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Sparsomycin and Its Analogues: A New Approach for Evaluating Their Potency as Inhibitors of Peptide Bond Formation

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SUMMARY

The ability of several sparsomycin analogues to inhibit peptide bond formation was studied *in vitro*. Peptide bonds are formed between puromycin (S) and the acetylPhe-tRNA of acetylPhe-tRNA/70 S ribosome/poly(U) complex (complex C), according to the puromycin reaction:

$$C + S \overset{K_s}{\rightleftharpoons} CS \overset{k_3}{\rightarrow} C' + P.$$

It was shown that the sparsomycin analogues, like sparsomycin itself, inhibit peptide bond formation in a time-dependent manner; they react with complex C according to the equation

$$C + I \overset{K_I}{\rightleftharpoons} CI \overset{k_6}{\underset{k_7}{\rightleftharpoons}} C^*I,$$

where C*I is a conformationally altered species in which I is bound more tightly than in CI. The determination of the rate constant k_7 for the regeneration of complex C from the C*I complex allows evaluation of these analogues as inhibitors of peptide bond formation. According to their k_7 values, these analogues are classified in order of descending potency as follows: *n*-pentyl-sparsomycin (4) > *n*-butyl-sparsomycin (3) ~ *n*-butyl-deshydroxy-sparsomycin (6) > benzyl-sparsomycin (2) > deshydroxy-sparsomycin (5) ~ sparsomycin (1) > *n*-propyl-desthio-deshydroxy-sparsomycin (7). The analogues with an aromatic or a larger hydrophobic side chain are stronger inhibitors of the puromycin reaction than are those with a smaller side chain or those lacking the bivalent sulfur atoms; replacement of the hydroxymethyl group with a methyl group does not affect the position of the compound in this ranking; compare the positions of compounds 1 and 3 with those of 5 and 6. In the case of compound 7, C*I adsorbed on cellulose nitrate disks was not sufficiently stable to allow examination by the method applied to the other analogues, probably due to a relatively large value of k_7. This analogue showed also time-dependent inhibition, but after the isomerization of CI to C*I, the kinetics of inhibition become complex, and C*I interacted further with puromycin, either as C*I or after its dissociation to C*.

Antibiotics acting as inhibitors of protein synthesis have been used extensively to study ribosomal structure and function. One such antibiotic is sparsomycin (1, Table 1), which inhibits peptide bond formation in both prokaryotic and eukaryotic cells by acting on the large ribosomal subunit (1, 2). Considerable efforts have been made to study the mechanism by which sparsomycin exercises this inhibition (3–8). With the use of the puromycin reaction, i.e., the reaction of complex C with excess puromycin (9), we have recently carried out a detailed analysis of the inhibition of this reaction by sparsomycin (8, 10). We found that, in agreement with other studies (2), sparsomycin binds initially to the ribosomal A site and competes with the antibiotic puromycin and, thus, with aminoacyl-tRNA. Inhibition by sparsomycin is increased when, in *in vitro* studies, it is preincubated with complex C. This is the preincubation effect (2, 11–14) and can be explained if we assume that the drug acts as a slow-binding inhibitor (8). Thus, sparsomycin (I) inhibits peptide bond formation between puromycin and AcPhe-tRNA of complex C (C) according to a mechanism in which the intermediate complex CI undergoes conformational changes to produce a new species, C*I. Sparsomycin is one of several antibiotics that we studied that behave as slow-binding inhibitors (8, 15, 16).

Sparsomycin has also been investigated as a potential antitumor agent (2). However, initial clinical trials indicated that sparsomycin might cause eye toxicity. Nevertheless, the successful total synthesis of sparsomycin prompted investigators to synthesize a large number of sparsomycin ana-

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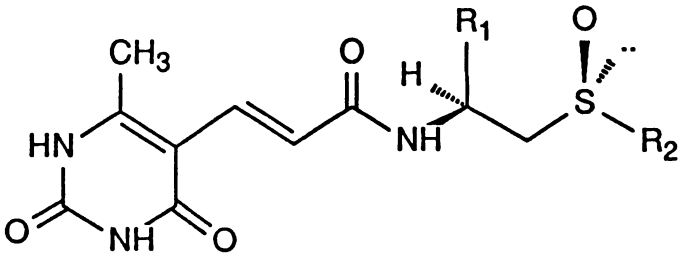
ABBREVIATION: Ac, acetyl; complex C, AcPhe-tRNA/70 S ribosome/poly(U) complex.

TABLE 1
Sparsomycin and its analogues



No.	Compound	R^1	R^2	$E^{1M}303$
1	sparsomycin[17]	CH_2OH	CH_2SCH_3	17,875
2	benzyl-sparsomycin[17]	CH_2OH	CH_2SCH_2Ph	20,130
3	n-butyl-sparsomycin[18]	CH_2OH	$CH_2S(CH_2)_3CH_3$	42,000
4	n-pentyl-sparsomycin[18]	CH_2OH	$CH_2S(CH_2)_4CH_3$	31,000
5	deshydroxy-sparsomycin[18]	CH_3	CH_2SCH_3	42,000
6	n-butyl-deshydroxy-sparsomycin[18]	CH_3	$CH_2S(CH_2)_3CH_3$	37,200
7	n-propyl-desthio-deshydroxy sparsomycin[18]	CH_3	$(CH_2)_2CH_3$	45,000

logues (17–19) in search of one or more analogues that will be free of toxic side effects while enhancing the cytostatic activity of the parent compound (20). Thus, several synthetic analogues have been studied, and their ability to inhibit protein synthesis was determined through a variety of methods (17, 18). These studies uncovered some of the functionally important parts of the sparsomycin molecule, while also indicating the existence of a hydrophobic region in the peptidyltransferase center, which plays a role in the interaction of some of the more active sparsomycin analogues with the ribosome (18, 21). These findings tacitly implied that the K_i values of the equation C + I ⇌ CI reflects the potency of the analogue (11, 17, 18).

In the current study, we examined the ability of the sparsomycin analogues **2–7** (Table 1) to act as inhibitors of ribosomal peptidyltransferase. We allowed these sparsomycin analogues to react with complex C and investigated the mechanism of inhibition of peptide bond formation by using the puromycin reaction. We studied whether these analogues behave as classic, competitive inhibitors or belong, like sparsomycin itself, to a class of inhibitors that exhibit time-dependent inhibition. Our analysis permitted the determination of rate constants rather than of equilibrium constants such as the K_i. The determination of these rate constants represents a more accurate measure of the potency of antibiotics than the use of K_i.

Experimental Procedures

Materials. L-[2,3,4,5,6-^{3}H]Phenylalanine was purchased from Amersham. Heterogeneous tRNA from *Escherichia coli* strain W, L-phenylalanine, poly(U), GTP (disodium salt), ATP (disodium salt), and puromycin dihydrochloride were purchased from Sigma Chemical Co. Cellulose nitrate filters (type HA, 24 mm diameter, 0.45 μm pore size) were purchased from Millipore Corporation. Sparsomycin (compound 1) was a gift from the Upjohn Co.

Sparsomycin analogues. The sparsomycin analogues **2–7** (Table 1) used in this study were synthesized by van den Broek *et al.* (17, 18).

***De novo* formation of complex C or of a mixture of complex C and the sparsomycin analogue complex.** Complex C was formed in a binding mixture that was prepared at 0°; 0.2 ml of mixture consisted of 20 μmol of Tris·HCl, pH 7.2, 20 μmol of NH_4Cl from a solution adjusted with NH_4OH to pH 7.2, 2 μmol of magnesium acetate, 64 μg of poly(U), 0.08 μmol of GTP, 6.4 A_{260} units of washed ribosomes, 80 μg of (protein) ribosomal wash (FWR fraction) and 65 pmol of Ac-[^{3}H]Phe-tRNA (2800 cpm/pmol) (9). Whenever required, one of the sparsomycin analogues was added in the binding mixture before the addition of the ribosomes; in this way, a mixture of complex C and of the sparsomycin analogue complex (CI + C*I) was obtained. In either case, incubation followed at 25° for 8 min, after which the reaction was stopped by placing the binding mixture in ice. Complex C or the mixture of complex C and sparsomycin analogue complex was separated from excess donor Ac-[^{3}H]Phe-tRNA and from the other molecules present in the binding mixture by dilution with ice-cold binding buffer (100 mM Tris·HCl, pH 7.2, 50 mM KCl, 10 mM $MgCl_2$, and 6 mM β-mercaptoethanol), filtration through cellulose nitrate filter disks, and three washes with binding buffer. These complexes were examined with the use of the puromycin reaction.

Formation of the nK_i sparsomycin analogue-modified complex C. *De novo*-formed complex C was preincubated at 25° with one of the sparsomycin analogues at the indicated concentrations expressed for each analogue in K_i units (nK_i, with n ranging from 0.25 to 40).

Puromycin reaction. The puromycin reaction was carried out with reactive complex adsorbed on a cellulose nitrate filter disk under three different conditions: (a) with the *de novo*-formed complex C or with the *de novo*-formed mixture of complex C and sparsomycin analogue complex (puromycin at the appropriate concentrations reacted with these complexes for various time intervals); (b) without preincubation of complex C with the sparsomycin analogues (puromycin reacted with complex C in a mixture also containing the sparsomycin analogue at the appropriate concentration, and the reaction was allowed to proceed at 25° for the time intervals indicated and then stopped by the addition of 1.0 ml of 1 M NaOH); and (c) after preincubation of complex C with the sparsomycin analogues (puromycin reacted with the nK_i-sparsomycin analogue-modified complex C). The preincubation takes place at 25° for an appropriate period of time, which depends on the information obtained from the inactivation plot for each analogue. A sufficient preincubation period before the puromycin reaction is carried out is seven times the $t_{1/2}$. Then, puromycin was added at the appropriate concentrations, and the reaction was allowed to proceed at 25° for the time intervals indicated and then stopped by the addition of 1.0 ml of 1 M NaOH.

First-order analysis of the puromycin reaction. The first-order analysis of the puromycin reaction in the absence of inhibitor has been described previously (9). The entire course of the reaction in

the absence of inhibitor obeys pseudo-first-order kinetics. In the presence of inhibitor, the first-order time plots are biphasic. The initial slope (k), i.e., the slope of the line going through the origin, is taken as the apparent first-order rate constant (initial slope analysis). The relationship between k and [S] follows the equation: $k=k_3[S]/(K's+[S])$, as described previously (22, 23).

Determination of the apparent k^I_{obs} from inactivation plots. To each one of a series of small beakers, 0.9 ml of reaction buffer (100 mM Tris·HCl, pH 7.2, 100 mM NH_4Cl, 10 mM $MgCl_2$, and 6 mM β-mercaptoethanol) containing the appropriate amount of each sparsomycin analogue was added so that the desired concentrations were achieved (final volume, 1 ml). The beakers were allowed to equilibrate at 25° for 5 min. At the time intervals indicated, the reaction between complex C and the sparsomycin analogue was started by the addition to each beaker of one half of a cellulose nitrate filter disk bearing complex C. After the desired reaction time had elapsed, 0.1 ml of 20 mM puromycin was added in the same buffer (containing the appropriate amount of each sparsomycin analogue so that its final concentration remained constant) and allowed to react for 30 sec. The percent $x/\alpha = x'$ for each time of exposure (t) was determined, and the log x' was plotted against t. The initial slope of this plot gave the apparent first-order rate constant (k^I_{obs}) for each concentration of sparsomycin analogue.

Regeneration of complex C from the *de novo*-formed sparsomycin analogue complex. The mixture of complex C and sparsomycin analogue complex C*I on cellulose nitrate filter disks was exposed to reaction buffer for various periods of time. Subsequently, the disk was removed, placed into cold binding buffer, filtered, and washed with the same buffer. The regeneration of complex C from C*I was monitored with the use of the puromycin reaction (2 mM puromycin for 2 min).

Results and Discussion

Time-dependent inhibition and the preincubation effect. The present study was conducted on selected sparsomycin analogues synthesized by van den Broek *et al.* (17, 18) (see Table 1). The inhibition of peptide bond formation by each sparsomycin analogue was studied in an *in vitro* system in which complex C was isolated on cellulose nitrate filter disks free of excess unbound AcPhe-tRNA. It then reacted with excess puromycin (S) according to the ribosome-catalyzed reaction of complex C and puromycin as shown in the following equation (9):

$$C + S \underset{}{\overset{K_s}{\rightleftharpoons}} CS \xrightarrow{k_3} C' + P \qquad (1)$$

A peptide bond is formed in AcPhe-puromycin (P), the determination of which monitors the progress of the reaction; C′ cannot revert back to C. Thus, the puromycin reaction can be analyzed as a pseudo-first-order reaction, giving logarithmic time plots that are linear.

In the presence of the sparsomycin analogue (I), we surmise that complex C reacts with I according to the following equation:

$$C + I \underset{}{\overset{K_i}{\rightleftharpoons}} CI \underset{k_7}{\overset{k_6}{\rightleftharpoons}} C^*I \qquad (2)$$

The sparsomycin analogues were studied by using the reactions of eqs. 1 and 2 taking place either simultaneously or separately.

Fig. 1 shows the time course of the reaction between complex C and 1×10^{-4} M puromycin in the absence or presence of 1×10^{-7} M sparsomycin analogue. In the absence of an

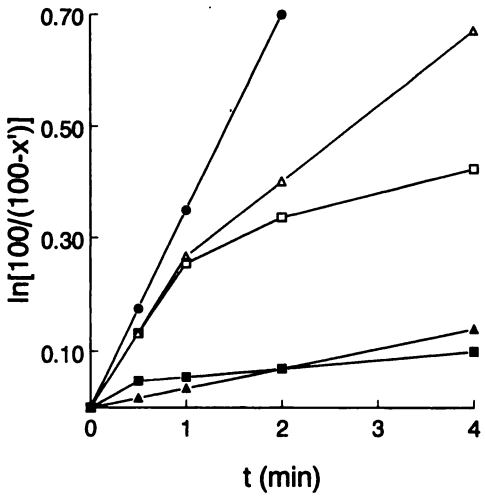


Fig. 1. First-order time plots for AcPhe-puromycin formation between complex C adsorbed on cellulose nitrate filter disks and puromycin at 1×10^{-4} M under the following conditions: in the absence of sparsomycin analogue (●); in the presence of a mixture of puromycin and benzyl-sparsomycin (△) or pentyl-sparsomycin (□) at a final concentration of 1×10^{-7} M; and after preincubation for 8 min at 25° with benzyl-sparsomycin (▲) or pentyl-sparsomycin (■) at 1×10^{-7} M and then reaction with puromycin.

analogue, a straight line is obtained until all of complex C has been converted to product AcPhe-puromycin. The reaction is over in <3 min. In the presence of analogues **2** and **4** at 1×10^{-7} M, the reactions 1 and 2 occur simultaneously, and biphasic time plots are obtained. The inhibition observed in the presence of **2** and **4** can be estimated from the initial slopes of these biphasic time plots. The degree of inhibition changes with time (time-dependent inhibition). The biphasic time plots point to the possibility of a slow step during the interaction of complex C with the sparsomycin analogue. When the analogue is preincubated with complex C, before the addition of puromycin, the inhibition is increased as shown in Fig. 1 (*two bottom lines*); this is the so-called preincubation effect, which can be accounted for by the slow equilibration between the reactive ribosomal complex and the inhibitor (8). All six sparsomycin analogues that we tested displayed this behavior, which can be explained by the assumption that these compounds, similar to sparsomycin itself, interact with complex C in the two-step reaction presented in eq. 2.

Determination of the apparent k^I_{obs} from the inactivation plot. To determine whether a slow step in the reaction between C and I does exist, we carried out the reaction of eq. 2 alone at several concentrations of I. The remaining active complex C was monitored at each time period with the puromycin reaction (2 mM puromycin for 30 sec). The inactivation plot for benzyl-sparsomycin at 1×10^{-6} M is shown in Fig. 2. Similar plots were obtained for all six sparsomycin analogues (data not shown). If the inactivation process is treated as a pseudo-first-order reaction, then it approaches equilibrium with an apparent k^I_{obs} that differs for each concentration of inhibitor. Fig. 2 (*inset*) depicts the initial slopes of a series of inactivation plots at various concentrations of compound **2**. For each concentration of the analogue, we obtained an apparent k^I_{obs}, the reciprocal of which, if plotted against the reciprocal of the concentration of the analogue (1/[I]), gives a straight line that meets the $1/k^I_{obs}$ axis at a

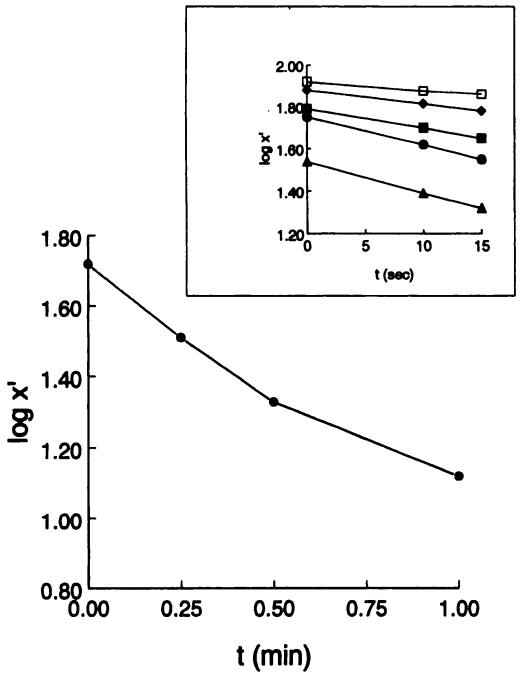


Fig. 2. Time plot for the inactivation of complex C by benzyl-sparsomycin. Complex C adsorbed on cellulose nitrate filter disks reacted with benzyl-sparsomycin at 1×10^{-6} M for the indicated periods of time. The percentage (x') of the remaining active complex C is then estimated through titration with puromycin at 2×10^{-3} M for 30 sec. *Inset*, initial slopes of inactivation plots obtained in the presence of benzyl-sparsomycin at the following concentrations: 0.1×10^{-6} M (□), 0.2×10^{-6} M (◆), 0.4×10^{-6} M (■), 1.0×10^{-6} M (●), and 2.0×10^{-6} M (▲).

point above zero. A representative plot is depicted in Fig. 3A for compound **2**. This characteristic feature could indicate that the conversion of complex C to C*I proceeds via formation of an intermediate complex CI, i.e., in two steps. In the absence of S, the relationship between k^I_{obs} and I predicted by the two-step mechanism is given by the equation $k^I_{obs} = k_7 + k_6[I]/(K_i + [I])$ (24–26), provided that the experimentally determined k^I_{obs} is equal to $k^I_{obs} - k_7$. If we assume that k_7 is much smaller than k_6, as we indeed find, the previous relationship becomes $k^I_{obs} = k_6[I]/K_i + [I]$, and the plot $1/k^I_{obs}$ versus 1/[I] gives, for **2**, values of $k_6 = 2.4$ min^{-1} and $K_i = 4.2 \times 10^{-7}$ M, which are very close to those reported recently (10) for the parent compound sparsomycin (**1**). Five of six of the compounds under investigation gave similar double-reciprocal plots, thus conforming to the two-step mechanism of eq. 2, which involves a rapid step followed by a slow step. Their k_6 and K_i values are given in Table 2.

However, in the case of *n*-pentyl-sparsomycin (**4**), the double-reciprocal plot tends to meet the $1/k^I_{obs}$ axis at a point very close to zero (Fig. 3B), giving an estimate of k_6 that is >5.5 min^{-1} and an apparent K_i of 1×10^{-5} M. If we assume that the straight line meets the $1/k^I_{obs}$ axis at zero, then the interaction between C and I would be consistent with the one-step mechanism of the following equation:

$$C + I \rightleftharpoons C^*I \qquad (3)$$

A plausible and simple explanation for this observation is that we are dealing with a variation of the two-step mechanism, in which the initial encounter complex CI is kinetically insignificant and C*I is the product of a conformational change of CI. Table 2 shows the values of k_6 and K_i for **4** obtained from such double-reciprocal plots. The rationale for the mechanism represented by eq. 3 is the same as that given recently for the interaction between another antibiotic, spiramycin, and complex C (10).

Regeneration of complex C from C*I. The *de novo*-formed mixture of complex C and the sparsomycin analogue complex C*I, isolated on cellulose nitrate filter disks, was first exposed to reaction buffer at 25° for the indicated time intervals. At the end of each exposure, the regenerated complex C was measured by reaction with puromycin (2 mM for 2 min). This method gave a pseudo-first-order rate constant for each analogue tested. Typical results are given in Fig. 4 for compound **2**. The intercept of the straight line with the vertical axis is a measure of preexisting complex C. The slope of the line is taken as a measure of the rate of regeneration, which is analogous to the value of the kinetic constant k_7. This value is equal to 0.04 min^{-1} (Table 2). It can also be seen (Table 2) that the values of the rate constants of regeneration differ by at least 1 order of magnitude ranging from 0.01 min^{-1} for compound **4** to 0.10 min^{-1} for compounds **1** and **5**. In the case of compound **7**, however, it was not possible to determine its k_7 with the methodology applied for compounds **1–6** because C*I adsorbed on cellulose nitrate filter disks was not sufficiently stable. A relatively large value for k_7 may contribute to this behavior. Based on the k_7 values, we can

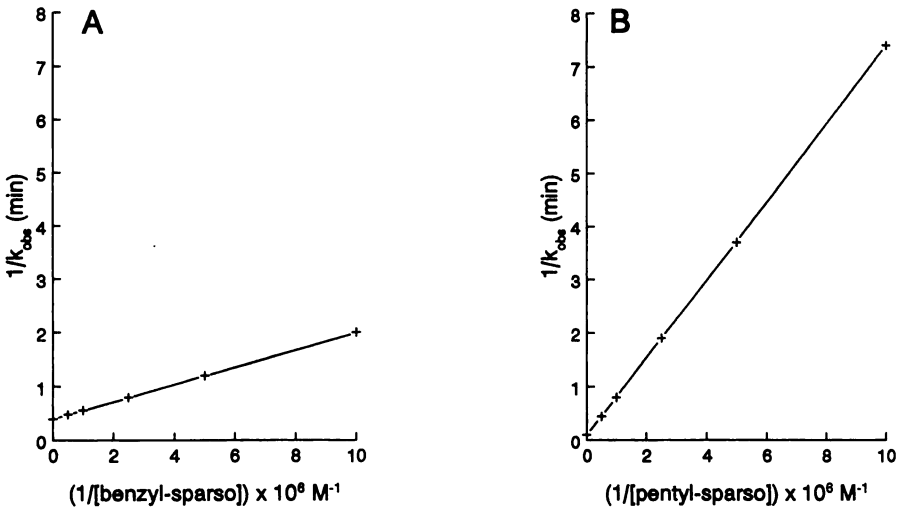


Fig. 3. Double-reciprocal plots ($1/k'_{obs}$ versus 1/[I]) for the inactivation of complex C by benzyl-sparsomycin (A) and pentyl-sparsomycin (B). The k'_{obs} values for each concentration of the sparsomycin analogues were taken from plots such as the inset of Fig. 2.

TABLE 2

Kinetic constants of sparsomycin and its analogues

The values of K_i and k_6 were determined from inactivation plots, and the values of k_7 were determined by exposure of the mixture C and C*I to reaction buffer and subsequent reaction with puromycin (2 mM for 2 min).

Compound	No.	K_i	k_6 min^{-1}/k_7 min^{-1}	
		M		
Sparsomycin	1	4.0×10^{-7}	2.6	0.10
Benzyl-sparsomycin	2	4.2×10^{-7}	2.4	0.04
n-Butyl-sparsomycin	3	4.2×10^{-7}	3.8	0.017
n-Pentyl-sparsomycin	4	1.0×10^{-5}	>5.5	0.01
Deshydroxy-sparsomycin	5	4.0×10^{-7}	5.5	0.10
n-Butyl deshydroxy-sparsomycin	6	4.6×10^{-7}	2.6	0.02
n-Propyl desthio-deshydroxy-sparsomycin	7	4.3×10^{-7}	2.5	0.49

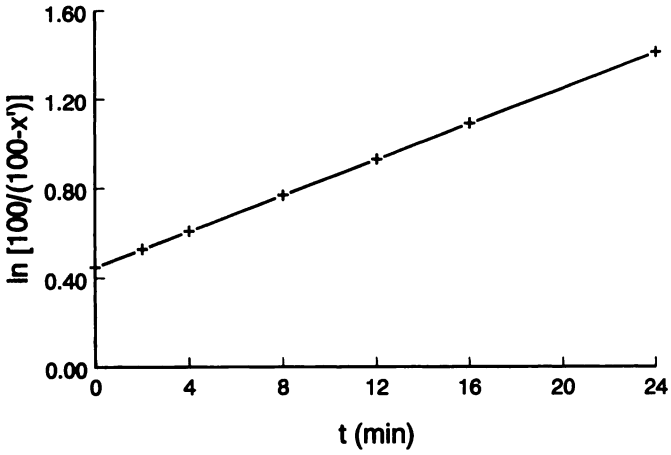


Fig. 4. Determination of the pseudo-first-order rate constant for the regeneration of complex C from the sparsomycin analogue complex C*I after exposure to reaction buffer for several time intervals and reaction with 2×10^{-3} M puromycin for 2 min. More details are given in the text.

now classify the six sparsomycin analogues and compare them with the parent compound. Thus, the analogues can be classified in order of descending potency: **4 > 3 ~ 6 > 2 > 5 ~ 1 > 7**.

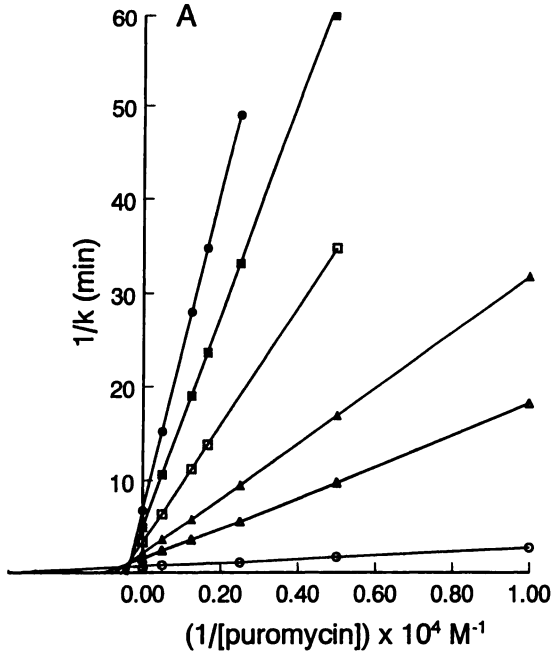
In the case of compound **4**, the K_i and k_6 values were determined on the assumption that **4** also follows the two-step reaction of eq. 2. However, it is possible that the inactivation of complex C by this analogue is adequately described with the one-step reaction of eq. 3. If this is the case, the value of 0.01 min^{-1} refers not to the k_7 but rather to k_{off} of the one-step reaction, and the real value for K_i would be much smaller.

Had we tried to classify these analogues on the basis of their K_i values, we would not have been able to differentiate them, except, perhaps, for compound **4**, which appears to react through formation of a kinetically insignificant encounter complex CI. Moreover, k_6 alone also cannot be used to distinguish most of these analogues; this is in agreement with the observation made by Merkler *et al.* (27) in their study with yeast AMP deaminase (EC 3.5.4.6); they reported that k_6 seems to be independent of inhibitor structure. It is the rate constant of regeneration k_7 that effectively differentiates the compounds under study according to their potency.

The low k_7 values and therefore the stability of five of six of the sparsomycin analogues on the cellulose nitrate filter disks allowed a kinetic analysis to be made based on the two-step mechanism (eq. 2 alone). This approach allowed us to avoid the kinetic complexity that exists when the puromycin reaction (reaction 1) is not used as a monitoring reaction but takes place simultaneously with reaction 2. The exception is compound **7**, of which the kinetics of inhibition of peptide bond formation were carried out through a method that allows examination of the further interaction of C*I with puromycin either in the continuous presence of the analogue (initial slope analysis) or after removal of the excess analogue (*de novo*-formed C*I complex).

Initial slope analysis. In Fig. 1, it is shown that without preincubation of C and I, the progress curves are biphasic for all the analogues tested, including **7**, whereas after preincubation, the time plots of most analogues become linear for ≤4 min. At several concentrations of puromycin and without preincubation with **7**, the initial slopes (k) gave linear double-reciprocal plots showing competitive kinetics from which a value of $K_i = 4 \times 10^{-7}$ M was confirmed. If C and I are preincubated before the addition of S, the kinetics of inhibition in the case of **7** are not competitive. With increasing concentrations of I, the kinetics become of the mixed-noncompetitive type, as shown by the double-reciprocal plot in Fig. 5A. The intercept replot ($1/k_{max}$ versus [I]) for the entire range of inhibitor concentrations, which includes the competitive as well as the mixed-noncompetitive phase, is not linear, as shown in Fig. 5B. This secondary plot becomes linear for values of [I] of >20 K_i, and the straight line, when extrapolated, cuts the vertical axis at 2.9. The reverse of this value (0.35 min^{-1}) corresponds to the hypothetical k_{max} in the absence of I. The fact that this value is much lower than the respective k_3 permits us to predict the existence of another species that reacts with S at a lower rate (k_3*). The slope replot for the mixed-noncompetitive phase becomes also linear at I concentrations of >20 K_i (data not shown). From this plot, the equilibrium constant K_s* for the interaction of C*I with S may be determined, and it is found to be 2.1 mM. One model suggested by this kinetic analysis is that given previously (22) when the kinetics of inhibition by blasticidin S were analyzed.

Experiments with the *de novo*-formed *n*-propyldesthio-deshydroxy-sparsomycin complex. When compound **7** is present during the binding reaction, a mixture of C and C*I is formed. In the reaction with puromycin, the progress curve for compound **7** (Fig. 6) becomes linear after 15 sec as the initially existing, more active component of the mixture, i.e., complex C, has disappeared. The linear part of the line obtained after 15 sec (Fig. 6, late slope) is retained at the same slope for ≤32 min (results not shown beyond 4 min). The late slope for **7** gives a value of k' = 0.66 min^{-1} and can be used to calculate k_7 on the condition that the excess I has been removed and that the species formed after the interaction of C and I are C and C*I. In this case, we may assume that this slope pertains to two pseudo-first-order parallel reactions with a common starting species, i.e., C*I (28). One of these reactions is the reversal of C*I toward free C (reaction 2), and the other one is the direct reaction of C*I with S (k_3* step). The sum of these two parallel reactions gives an overall value of $k' = k_7 + k_3*[S]/(K_s* + [S])$. Because [S] = 2 mM and K_s* = 2.1 mM, then $k' = k_7 + k_3*/2$. From this, it follows that k_7 = 0.66-0.17 = 0.49 min^{-1}, which is a relatively large k_7 value for compound **7**. This large value must be taken into consideration, and the k_6 and K_i values for



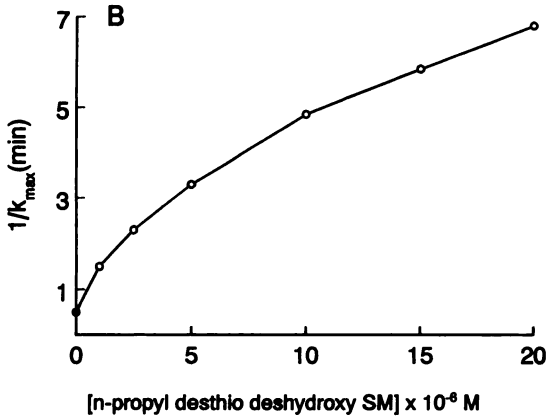


Fig. 5. A, Double-reciprocal plots (1/*k* versus 1/[puromycin]) for the puromycin reaction after preincubation of complex C for 8 min at 25° with *n*-propyl-desthio-deshydroxy-sparsomycin at a final concentration of 1×10^{-6} M (△), 2.5×10^{-6} M (▲), 5×10^{-6} M (□), 10×10^{-6} M (■), and 20×10^{-6} M (●). The double-reciprocal plot in the absence of the analogue (○) is also presented. B, Intercept replot ($1/k_{max}$ versus [*n*-propyldesthio-deshydroxy-sparsomycin]). The data were taken from double-reciprocal plots such as the one shown in A. The 1/*k* axis intercepts of these plots were replotted against the concentration of the analogue. The point plotted on the $1/k_{max}$ axis was taken from the intercept of the line in the absence of inhibitor (control).

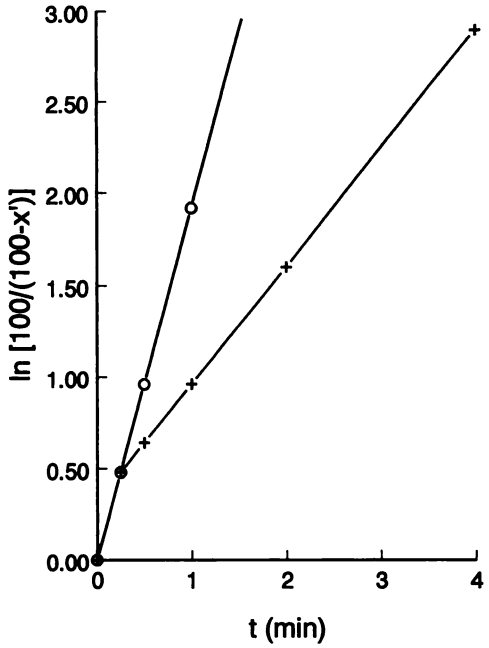


Fig. 6. First-order time plot for the puromycin reaction obtained with 1×10^{-6} M *n*-propyl-desthio-deshydroxy-sparsomycin in the binding reaction. The mixture of complex C and the sparsomycin analogue complex C*I formed in this way was adsorbed on cellulose nitrate filter disks and then reacted with 2×10^{-3} M puromycin for the indicated time intervals (+). The first-order time plot obtained in the absence of the analogue is also shown (○, control).

compound **7** should be determined from the plot of $1/(k^I_{obs} - k_7)$ versus 1/[I] instead of $1/k^I_{obs}$ versus 1/[I]. However, this calculation does not significantly change the values of k_6 and K_i shown in Table 2. With regard to the ratio $k_6/k_7 = 5$, although it is small, it permits the characterization of the analogue as slow-binding inhibitor. There have been reports in the literature of slow-binding inhibitors with values similar to that of the k_6/k_7 ratio (16, 27).

Conclusion. We studied the kinetics of inhibition of peptide bond formation by six synthetic analogues of sparsomycin. It is useful to unravel the mechanism of action of these analogues, especially as some of them may be found to have antitumor activity. A recent study assigned a role for one such analogue as a modifier of antitumor responses in cancer chemotherapy (20). All six analogues studied in this report show time-dependent inhibition that is similar to that exhibited by the parent compound, sparsomycin. Their potencies have been determined on the basis of the rate constant of regeneration k_7. The following conclusions may be drawn: (a) analogues with an aromatic (**2**) or an extended alkyl side chain (**3** and **4**) exert a higher degree of inhibition of the puromycin reaction than does sparsomycin (**1**). In fact, the longer the chain, the higher is the inhibitory activity. This is in agreement with the finding (17, 18) that replacement of the SCH_3 group of **1** with extended alkylthio groups causes an increase in the biological activity of the drug. However, not only the hydrophobicity but also the shape, size, and polarity of the substituent are important (2, 17, 18). (b) Replacement of the hydroxymethyl group by a methyl group to form the deshydroxy-sparsomycin analogues **5** and **6** does not seem to affect the above classification. Accordingly, **5** appears similar in potency to **1**, whereas **6** is a stronger inhibitor than **1** and comparable in activity with its counterpart, **3**. (c) The lower activity of **7** has to be attributed to its structure, which differs from those of the other analogues by the absence of the bivalent sulfur atom.

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