation was supported by Research Grant AI 11603, Research Career Development Award GM 00245, and Biomedical Research Support Grant 1 507 RR-05456 from the National Institute of Health and research grants from the University of Wisconsin Graduate School. This paper is the seventh in a series of aminoglycoside inactivating enzymes.

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*** This enzyme is designated AAC(3)-1 according to proposals by the Plasmid Group Nomenclature[2].

groups. Combining this information with the current knowledge of the thermodynamics of substrate and inhibitor binding[6], a multisubstrate analog appeared to be the most promising design for an inhibitor. The design consists of antibiotic and coenzyme moieties (to take advantage of synergistic and sequential binding), linked together within the 2-deoxystreptamine ring (to minimize binding interference). The lack of acyl transfer specificity suggests enzymatic synthesis as a means for accomplishing specific linkage of multisubstrate analogs. This report describes the synthesis of an inhibitory acetylgentamicin-CoA multisubstrate analog, based on an intermediate enzymatic synthesis of 3-N-chloroacetylgentamicin as an example of this design.

Materials

Acetyl-CoA, CoA, and phosphotransacetylase were purchased from P-L Biochemicals, chloroacetic anhydride was from Eastman. Tobramycin was a gift from Dr. MARVIN GORMAN of Eli Lilly and Company and gentamicin C_{1a} was a gift from Dr. GERALD WAGMAN of Schering Corporation. DEAE-Sephadex and Sephadex G-10 were from Pharmacia Fine Chemicals. Bio-Gel CM-2 was from Bio-Rad. Gentamicin acetyltransferase I was prepared and assayed as described previously[7]. Tobramycin was used as a substrate for kinetic studies because it displays linear initial velocity kinetics[4].

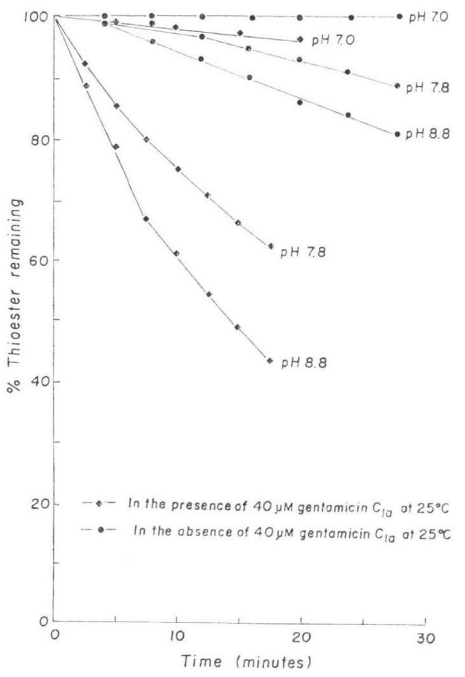
Results

Preparation and Instability of Chloroacetyl Coenzyme A*

Chloroacetyl-CoA was prepared by dissolving 9.83 mg of CoA in 7 ml of distilled dimethylformamide by vigorous stirring under nitrogen. The reaction mixture was then cooled on ice and 2.5 mg of chloroacetic anhydride added with stirring. After 15 minutes, 4 ml of deionized water was added and the reaction mixture frozen and lyophilyzed. The dried powder was dissolved in 1 ml of water, desalted on Sephadex G-10, lyophilized, and stored as a dry powder. The product was enzymatically active with both gentamicin acetyltransferase I (Cl-acetyl-CoA activity = 0.25 × acetyl-CoA activity) and phosphotransacetylase at pH 7.0, and represented a 60% yield of chloroacetyl-CoA based on enzymatic assay.

The instability of chloroacetyl-CoA is shown in Fig. 1, indicated by loss of absorbance at 232 nm due to the thioester bond[9]. Loss due to hy-

Fig. 1. Non-enzymatic loss of chloroacetyl-CoA. 20 mM phosphate buffer was used at pH 7.8 and 8.8. Absorbance measurements were performed at 232 nm.



* Preparation of chloroacetyl coenzyme A was first attempted by the method of SIMON and SHEMIN[8] where the acetic anhydride was replaced with chloroacetic anhydride . However, chloroacetic anhydride was found to hydrolyze too rapidly in aqueous solution to react sufficiently with CoA, and attempts to increase the yield of chloroacetyl-CoA by adding large excesses of the anhydride caused the formation of a variety of side products.

drolysis is undetectable at pH 7.0, but progressively increases in rate at higher values of pH. The presence of 40 mM gentamicin increases the rate of loss of absorbance, both at pH 7.0 and higher values of pH.

Preparation and Purification of Acetylgentamicin-CoA

An excess of chloroacetyl-CoA was dissolved in 0.2 ml H_2O and quickly added to 3 mg of purified gentamicin acetyltransferase I (specific activity = 3 units/mg at pH 6.8) and 10 mg of gentamicin C_{1a} dissolved in 0.9 ml of 0.1 M KPO_4^- buffer of pH 6.8 at room temperature. After stirring for 45 minutes, nitrogen was bubbled through the reaction mixture for 10 minutes followed by an addition of 10 mg of CoA. The pH was adjusted to 7.8 with 2 M $KHCO_3$. The reaction mixture was kept under nitrogen overnight at room temperature, desalted on Sephadex G-10 equilibrated with 10 mM potassium acetate at pH 5.5, and applied to a DEAE-Sephadex column equilibrated with the same buffer. This column was washed with 50 mM KCl in the potassium acetate buffer, and eluted in a linear KCl gradient from 50 to 400 mM, in buffer. Two fractions (I and II) eluted in the gradient as detected by absorbance at 254 nm and shown in Fig. 2. The column was then eluted with buffered 1 M KCl, producing on large fraction (III). When 20 μl aliquots of each fraction was tested for inhibition of a standard gentamicin acetyltransferase I assay, fractions I and III showed no inhibition, whereas fraction II gave >90% inhibition. The material in fraction II was ninhydrin-positive. The material in fraction III reacted with 5,5′-dithiobis(2-nitrobenzoic acid), indicative of a free sulfhydryl group, and was determined to be CoA by reaction with phosphotransacetylase and acetyl phosphate.

Fig. 2. Elution profile of the acetylgentamicin-CoA analog reaction mixture from DEAE-sephadex. The column was eluted with a 60-ml linear gradient from 0.05 M~0.4 M KCl followed by 1 M KCl. The column was 1×30 cm and was eluted at 0.5 ml/min.

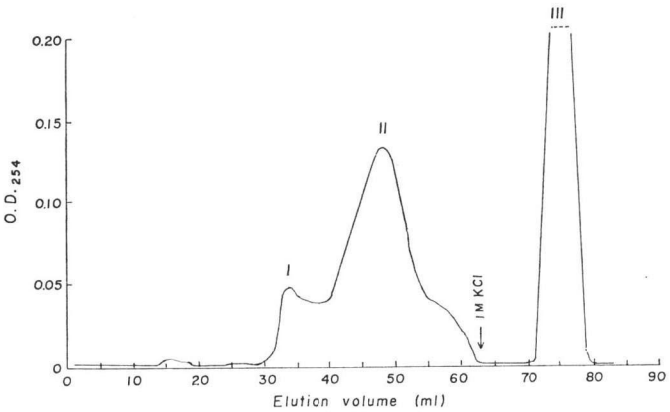
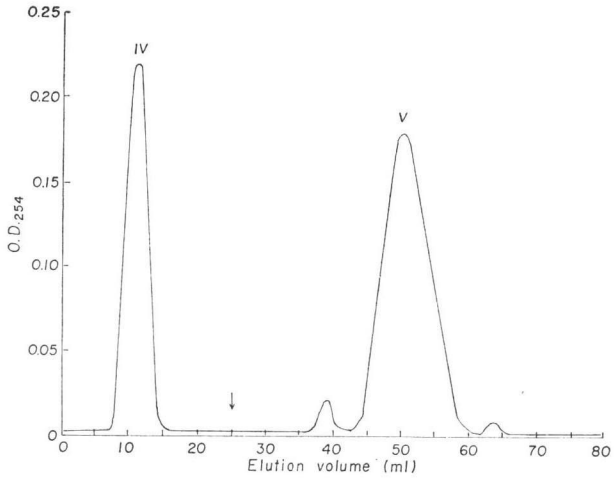


Fig. 3. Elution profile of fraction II from CM-polyacrylamide. The column was washed to baseline and then eluted (denoted by arrow) with a 100-ml linear gradient from 0~1 M NaCl. The column was 1×30 cm and was eluted at 0.5 ml/min.



The material in fraction II was lyophilized, redissolved in 1.5 ml H_2O, and desalted on Sephadex G-10 that had been equilibrated with 20 mM sodium acetate, pH 3.8. The desalted material was then

applied to a Bio-Gel CM-2 column equilibrated with the sodium acetate buffer. Fraction IV eluted in buffer, and fraction V, as well as other minor components, eluted in a linear gradient of 0 to 1 M NaCl in sodium acetate buffer (Fig. 3). An aliquot of fraction IV inhibited a standard assay only 15%, whereas an aliquot of fraction V was found to inhibit >90%. Fraction V was ninhydrin positive and absorbed ultraviolet light. Assuming the acetylgentamicin-CoA analog has the same extinction coefficient as CoA at 259 nm, 0.89 mg of the multisubstrate analog was present in fraction V.

Kinetics of Inhibition

Assays initiated by the addition of enzyme produced non-linear reaction velocity tracings in the presence of acetylgentamicin-CoA. The tracings were smooth curves revealing a time-dependent onset of inhibition. Tangents to the curved tracings were drawn at 15-second intervals. The slopes of these tangents versus time produced linear first order plots shown in Fig. 4. Apparent rate constants for the disappearance of enzyme activity at four inhibitor concentrations were obtained from the slopes of these plots. The dependence of these apparent rate constants on inhibitor concentration was analyzed in a reciprocal plot as shown in Fig. 5.

Assays initiated by the addition of substrates following pre-incubation of enzyme and inhibitor also produced non-linear reaction velocity tracings. Concentrations of enzyme and inhibitor which gave no detectable inhibition of initial rates, displayed no detectable enzyme activity after a 5-minute pre-incubation at higher concentrations. Enzyme activity then appeared over a period of minutes following a 500-fold dilution into an assay mixture containing saturating levels of substrates, producing another smoothy curved reaction velocity tracing and revealing a time-dependent reversal of inhibition. A first order plot of the slopes of tangents to the second curved tracing was linear as shown in Fig. 6.

Fig. 4. First order plot of the disappearance of gentamicin acetyltransferase I enzymatic activity. Enzyme was added to an assay mixture containing substrates and varied concentrations of acetyl-gentamicin-CoA. Acetyl-CoA and tobramycin were 15 μM and enzyme was 34 nM. (V_i = inhibited velocity).

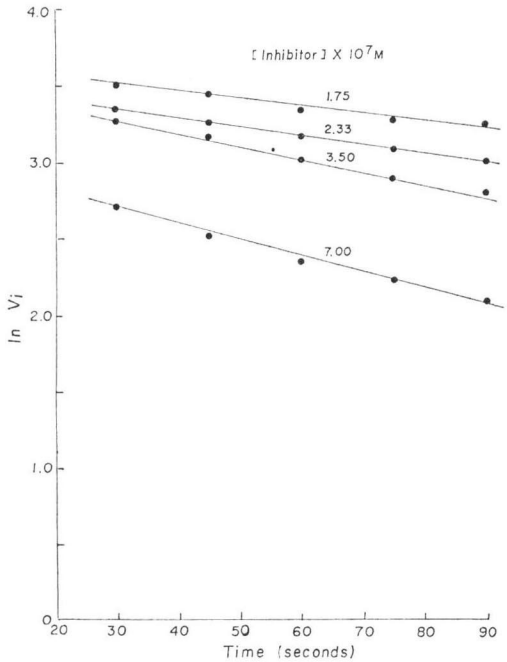
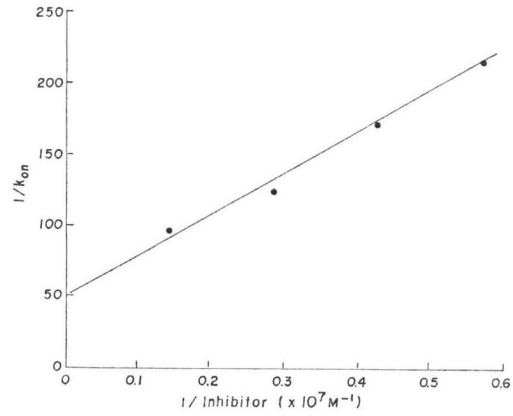


Fig. 5. Reciprocal plot of apparent k_{on} versus inhibitor concentration. Values of k_{on} were determined from the slopes of Fig. 4.



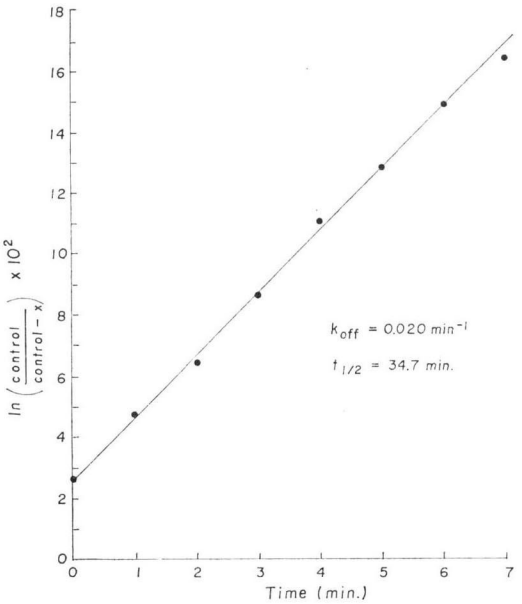
In Vivo Inhibitor Activity

Escherichia coli C-600 containing R factor JR88, from which gentamicin acetyltransferase I was isolated[7], were plated and subjected to agar-diffusion assays of gentamicin in the presence and absence of acetylgentamicin-CoA. No differences in the zones of inhibition were detectable.

Discussion

Specific synthesis of the multisubstrate analog is accomplished by taking advantage of the lack of acyl transfer specificity of gentamicin acetyltransferase.* By substituting chloroacetyl-CoA for acetyl-CoA in the enzymatic acylation of gentamicin, it appears possible to incorporate a chloroacetyl moiety at the appropriate 3-amino group of the deoxystreptamine of gentamicin, to which the sulfur of CoA can be covalently bonded by nucleophilic displacement of the chlorine. The proposed structure of the acetylgentamicin-CoA multisubstrate analog prepared in this manner is shown in Fig. 7. Evidence for this structure beyond the route by which it was prepared lies in the binding of the compound to both anionic and cationic ion-exchange resins, its absorbance at 259 nm, and its positive reactivity with ninhydrin.

Fig. 6. First order plot of the appearance of gentamicin acetyltransferase I enzymatic activity. Enzyme was preincubated with the acetylgentamicin-CoA analog for 5 minutes and diluted 500-fold into an assay mixture. Control assays were run using enzyme with no inhibitor and enzyme to which inhibitor and subtrates were added at the same time. The two controls gave identical rates. Control-X represents the difference in slopes between inhibited and uninhibited enzyme. Final concentrations were: Acetyl-CoA, 54 μM; tobramycin, 30 μM; enzyme, 32 nM, and inhibitor, 160 nM.



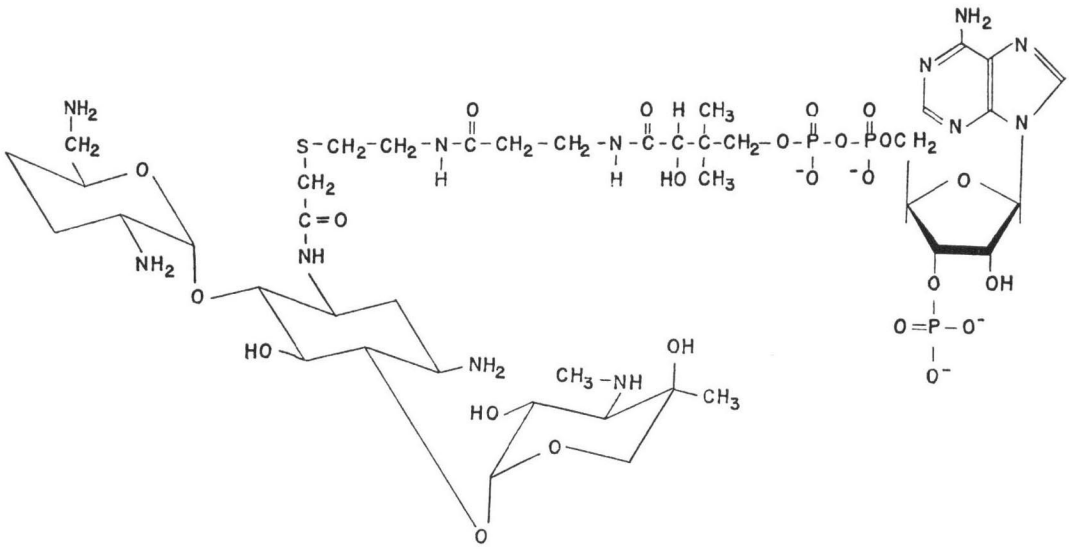
Efficient synthesis requires minimizing non-specific loss of chloroacetyl-CoA. The pH dependence of disappearance of thioester absorbance indicates a base catalyzed hydrolysis. The increased rate of disappearance of the thioester absorbance in the presence of gentamicin suggests non-specific esterification of amino groups of gentamicin due to the increased reactivity of the thioester in chloroacetyl-CoA.** Because both hydrolysis and non-specific esterification are similarly pH dependent (Fig. 1), loss of chloroacetyl-CoA is minimized by conducting the enzymatic esterification at slightly acidic pH. Efficient synthesis also requires minimizing non-specific alkylation of amine and hydroxyl groups of gentamicin and CoA by the alkyl halide intermediate, in competition with the alkylation of the sulfur of CoA. The presence of gentamicin acetyltransferase I during this chemical step supposedly serves to bring the reactants together at the active site of the enzyme. In a similar synthesis, the rate of alkylation of the sulfur of CoA by bromoacetylcarnitine is increased by approximately 10^3 fold by the presence of carnitine acetyltransferase[10]. Thus, use of the enzyme serves both to increase rates and to convey specificity to two reactions in the synthesis.

The time-dependent onset and reversal of inhibition of gentamicin acetyltransferase I by acetyl-

* A certain irony may be noted in the procedure. Current thinking on the origin of the bacterial antibiotic-inactivating enzymes holds that the plasmid genes may have derived from antibiotic-producing organisms, where the enzymes function in biosynthesis of antibiotics. Thus, the first function of this enzyme is production of antibiotics, the second is protection against antibiotics, and the present usage is inhibition of protection.

** Acetyl-CoA itself will slowly and non-specifically acylate the amine groups of aminoglycoside antibiotics (WILLIAMS and NORTHROP, unpublished results).

Fig. 7. Proposed structure of the acetylgentamicin-CoA analog inhibitor.



gentamicin-CoA is consistent with the minimal mechanism shown in eq. 1.

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^* \tag{1}$$

Enzyme and inhibitor first form a diffusion controlled complex (EI) which then slowly relaxes into a tightly bound complex (EI^*) from which the inhibitor cannot directly dissociate. The inhibition or dissociation constant for this mechanism is defined as

$$K_I = \frac{k_4}{k_3} \cdot \frac{k_2}{k_1} \tag{2}$$

During assays initiated by the addition of enzyme, EI^* is not initially present and the mechanism of eq. 1 reduces to eq. 3.

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \overset{k_3}{\longrightarrow} EI^* \tag{3}$$

At low concentrations of inhibitor, the onset of inhibition depends upon the rate of EI^* formation (k_{on}), which is a pseudo-first order process at fixed inhibitor and enzyme concentration, described by eq. 4[11].

$$k_{on} = \frac{k_3[I][E_t]}{\frac{k_2}{k_1} + [I]} \tag{4}$$

Apparent values for k_{on} (Fig. 4) may be graphically analyzed in a reciprocal plot versus inhibitor concentration (Fig. 5) according to the reciprocal form of eq. 4[9].

$$\frac{[E_t]}{k_{on}} = \frac{k_2/k_1}{k_3[I]} + \frac{1}{k_3} \tag{5}$$

Eq. 5 predicts a linear relationship, as observed, with a vertical intercept equal to $1/k_3$ and horizontal intercept of $-k_1/k_2$. Values obtained for k_3 and k_2/k_1, were 1.9×10^{-2} sec^{-1} and 5.3×10^{-7} M, respectively.

During assays initiated by the addition of substrates following pre-incubation of enzyme and inhibitor, free enzyme is not initially present. The mechanism of eq. 1 then reduces to eq. 6.

$$E + I \underset{k_2}{\longleftarrow} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^* \tag{6}$$

Enzymatic activity is proportional to the free enzyme concentration, whose appearance is determined

by the rate of release of inhibitor, a first order process described by eq. 7.

$$k_{off}=\frac{k_2 \cdot k_4}{k_2+k_3} \tag{7}$$

which reduces to

$$k_{off}=k_4 \tag{8}$$

when $k_2 \gg k_3$, the condition necessary for time dependent onset of tight binding. From the slope of the plot in Fig. 6, a value of 3.3×10^{-4} sec^{-1} was obtained for k_4, corresponding to a half-life of 34.7 minutes for the breakdown of the enzyme-inhibitor complex.

The inhibition constant defined by eq. 2 and calculated from the values obtained for k_2/k_1, k_3, and k_4 is 9.2×10^{-9} M. However, this represents only an apparent value because the mechanism in eq. 1 does not include a description of the effects of substrate binding. Assuming that substrate binding is strictly competitive versus inhibitor, and using substrate concentrations of Fig. 4 and substrate kinetic constants previously determined[4], the lower limit for the corrected inhibition constant becomes:

$$K_I=\frac{K_{I(app)}}{1+\dfrac{[A][B]}{Kia \cdot Kb}}=\frac{9.2\times10^{-9}\,\text{M}}{1+\dfrac{(1.5\times10^{-5}\,\text{M})(1.5\times10^{-5}\,\text{M})}{(8\times10^{-6}\,\text{M})(1.7\times10^{-6}\,\text{M})}}$$

$$K_I=5.2\times10^{-10}\,\text{M} \tag{9}$$

At the other extreme, assuming that substrate binding is strictly non-competitive versus inhibitor, the upper limit for the corrected inhibition constant becomes:

$$K_I=\frac{K_{I(app)}}{1+\dfrac{[A][B]}{Ka[B]+Kb[A]+Kia\,Kb}}$$

$$K_I=\frac{9.2\times10^{-9}\,\text{M}}{1+\dfrac{(1.5\times10^{-5}\,\text{M})(1.5\times10^{-5}\,\text{M})}{(1.6\times10^{-6}\,\text{M})(1.5\times10^{-5}\,\text{M})+(1.7\times10^{-6}\,\text{M})(1.5\times10^{-5}\,\text{M})+(8\times10^{-6}\,\text{M})(1.7\times10^{-6}\,\text{M})}}$$

$$K_I=2\times10^{-9}\,\text{M} \tag{10}$$

Because the multisubstrate analog resembles both substrates, it is likely that the inhibition is of the mixed type with binding to free enzyme being much more favorable than binding to either an enzyme-tobramycin or an enzyme-acetyl CoA complex, because of entropy considerations[6]. This places the inhibition constant near the lower value of the limits set by eq. 9 and 10, and nearly 1,000-fold lower than the dissociation constants of the best known substrates or inhibitor[5]. Thus the acetylgentamicin-CoA multisubstrate analog is a potent, tight-binding inhibitor of gentamicin acetyltransferase I.

Unfortunately, the inhibitor has no effect on the antibiotic activity of gentamicin against R-factor resistant *E. coli*. This failure is probably due to a failure of the inhibitor to penetrate the bacterial membrane, since nucleotides do not readily cross the membrane. This in turn suggests that the antibiotic inactivating enzyme is not located in the periplasmic space between the cell membrane and cell wall, but rather is buried within the cell membrane, a possibility consistent with the variable results of releasing the antibiotic inactivating enzymes from resistant bacteria by osmotic shocking procedures[7,12,13]. Nevertheless, the effectiveness of the inhibitor against the purified enzyme demonstrates that inhibitors of resistance can be designed and that the approach merits further study to obtain compounds which will penetrate bacterial membranes.

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